Institute for Animal Productions in the Tropic and Subtropics

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Jatropha meal and protein isolate as a protein source in aquafeed

Dissertation

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Dedicated

To

My Dad

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Declaration

I hereby declare that this work, submitted to the Faculty of Agricultural Sciences, University of Hohenheim, Stuttgart, Germany, for the award of Dr.sc.agr. is the result of original work carried out by myself under the guidance of Prof. Dr. Klaus Becker. Any assistance or citation of other work has been duly acknowledged. I further declare that the results of this work have not been submitted for the award of any other degree or fellowship.

Stuttgart, 22nd July, 2011   Vikas Kumar

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List of abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANFs	Antinutrtional factors
BUN	Blood urea nitrogen
DJKM	Detoxified *Jatropha curcas* kernel meal
DJPI	Detoxified Jatropha protein isolates
Ex	Expeller
GH	Growth hormone
H-JPKM	Heated *Jatropha platyphylla* kernel meal
IGF-1	Insulin-like growth factor-1
JCM	*Jatropha curcas* meal
JPI	Jatropha protein isolate
JPKM	*Jatropha platyphylla* kernel meal
LD	Lethal dose
LPC	Lupin protein concentrate
MCV	Mean cell volume
ND	Not detected
NL	Narrow-leaf
NSP	Non-starch polysaccharides
PPC	Potato protein concentrate
RBC	Red blood cells
RIPs	Ribosome-inactivating proteins
SE	Solvent-extracted
SPC	Soybean protein concentrate;
SPI	Soy protein isolate
SSF	Solid state fermentation
TBIL	Total bilirubin

1. Summary

Aquaculture is growing rapidly at an average rate of 8.8% per year since 1970, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems. This high growth of aquaculture has resulted from a large increase in demand for fish as food. Global aquaculture feed industries are heavily dependent on fish meal protein because of its balanced amino acid and fatty acid composition and palatability. Recently, its increasing cost, and decreasing availability in the market have stimulated several studies on its partial or complete substitution with alternative protein sources. Plants are widely available and reasonably priced. The use of plant protein sources in aquafeeds should therefore be considered. Much work had been done towards the replacement of fish meal with a number of different plant ingredients, including soybean meal, lupin, peas, rapeseed meal and sunflower. Among plant ingredients soybean meal is currently the most commonly used plant protein source in fish feeds. However, use of soybean meal in fish diet may not be sustainable because firstly it is a good source of protein for human and consequently it competes with human food and secondly, its availability has been affected by discontinuous supply because of change in global climate. Therefore, there is an urgent need to identify other protein rich plant resources that could substitute for fish meal and soybean meal in fish and shrimp diets.

     Jatropha curcas L. (physic nut) is a drought-resistant shrub or tree, which is widely distributed in wild or semi-cultivated areas in Central and South America, Africa, India, China and South East Asia. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. Its seeds have been extensively investigated as a source of oil. Jatropha seed kernel contains 55-60% oil that can be transformed into good quality of biodiesel upon transesterification and used as a substitute for diesel. The kernel meal obtained after oil extraction is an excellent source of nutrients and about 60-66% crude protein. The principle of isoelectric precipitation was used to obtain Jatropha protein isolate from Jatropha seed cake. Jatropha protein isolate contains about 81-85% crude protein. The content of essential amino acids (except lysine) is higher in *Jatropha* kernel meal and Jatropha protein isolate than in soybean meal and soy proteins isolate. However the presence of high concentrations of antinutrients like trypsin inhibitor, lectin and phytate and the major toxic components phorbol esters restricts their use in aquafeed. Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for

detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction and destruction of phorbol esters using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

Jatropha platyphylla is a multipurpose and drought-resistant shrub, belonging to the family *Euphorbiaceae*. It is available in Mexico where it is known as "sangregrado" and its seeds are rich in oil and protein. *Jatropha platyphylla* kernel meal (JPKM) obtained after oil extraction contains 70-75% crude-protein. The kernel meal is free of phorbol esters, the main toxins present in other Jatropha species. However, it contains phytate and trypsin-inhibitor. The levels of essential amino acids (except lysine) are higher in JPKM than in soybean meal. The kernel meal was heat treated to remove heat labile antinutrient such as trypsin inhibitor.

Main aim of the present thesis was to use the detoxified *Jatropha curcas* kernel meal (DJKM) and detoxified protein isolates (DJPI), and heated *Jatropha platyphylla* kernel meal (H-JPKM) as protein source in fish and shrimp diets.

On feeding different levels (50% and 75% replacement of fish meal protein) of DJKM and soybean meal to common carp for eight weeks and comparing their performance with a group fed on fish meal protein, it was observed that 50% fish meal protein can be replaced by DJKM without compromising growth performance and health of fish. This study also revealed that DJKM is a good protein source and better than soybean meal for a common carp diet. A long term (16 weeks) feeding trial with common carp demonstrated that DJKM can replace 62.5% fish meal protein with no adverse effects on growth and health.

Routine metabolic rate, energy expenditure per g protein fed, energy expenditure per g protein retained in the body and each component of the energy budget (energy expenditure, energy retention, metabolisable energy ingested and apparently unmetabolised energy) did not differ significantly among the plant protein (DJKM, H-JPKM and soybean meal) and fish meal fed groups for common carp and Nile tilapia. This study reveals that fish do not have to spend more energy to utilize plant protein compared to fish meal.

Detoxified Jatropha kernel meal was fed to rainbow trout and H-JPKM was fed to Nile tilapia to replace 50% and 62.5% of fish meal protein. It was observed in rainbow trout that DJKM could replace 50% of the fish meal protein without compromising growth performance or nutrient utilization compared to a control diet but in Nile tilapia H-JPKM could replace fish meal protein at both the 50% and 62.5% levels.

In another study in which 25% and 50% of fish meal protein were replaced by DJKM in white leg shrimp, higher body mass gain was observed in DJKM fed groups than in the control group. However, lower feed conversion ratio was observed in DJKM fed groups compared with the control group, while protein efficiency ratio exhibited an opposite trend. In another study, it was found that 75% of fish meal protein can be replaced by DJPI in common carp diets without compromising growth performance and nutrient utilization parameters.

In terms of biochemical and haematological parameters, it was observed that increase in plant protein (DJKM, H-JPKM and DJPI) content in fish diets at levels given above led to increases in red blood cells count, haemoglobin and hematocrit content, and decreased cholesterol and triglyceride concentrations in plasma when compared to a control. However, metabolic enzymes such as alkaline phosphatase and alanine transaminase activities, and total bilirubin, urea nitrogen and creatinine concentration in blood were within normal ranges, suggesting that the DJKM, H-JPKM and DJPI based diets did not have any harmful effect on liver or kidney function.

The complexity of natural phytates in plant ingredients are complex and their functional properties are dependent on their structures, so phytates were isolated from Jatropha kernel meal to evaluate their effects. This approach resulted in the attribution of the observed effects exclusively to Jatropha phytate. The effects of addition of phytase were also investigated, to confirm that the effects obtained on addition of the isolated phytate are solely due to Jatropha phytate, because the addition of phytase should counteract the effects of isolated phytate if that is the case. The study using phytase was also conducted to evaluate whether the phytase units added in the diet were sufficient for the phytate levels investigated or not. The phytate rich fraction was isolated from defatted kernel meal using organic solvents (acetone and carbon tetrachloride). It had 66% phytate and 22% crude protein. The supplementation of partially purified Jatropha phytate in the fish diet produced negative effects on growth performance, nutrient utilization and digestive physiology in Nile Tilapia. Inclusion of Jatropha-phytate in the fish diet also adversely influenced the biochemical changes such as metabolic enzymes and electrolytes/metabolites. The salient changes included decreased red blood cell count and hematocrit content, decreased cholesterol and triglyceride concentrations in plasma and decreased blood glucose levels, when compared with the control. The adverse changes observed may be due to the interaction of phytate with minerals and enzymes in the gastro intestinal tract, resulting in poor bioavailability of

minerals. Phytate concentrations used in the present study (1.5 and 3.0%) correspond to 16.5% and 33.0% of DJKM in the fish diet. It was concluded that DJKM at levels > 16.5% in the diet would be detrimental to Nile tilapia. Addition of phytase (1500 FTU/kg) to the phytate containing diets would mitigate the adverse effects of at least 3% Jatropha phytate (or 33% DJKM) in the diet. Thus, efficient utilization of phytate containing DJKM in animal/fish diets would involve supplementation of the diet with exogenous enzyme phytase.

   Over all these studies demonstrate that DJKM, H-JPKM and DJPI can replace 50%, 62.5% and 75% fish meal protein respectively without affecting growth and health of fish. This work enlarges the portfolio of plant protein sources that can be used in fish and shrimp feeding, and opens new market opportunities for the use of a new feed resource. Further studies with DJKM, H-JPKM and DJPI based diets on a larger scale and under commercial pond conditions are suggested.

2. Zusammenfassung

Seit 1970 wächst der Aquakultursektor mit einer durchschnittlichen jährlichen Wachstumsrate von 8,8% rapide an im Vergleich zum Fischfang mit nur 1,2% und fleischproduzierenden Betrieben mit nur 2,8%. Diese hohe Wachstumsrate ist auf die erhöhte Nachfrage für Fisch als Nahrungsmittel zurück zu führen. Weltweit hängt die Aquakultur Futterindustrie auf Grund des ausgewogenen Aminosäure- und Fettsäuregehalts und der Schmackhaftigkeit sehr stark von Fischmehlprotein ab. Dessen steigende Kosten und sinkende Verfügbarkeit auf dem Markt hat in den letzten Jahren zu vielen Studien über teilweisen oder kompletten Ersatz von Fischmehl im Futter durch alternative Proteinquellen geführt. Pflanzen sind überall verfügbar und preisgünstiger. Deshalb sollte die Nutzung von pflanzlichen Proteinen in der Aquakultur ins Auge gefasst werden. Es wurden bereits viele Arbeiten durchgeführt, um Fischmehl durch verschiedene Pflanzeninhalte zu ersetzen, z.B. über Sojamehl, Lupine, Erbsen, Rapssamenmehl und Sonnenblumen. Unter den pflanzlichen Proteinträgern ist das Sojamehl momentan die meist genutzte Proteinquelle für Fischfutter. Allerdings erweist sich Sojamehl im Fischfutter nicht als nachhaltig. Erstens, weil es mit der menschlichen Nahrung konkurriert und zweitens weil dessen Verfügbarkeit durch Veränderungen des globalen Klimas unzuverlässlich geworden ist. Aus diesen Gründen besteht ein dringender Bedarf andere proteinreiche Pflanzenprodukte zu identifizieren, welche Fischmehl und Sojamehl im Fischfutter ersetzen können.

Jatropha curcas L. (Purgiernuss) ist ein dürreresistenter Busch oder Baum, welcher in Zentral und Südamerika, Afrika, Indien, China und Südostasien weit verbreitet ist. Es handelt sich um eine robuste Pflanze, welche auf degradierten Böden gedeiht und geringe Anforderungen an Nährstoffe und Wasser hat. Ihre Samen wurden ausgiebig als Ölquelle untersucht. Jatropha Samenkerne enthalten 55-60% Öl, welches durch Umesterung in Kraftstoff guter Qualität umgewandelt werden kann und als Biodiesel Verwendung findet. Das Kernmehl, welches nach der Extraktion gewonnen wird ist eine exzellente Nährstoffquelle und enthält ca. 60-66% Rohprotein. Um das Jatropha Proteinisolat aus dem Jatropha Samenkuchen zu erhalten wurde das Prinzip der isoelektrischen Ausfällung angewandt. Jatropha Proteinisolat enthält ca. 81-85% Rohprotein. Der Gehalt an essentiellen Aminosäuren (außer Lysin) ist im Jatropha Kernmehl und im Jatropha Proteinisolat höher als im Sojamehl und Sojaproteinisolat. Allerdings wird die Verwendung als Aquafutter durch die hohe

Konzentration von Antinutritiven, wie Trypsin Inhibitor, Lektin, Phytase und die größten toxischen Bestandteile, den Phorbolestern, eingeschränkt. Hitzelabile Antinutritive, wie Proteaseinhibitoren und Lektine sind relativ einfach durch feuchte Erhitzung zu inaktivieren. Eine Methode das Jatropha Kernmehl zu detoxifizieren wurde in unserem Labor entwickelt. Diese basiert auf der Extraktion und Zerstörung der Phorbolester unter Anwendung von organischen Lösungsmitteln und der Inaktivierung von Trypsin und Lektin durch Wärmebehandlung.

Jatropha platyphylla ist ein Dürre resistenter Busch mit vielen Anwendungsmöglichkeiten, der zur Familie der *Euphorbiaceaen* zählt. Die Pflanze gedeiht in Mexiko, wo sie als „sangregrado" bekannt ist. Ihre Samen sind reich an Öl und Protein. *J. platyphylla* Kernmehl (JPKM), welches aus der Extraktion gewonnen wird, enthält 70-75% Rohprotein.

Das Kernmehl ist frei von Phorbolestern und Lektinen, den am meisten vorkommenden giftigen Substanzen in anderen Jatropha Arten. Allerdings enthält es Phytase und Trypsininhibitoren. Der Gehalt an essentiellen Aminosäuren (außer Lysin) ist im JPKM höher als im Sojamehl. Das Kernmehl wurde wärmebehandelt, um hitzelabile Antinutritive, wie Trypsininhibitoren zu entfernen.

Das Hauptziel der vorliegenden Thesis war es, das entgiftete *Jatropha curcas* Kernmehl (DJKM) und die entgifteten Proteinisolate (DJPI), sowie auch das wärmebehandelte *Jatropha platyphylla* Kernmehl (H-JPKM) als Proteinquelle für Fisch- und Garnelendiäten zu verwenden.

Bei Fütterung von verschiedenen Zusammensetzungen (50% und 75% Ersetz von Fischmehl Protein) von DJKM und Sojamehl an Karpfen für acht Wochen wurde im Vergleich zu einer Gruppe, die mit Fischmehl gefüttert wurde festgestellt, dass 50% Fischmehl Protein durch DJKM ohne Beeinträchtigung von Wachstumsrate und Gesundheit der Fische ersetzt werden kann. Diese Studie zeigte ebenfalls, dass DJKM eine gute Proteinquelle darstellt und im Vergleich für Karpfenfutter besser ist, als Sojamehl. Ein Langzeit Fütterungsexperiment (16 Wochen) mit Karpfen demonstrierte, dass 62,5% des Fischmehls mit DJKM ohne negative Einflüsse auf Wachstum und Gesundheit ersetzt werden können.

Der Grundumsatz, Energieaufwand pro g gefüttertes Protein, Energieverbrauch pro g Proteinansatzund jede Komponente der Energiebilanz (Energieaufwand, Energieansatz, umsetzbare Energie und scheinbar ungenutzte Energie) unterschieden sich nicht signifikant zwischen den verschiedenen mit pflanzlichen Proteinen (DJKM, H-JPKM und Sojamehl) und Fischmehl gefütterten Gruppen bei Karpfen und Niltilapien.

Diese Studie zeigt, dass Fische nicht mehr Energie verbrauchen müssen, um pflanzliches Protein zu nutzen.

Detoxifiziertes Jatrophamehl wurde an Regenbogenforellen und H-JPKM an Niltilapien verfüttert, um 50% und 62,5% des Fischmehls zu ersetzen. Es konnte beobachtet werden, dass DJKM 50% des Fischmehl Proteins ersetzen kann, ohne das Wachstum oder die Nährstoffnutzung im Vergleich zur Kontrolle zu beeinflussen. Im Fall von Niltilapien konnte Fischmehl zu 50% und 62,5% durch H-JPKM ersetzt werden.

In einer weiteren Studie, in der 25% und 50% des Fischmehl Proteins durch DJKM im Futter von pazifischen Riesengarnelen ersetzt wurden, wurde ein höherer Zuwachs an Körpermasse in den mit DJKM gefütterten Gruppen im Vergleich zur Kontrolle festgestellt. Allerdings konnte auch eine niedrigere Futterverwertungsrate in den mit DJKM gefütterten Gruppen festgestellt werden, während sich die Proteinverwertung in die andere Richtung bewegte. In einer weiteren Studie wurde gezeigt, dass 75% des Fischmehls mit DJPI in Karpfendiäten ersetzt werden kann, ohne das Wachstum und die Nährstoffverwertung zu beeinflussen.

Bezüglich der biochemischen und hämatologischen Parameter wurde beobachtet, dass der erhöhte Gehalt an pflanzlichen Proteinen (DJKM, H-JPKM und DJPI) in Fischdiäten mit den o.g. Größen die Anzahl der roten Blutkörperchen sowie den Hämoglobingehalt und Hämatokrit erhöht und die Cholesterin- und Triglyzerin-Konzentrationen im Plasma im Vergleich zur Kontrolle verringert. Allerdings bewegten sich die Aktivitäten von metabolischen Enzymen, wie alkalische Phosphatase und Alanin-Aminotransferase, sowie gesamtes Bilirubin, Harnstoff-Stickstoff- und Kreatinin-Konzentrationen innerhalb der normalen Bandbreite, was vermuten lässt, dass die Diäten mit DJKM, H-JPKM und DJPI keinen nachteiligen Einfluss auf die Leber- und Nierenfunktionen haben.

Die Komplexität von natürlichen Phytasen in pflanzlichen Inhaltsstoffen ist vielschichtig und deren Funktionalität ist von ihren Strukturen abhängig. Deshalb wurden Phytasen aus dem Jatrophamehl isoliert, um deren Einfluss zu beurteilen. Dieser Ansatz beweist, dass die beobachteten Ergebnisse einzig in Jatropha-Phytinsäure beobachtet werden konnten. Der Einfluß von Phytase wurde ebenfalls untersucht. Dies geschah um zu bestätigen, dass die durch die Zugabe von isolierter Phytinsäure erzielten Effekte, vollkommen der Jatropha-Phytinsäure zugeordnet werden können, da die Zugabe von Phytase dem Effekt der isolierten Phytinsäure in diesem Fall gegensteuern würde.

Ein weiteres Ziel der Studie war es, zu unteruchen ob die dem Futter beigesetzten Phytasegehalte ausreichen würden um den negativen Effekte bei gegebenem Phyinsäuregehalt gegenzusteuern. Die Phytinsäurereichen Fraktionen wurden aus dem entfetteten Kernmehl mit organischen Lösungsmitteln isoliert (Azeton, Tetrachlormethan) und enthielten 66% Phytinsäure und 22% Rohprotein. Die Supplementierung von teilweise gereinigter Jatropha Phytinsäure im Fischfutter wirkte sich negativ auf das Wachstum, die Nährstoffnutzung und Verdauung in Niltilapien aus. Zusatz von Jatropha-Phytinsäure im Fischfutter beeinflusste die biochemischen Prozesse, wie metabolische Enzyme und Elektrolyten/Metaboliten negativ. Die typischen Veränderungen bezogen sich auf verringerte Anzahl der roten Blutkörperchen und Hämatokrit, niedrigere Cholesterin- und Triglycerid-Konzentrationen im Plasma und niedrigerer Blutzuckerspiegel, im Vergleich zur Kontrolle. Diese negativen Effekte könnten mit der Interaktion von Phytinsäure mit Mineralien und Enzymen im Verdauungstrakt zusammenhängen, was zu einer schlechten biologischen Verfügbarkeit von Mineralien führt. Die in der vorgelegten Studie verwendeten Phytinsäure-Konzentrationen (1.5 und 3.0%) entsprechen zu 16.5% und 33.0% denen von DJKM im Fischfutter. Das Fazit hieraus ist, dass die Zugabe von DJKM > 16.5% im Fischfutter für Niltilapien abträglich wäre. Die Zugabe von Phytase (1500 FTU/kg) zu den Diäten die Phytate enthalten würde diese negativen Effekte negieren. Um eine effiziente Nutzung von DJKM in Fisch-/Tierfutter, welches Phytinsäure enthält, zu gewährleisten muss das Futter mit dem exogener Phytase supplementiert werden.

Zusammenfassend demonstrieren diese Studien, dass DJKM, H-JPKM und DJPI Fischmehl zu 50%, 62,5% und 75% ersetzen kann ohne das Wachstum und die Gesundheit der Fische zu beeinträchtigen. Diese Arbeit erweitert das Portfolio von pflanzlichen Proteinquellen, die zur Fütterung von Fischen und Garnelen verwendet werden können und öffnet neue Vermarktungsmöglichkeiten für eine neue Futterressource. Weitere Studien mit DJKM, H-JPKM und DJPI basierenden Diäten auf einer größeren Ebene und unter kommerziellen Teichkonditionen sollten durchgeführt werden.

Thesis structure: This thesis is presented in 15 chapters as shown below
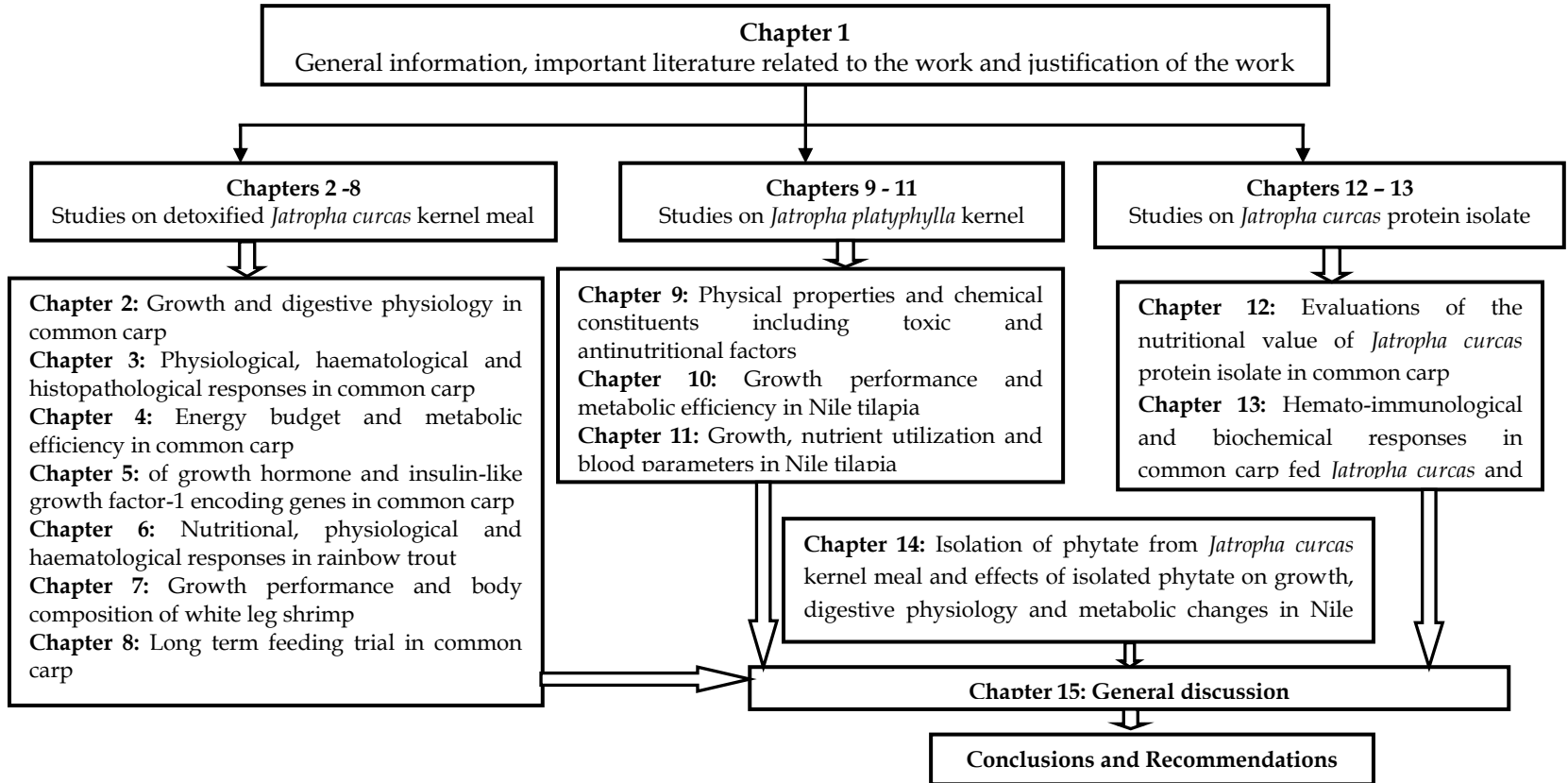
Figure 1: Structure of the thesis

Chapter 1

Section 1

General Introduction

1.1 Global Overview of Aquaculture

Conventionally, the oceans were considered limitless and thought to harbour enough fish to feed an ever-increasing human population. However, the demands of a growing population, particularly in developing countries, now far outstrip the sustainable yield of the sea (Tidwell and Allan, 2001; Agbo, 2008). Global capture fisheries production especially marine fisheries resources are being exploited to their maximum or beyond the level of sustainability. Simultaneously, the worldwide demand for fish is increasing so it is becoming doubtful whether the global demand for fish products can be met in the future (ICTSD, 2003; Agbo, 2008). Aquaculture is seen as the best solution to bridge the gap between demand for food fish and supply.

Total fish production has been increasing for the last 60 years and in 2008, the total production (from capture fisheries and aquaculture) was about 142 million tonnes (mt) (Table 1 and Figure 2) out of which 115 mt was used as human food (FAO, 2010). The reported global production of food fish from aquaculture, including finfishes, crustaceans, molluscs and other aquatic animals for human consumption reached 52.5 mt (46% of total food fish supply) in 2008 (FAO, 2010). The aquaculture production supplied the rest of the world with 26.7% of its food fish, up from 4.8% in 1970 (FAO, 2010). In 2007, fish accounted for 15.7% of the global population's intake of animal protein and 6.1% of all protein consumed (FAO, 2010). Globally, fish serves as a source of protein for more than 1.5 to 3.0 billion people with almost 15 to 20% of their average per capita intake of animal protein (FAO, 2010).

The contribution of aquaculture towards the total production of capture fisheries and aquaculture continued to grow, rising from 34.5% in 2006 to 36.9% in 2008 (FAO, 2010). In the period 1970–2008, the production of food fish from aquaculture increased at an average annual rate of 8.3%, while the world population grew at an average of 1.6%/year (FAO, 2010). The worldwide aquaculture development on the one hand and the expansion in global population on the other resulted in a more than tenfold increase in average annual per capita supply of fish food for human consumption from 0.7 kg in 1970 to 7.8 kg in 2008, at an average rate of 6.6 % per year (FAO, 2010).

Table 1: World fisheries and aquaculture production and utilization (Source: FAO, 2010)

	2004	2005	2006	2007	2008	2009
Production (Million tonnes)						
Inland						
Capture	8.6	9.4	9.8	10.0	10.2	10.1
Aquaculture	25.2	26.8	28.7	30.7	32.9	35.0
Total inland	33.8	36.2	38-5	40.6	43.1	45.1
Marine						
Capture	83.8	82.7	80.0	79.9	79.5	79.9
Aquaculture	16.7	17.5	18.6	19.2	19.7	20.1
Total marine	100.5	100.1	98.6	99.2	99.2	100.0
Total capture	92.4	92.1	89.7	89.9	89.7	90.0
Total aquaculture	41.9	44.3	47.4	49.9	52.5	55.1
Total World fisheries	134.3	136.4	137.1	139.8	142.3	145.1
Utilization						
Human consumption	104.4	107.3	110.7	112.7	115.1	117.8
Non-food uses	29.8	29.1	26.3	27.1	27.2	27.3
Population (billions)	6.4	6.5	6.6	6.7	6.8	6.8
Per capita food fish supply (kg)	16.2	16.5	16.8	16.9	17.1	17.2
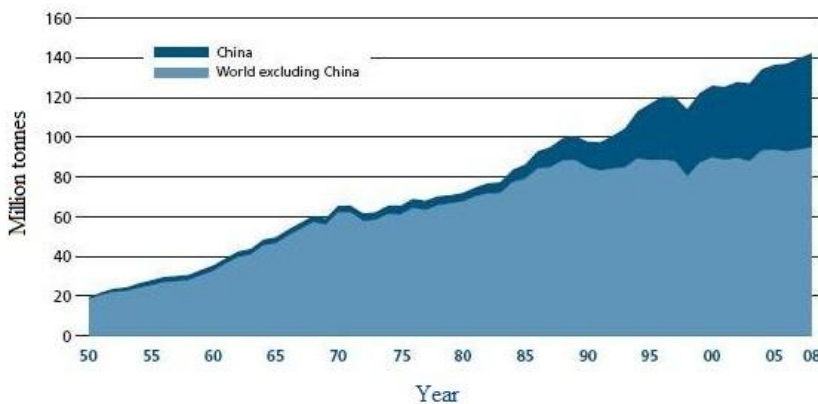
Figure 2: World capture fisheries and aquaculture production (FAO, 2010)

However, the growth patterns in aquaculture production are not uniform among the regions, as presented in Figure 3 (FAO, 2010). Latin America and the Caribbean shows the highest average annual growth (21.1%), followed by the Near East (14.1%) and Africa (12.6%) (Figure 3). China's aquaculture production has increased at an average annual growth rate of 10.4% in the period 1970–2008. Since 2000, the average annual growth of fish production in Europe and North America has slowed substantially to 1.7% and 1.2% respectively (Figure 3, FAO, 2010). The leading countries (e.g., France, Japan and Spain) in aquaculture development have shown decreasing production tends in the last decade (FAO, 2010).
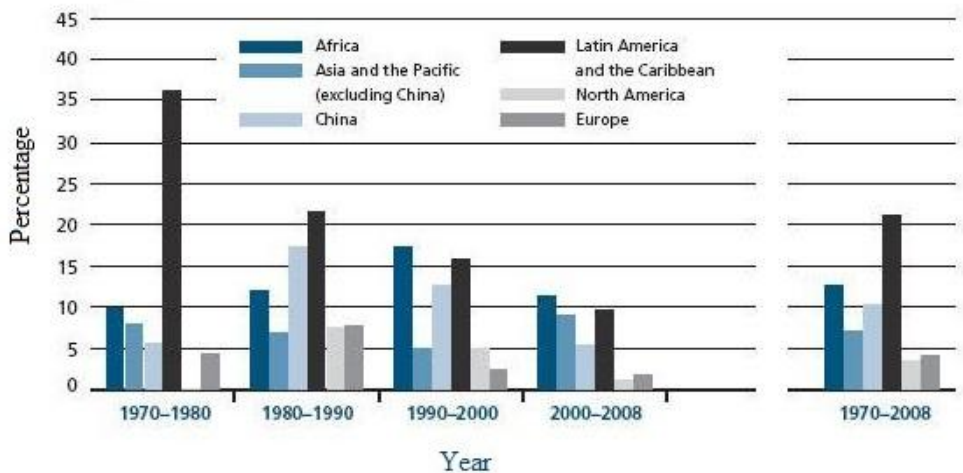
Figure 3: World aquaculture production: annual growth by region since 1970 (FAO, 2010)

The freshwater fishes continued to dominate with a production of 28.8 mt (54.7%) valued at 40.5 billion US$ (41.2%), followed by molluscs (13.1 mt), crustaceans (5 mt), diadromous fishes (3.3 mt), marine fishes (1.8 mt) and other aquatic animals (0.6 mt) in 2008 (Figure 4, FAO, 2010). The production of freshwater fishes was dominated by carps (*Cyprinidae*, 20.4 mt, or 71.1%). A small portion (2.4%) of freshwater fishes was cultured in brackish water, including tilapia farmed in Egypt in 2008. In 2008, the largest producer of all carps was China (70.7%) followed by India (15.7%) and rest 10.2% of all carps were produced by Bangladesh, Myanmar, Viet Nam, Indonesia and Pakistan (FAO, 2010).

A major determinant of successful growth and intensification of aquaculture production depends on aquafeed. It accounts for a major part

(30-75%) of the total operational cost of an average fish farm (Rumsey, 1993; El-Sayed, 2004; Agbo, 2008).

1.2 Protein source in aquafeed

In general, fish meal has been the major protein source in aquafeeds for many reasons such as high protein content, rich source of essential amino acids, high nutrient and energy digestibility, lack of antinutrients, essential fatty acids, energy and minerals. It is also highly palatable and digestible to most freshwater and marine fishes (Watanabe et al. 1997; Glencross et al., 2007). Fish meal production has remained relatively stable averaging 6.07 mt over the past two decades (Tacon et al., 2006) so it is unlikely that it will continue to be able to meet the demands of an expanding aquafeed industry. Thus the main challenges for the aquafeed industry are to reduce the inclusion rate of fishmeal in aquafeeds and to identify economically viable and environmentally friendly alternatives (Gatlin et al., 2007; Agbo, 2008). To be a viable alternative, a candidate ingredient must possess certain characteristics including wide availability and a competitive price. It must also be easy to handle ship and store. Several materials have been tested as alternative protein sources, such as animal by-products, single cell proteins including micro algae, bacterial single cell protein and yeast (El-Sayed, 1994; Perera et al., 1995; Mazurkiewicz, 2009), and plant proteins (Guillaume and Métailler, 2001; Hasan et al., 1997). Plants are widely available and reasonably priced; the use of plant protein sources in aquafeeds should be considered. There are a wide variety of plant protein ingredients that could be promising candidates for the replacement of fish meal as a protein source in fish feeds. These include oilseeds (soybean, Jatropha, cottonseed and canola), legumes (lupin and pea), cereal grains (corn and wheat) and protein concentrate (soy, canola and lupin). The comparative proximate and essential amino acid compositions of different aquafeed ingredients are summarized in the Table 2.
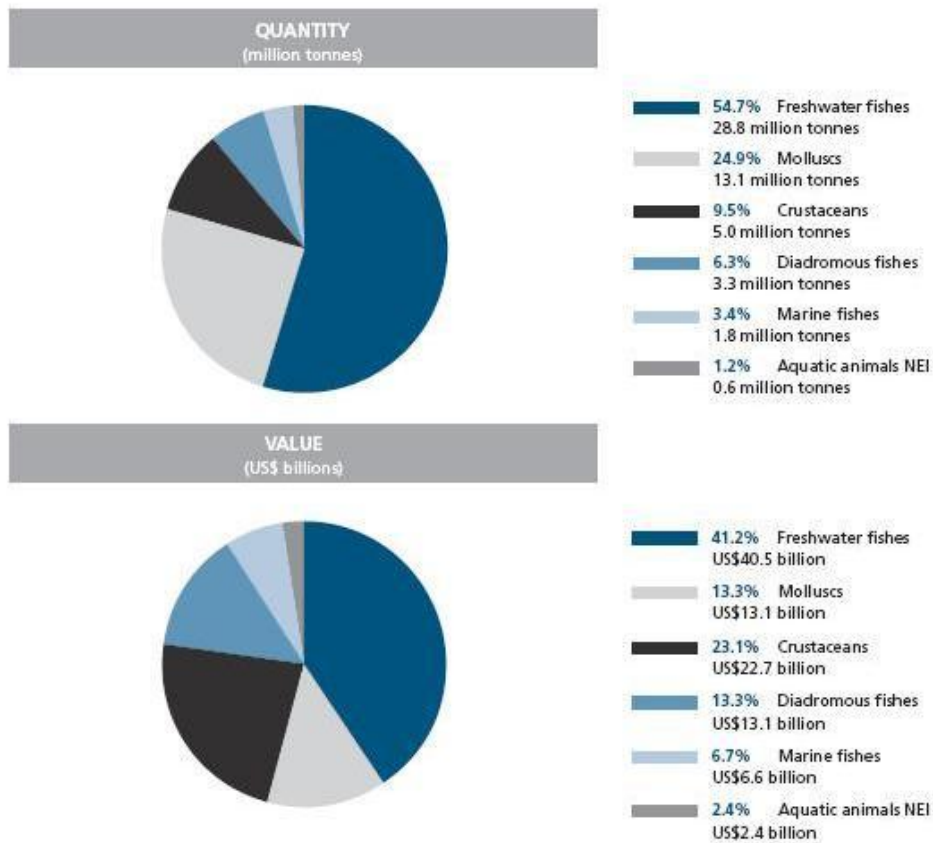
Figure 4: World aquaculture production: major species groups in 2008 (FAO, 2010)

The quality of the protein in plant protein ingredients depends largely on how the ingredients are processed. Ground whole seeds like full-fat soybean meal contain relatively low quality protein while the quality of protein in concentrated protein products, like soy, canola, lupin and Jatropha protein concentrate is generally much higher. It is likely that a combination of plant-derived feed ingredients will be required to replace fish meal. Supplements (amino acids and flavourings) and exogenous enzymes (phytase and cellulase) will also be needed to produce aquafeeds that minimize the content of fish meal whilst supporting the growth rates necessary for economic production of farmed fish.

Table 2: Typical composition of commonly used aquaculture feed ingredients (Source: Miller and Young, 1977; Nwokolo, 1987; NRC, 1982; 1998; Glencross et al.; 2007; Makkar et al., 2008; Makkar and Becker, 2009).

Ingredients	Proximate composition (g kg^{-1} on dry matter)					Essential amino acids (g kg^{-1} on dry matter)								
	Dry matter	Crude protein	Total lipid	Ash	Gross energy*	Arginine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenylalanine	Threonine	Valine
Fish meal[1]	917	770	68	142	21.3	43	25	28	55	46	21	29	32	34
Fish meal[2]	920	720	84	104	21.6	38	19	25	46	43	18	23	26	32
SE soybean meal	909	518	47	69	19.6	42	14	23	44	28	9	27	24	24
SE Canola meal	962	431	22	86	19.6	32	26	3	25	41	30	27	16	78
Ex canola meal	898	381	136	66	23.1	39	28	3	28	46	37	29	18	66
Yellow lupin	903	547	87	44	20.9	61	15	20	45	23	4	21	20	19
NL lupin	885	415	53	33	20.4	47	10	15	29	14	3	16	16	14
Peanut meal	928	481	13	58	20.3	67	16	19	32	20	5	29	14	20
Sunflower meal	930	422	29	76	20.7	36	11	17	26	12	7	20	13	23
Corn gluten meal	900	602	18	21	21.1	19	13	25	102	10	14	38	21	28
Cotton seed meal	900	414	18	64	20.6	45	12	13	25	17	7	22	14	18
Pea meal	928	252	13	38	16.4	19	11	14	41	27	6	19	17	14
Rapeseed meal	900	385	39	67	20.8	21	11	14	24	20	8	16	15	17
Wheat gluten	937	856	13	9	21.1	43	21	43	69	16	17	49	24	43
Wheat meal	941	145	16	14	18.7	6	3	4	9	3	7	2	4	5
DJKM	945	665	11	137	18.3	70	22	27	47	23	11	30	22	32
PPC	910	738	15	20	20.9	38	17	71	76	58	17	49	43	49
JPI	945	808	97	10	21.3	86	24	34	56	19	39	12	26	59
LPC	942	690	93	31	22.2	78	15	27	51	25	5	28	23	23
SPC	939	590	54	79	20.3	45	15	26	48	28	9	30	25	27
SPI	957	922	10	38	22.0	69	24	36	68	52	43	12	31	37

*MJ/kg; [1]Chilean anchovetta meal; [2]Herring; [3]Lupinus *luteus* (cv.Wodjil) kernel meal. NL lupin, narrow-leaf lupin *Lupinus angustifolius* (mixed cultivars) kernel meal; LPC, *Lupinus angustifolius* (mixed cultivars) protein concentrate; SE, solvent-extracted; Ex, Expeller. DJKM, detoxified Jatropha kernel meal; SPC, soybean protein concentrate; SPI, Soy protein isolate; JPI, Jatropha protein isolate; PPC, potato protein concentrate and LPC, Lupin protein concentrate.

1.3 Limitations to the utilization of plant ingredients

High levels of inclusion of plant protein in fish diets have frequently been reported to result in reduced growth due to poor palatability, high crude fibre, reduced digestibility of nutrients and energy, imbalance of essential amino acids and the presence of antinutritional factors (Francis et al., 2001).

1.3.1 Palatability and/or acceptability of plant derived feed stuffs

It is important in feed development to ascertain the acceptability of the feed ingredients, particularly as the texture and palatability or taste could change as increasing levels of especially plant ingredients are incorporated (Ogunji, 2004; Agbo, 2008). According to Houlihan et al. (2001) palatability of feed is a major factor determining feed acceptance because, irrespective of how digestible the nutrients and energy from a particular ingredient are, if the ingredient reduces feed intake, it is of limited use in a feed formulation. Feed palatability has been defined by Glencross et al. (2007) as acceptable to the taste or sufficiently agreeable in flavour to be eaten. Feed acceptance depends upon a variety of chemical, nutritional and physical characteristics, all of which can be influenced by the choice of feed ingredients and processing conditions used in the manufacture of feed (Jobling et al., 2001; Agbo, 2008). The ability of fish to detect and ingest a feed can be affected by its physical property– pellet density (sinking rate), size (shape, diameter and length), colour (contrast), and texture (hardness) and chemical property (composition) of the feed, which largely depends upon the types of ingredients used (Jobling et al., 2001; Agbo, 2008). It is known that many plants contain chemicals (secondary compounds/metabolites) that defend them against attack from herbivores (Hay and Fenical, 1988; Houlihan et al., 2001; Agbo, 2008). Secondary compounds produced by plants include terpenes, polyphenolics, alkaloids, a range of aromatic compounds and amino acid derivatives and some antinutritional factors (Becker and Makkar, 1999). Consequently, utilizing plant-derived feed ingredients for fish feed production may give rise to palatability problems.

1.3.2 Fibre and amino acids content in plant derived feed stuffs

Fibre provides physical bulk to feed and may improve palatability (NRC, 1993; Agbo, 2008). Small amounts of dietary fibre have been reported to improve efficiency of protein utilization in experimental diets and gastric

evacuation time of fish (Buhler and Halver, 1961; Hilton et al., 1983). However, monogastric animals including fish are generally unable to digest fibre because they cannot produce the enzyme cellulase (Bureau et al., 1999; Agbo, 2008). In diets for fish, it is not desirable to have a fibre content exceeding 8-12% because an increase in fibre content would lead to a decrease in the proportion of usable nutrients in the diet. Excessive fibre content could also result in decrease in total dry matter and nutrient digestibility of the diet resulting in poor performance (De Silva and Anderson, 1995; Agbo, 2008). Because fibre is indigestible, it adds to the faecal waste which affects the water quality and hence fish performance (Lovell, 1998; Agbo, 2008).

Amino acid content is also one of the major factors affecting the quality of fish feeds. Amino acids are the basic subunits of proteins. The requirements for amino acids in animals are well defined in various sets of recommendations such as those of NRC (1993). Amino acids requirements vary depending on the species and age of animals (Agbo, 2008). According to De Silva and Anderson (1995) the essential amino acids profiles of plant ingredients used for feed formulation are usually poor, implying they are deficient of one or more essential amino acids, compared to the requirements of the fish. Generally, plant ingredients are deficient of lysine and methionine therefore; plant protein based diets should be supplemented with lysine and methionine. Amino acid compositions of different plant feed ingredients are presented in Table 2.

1.3.3 Antinutrients in plant derived feed stuffs

Antinutrients have been defined as substances which by themselves, or through their metabolic products in living systems, interfere with food utilisation and affect the health and production of animals (Makkar, 1993). They could be broadly divided into four groups (Francis et al., 2001; Agbo, 2008):

I.   Factors affecting protein utilisation and digestion, such as protease (trypsin) inhibitors, tannins and lectins
II. Factors affecting mineral utilisation, which include phytates, gossypol pigments, oxalates and glucosinolates
III. Antivitamins
IV. Miscellaneous: Mycotoxins, mimosine, cyanogens, nitrate, alkaloids, photosensitizing agents, phytooestrogens and saponins.

Classifications of major anti-nutritional factors commonly present in plant-derived feedstuffs used in aquafeeds are summarized in Table 3. Dietary

effects of purified antinutrients in different fish and shrimp species are presented in Table 4. Usually, presence of antinutrients in the aquafeed adversely affects the nutrient utilization that lead to lower growth performance of fish and shrimp (Francis et al., 2001).

Table 3: Classification of major anti-nutritional factors commonly present in plant-derived feedstuffs used in aquafeed (Francis et al., 2001).

Antinutritional factors	Plant derived nutrient source	Means of alleviation
Interaction with protein nutrition		
Protease inhibitors	Soybean, Jatropha kernel meal, Rapeseed meal, Lupin seed meal, Pea seed meal, Sunflower oil cake, Alfalfa leaf meal, Sesame meal	Heat, autoclaving
Heamagglutinins (lectins)	soybean, Jatropah kernel meal, Pea seed meal	Heat, autoclaving
Saponins	Peas, alfalfa, Jatropah kernel meal, Lupin seed meal, Pea seed meal, Sunflower oil cake, Alfalfa leaf meal	
Polyphenols (Tannins)	Tannins, sorghum, Jatropah kernel meal, Pea seed meal, Mustard oil cake	
Chlorogenic compounds	Supplementary methionine or choline	
Interaction with mineral availability		
Phytic acid	Soybean, Jatropah kernel meal, Pea seed meal, Cottonseed meal, Sesame meal,	Supplementation, use of phytase
Oxalic acid	leaf proteins	Heat treatment
Glucosinolates	Rapeseed, Mustard oil cake	Genetic improvement of plants with low content
Gossypol	Cottonseed meal	Genetic improvement of plants
Interaction with Vitamin availability		
Vit A (lipoxygenase)	Soybean,	Heat treatment
Vit D	Soybean	Autoclaving
Vit E (oxidase)	Kidney beans	Autoclaving, addition of Vit E
Anti-nicotinic acid	(niacinogen) corn	

Table 3 continued

Anti-pyridoxine	Linseed meal	Water extraction, heating
Anti-vitamin B12	Raw soybean	Heat treatment
Cyanogens	Cassava, sorghum, Pea seed meal,	Heat treatment
Mimosine	Leucaena leaf meal	-
Arginase inhibitor	Sunflower oil cake	-
cyclopropenoic acid	Cottonseed meal	-
antivitamins,	Alfalfa leaf meal, Cottonseed meal, Pea seed meal, Soybean meal	-
Alkaloids	Lupin seed meal	-
Phytoestrogens	Soybean meal, Lupin seed meal,	-
Allergens	Soybean meal	-

Much research has been done on the replacement of fish meal with different plant ingredients, including soybean meal, lupin, peas, rapeseed meal; mustard, sesame and groundnut oil cakes, and sunflower meal (Kaushik et al., 1995; Hasan et al., 1997; Vielma et al., 2002). Currently, soybean meal is the most commonly used plant protein source in aquafeed (Yue and Zhou, 2009). However, soybean meal competes with human food and hence there is a need to identify other protein rich plant resources that could be used in aquafeed. The non-edible oil seed meal such as jatropha, karanja, simarouba and jojoba could potentially replace the soybean meal and/or fish meal protein source. Among them, Jatropha stands out as the most promising crop due to its wide climatic adaptability and oil rich seeds which could be used for quality biodiesel production.

Table 4: Dietary effects of purified antinutritients in different fish and shrimp species.

Nature of antinutrients	Fish/shrimp species	Antinutritients inclusion	Effects	References
Gossypol acetate	Fingerling trout	0.025, 0.1 or 0.2%	At the first two levels, fish accepted the feeds well but accumulated gossypol in the vascular tissues; complete suppression of feed intake at 2000 ppm level	Roehm et al. (1967)
Gossypol acetate	Rainbow trout	0.1%	Growth depression and high mortality; thickening of the glomerular basement membrane and fatty degeneration of the liver	Herman (1970)
Gossypol acetate	Tilapia	0.1 or 0.2%	No significant differences in growth or feed conversion	Robinson et al. (1984)
Sterculic acid	Rainbow trout	0.01 or 0.02%	Growth inhibition; alterations in liver fatty acid composition and abnormal glycogen deposition in liver	Roehm et al. (1970)
Phytoestrogens-formononetin, daidezin, genistein and equol	Yearling Siberian sturgeon	0.05 to 0.5 mg per g body mass	Except formononetin, all induced hepatic synthesis of vitellogenin	Pelissero et al. (1991)
Soybean protease inhibitors	Rainbow trout	0.37, 0.74, 1.11or 1.48%	Increased trypsin inhibition with increased inclusion; this was partly compensated by increased enzyme secretion and absorption by the intestine; the compensation was complete at lower levels of the inhibitor	Krogdahl et al. (1994)
Quillaja bark saponin	Chinook salmon	0.15 or 0.30%	Growth depression only at the higher level; abnormal intestinal morphology at both levels	Bureau et al. (1998)
Quillaja bark saponin	Rainbow trout	0.15 or 0.30%	Growth depression only at the higher level; abnormal intestinal morphology at both levels	Bureau et al. (1998)
Purified alcohol extract	Chinook	1 or 0.3%	Suppression of feed intake and growth in both cases	Bureau et al. (1998)

	salmon			
of soybean meal (PAES) or soy protein isolate (SPI) active principle being soy saponins	salmon			
PAES (1) or SPI (2)., active principle being soy saponins	Rainbow trout	1 or 0.3%	Depression in growth and moderate intestinal damage when fed 1; No significant effect when fed 2 (only 2 weeks duration).	Bureau et al. (1998)
Soy saponin (97% soy saponin B)	Channel catfish	2600 mg per kg	No effects on feed intake, growth performance and nutrient utilization.	Twibell and Wilson (2004)
Phorbol esters	Common carp	3.75 to 1000 ppm	Feed rejection, faecal mucus production, significant suppression in growth starting from 31 ppm onwards	Becker and Makkar (1998)
Tannic acid (hydrolysable tannin) and quebracho tannin (condensed tannin)	Common carp	2%	Condensed tannin did not affect the performance of fish but hydrolysable tannin had adverse (condensed tannin) effects after 28 days when it completely suppressed feeding	Becker and Makkar (1999)
Calcium phytate and Sodium phytate	Rainbow trout	0.5%	Inclusion of calcium and sodium phytate in diet leads to depression in growth and feed conversion efficiency	Spinelli et al. (1983)
Sodium phytate	Juvenile Chinook salmon	0.16, 0.65 and 2.58%	2.58% group showed depressed growth, feed and protein conversion and thyroid function; while, other not. Other treatments exhibit similar performance as control.	Richardson et al. (1985)
Sodium phytate	Blue tilapia	1.5%	Weight gain was generally improved; however, feed efficiency was not affected.	McClain et al.(1988)
Sodium phytate	Channel catfish	1.1 and 2.2%	2.2% phytate significantly reduced weight gain and feed efficiency compared with that with $\leq$1.1% phytate in diet	Satoh et al. (1989)

Table 4 continued

Sodium phytate	Channel Catfish	0.5 and 1.5%	No adverse effect on weight gain and feed efficiency	Gatlin and Phillips (1989)
Sodium phytate	*Penaeus japonicas* and *Penaeus vannamei*	0.75, 0.94, 1.19 and 1.44%	No effect on growth rate or survival rate of *P. Japonicas* even at 1.44%. In contrast, growth rate of *P. Vannamei* was strongly depressed by the presence of even 0.75% phytate. Feed conversion was markedly increased in both species when sodium phytate added at $\geq$ 0.75%.	Civera and Guillaume (1989)
Sodium phytate	Common carp	0.5 and 1.0%	Depression in growth, feed utilization and protein digestibility; effect was exacerbated by simultaneous increases in dietary calcium and magnesium levels. Intestinal epithelium showed abnormalities.	Hossain and Jauncey (1993)
Sodium phytate	Mrigal	0.5, 1.0, 1.5, 2.0 and 2.5%	Growth performance and nutrient utilization were significantly reduced at > 1% levels.	Usmani and Jafri (2002)
Sodium phytate	Atlantic salmon	1%	No significant effect on feed intake or weight gain; significantly reduced protein digestibility although there was no reduction in trypsin activity.	Sajjadi and Carter (2004)
Sodium phytate	Atlantic salmon	0.1, 0.21, 0.47, 1.0 and 2.07 %	Reduction in feed intake, growth, feed conversion efficiency, nutrient digestibilities and nutrient retentions at 2.07% level only.	Denstadli et al. (2006)
Sodium phytate	Japanese Flounder	0.51, 1.04, 1.35, and 2.06%	Reduction in growth performances and nutrient utilization at ≥1.35% level.	Laining et al., (2010)
Jatropha phytate	Nile tilapia	1.5 and 3.0%	Growth performance, nutrient utilization and digestive enzymes activities were significantly reduced at $\geq$ 1.5% levels.	Unpublished data

1.4 Jatropha

1.4.1 Botanical and agroclimatic description

The genus Jatropha belongs to the tribe Joannesieae of Crotonoideae in the Euphorbiaceae family (well known for its toxicity) and contains approximately 175 known species. Jatropha species for which the toxicity has been widely studied are *Jatropha curcas, Jatropha elliptica, Jatropha glauca, Jatropha gossypifolea, Jatropha aceroides, Jatropha tanoresisi, Jatropha macarantha, Jatropha integerrima, Jatropha glandulifera, Jatropha podagrica and Jatropha multifida* (Makkar and Becker, 2009; Devappa et al., 2010a,b, 2011). Among those species, *Jatropha curcas* (toxic genotype) are the most studied resulted from its oil (as a source of biofuel) and byproducts/coproducts utilization (Makkar and Becker, 2009). A non-toxic genotype of *J. curcas* has been also recorded, which is available only in Mexico (Makkar and Becker, 2009). *J. curcas* (toxic genotype) is found in the parts of tropical America (central and southern regions), and many tropical/sub-tropical parts of Africa and Asia, specifically India and China. It is believed that the species were introduced into other regions from the Caribbean, where it was used during the Mayan period (Schmook and Seralta-Peraza, 1997; Gaur, 2009), by sailors on Portuguese ships travelling via the Cape Verde islands and Guinea Bissau (Heller, 1996). Presently the name of *Jatropha curcas* is *J. curcas* Linnaeus (Euphorbiaceae). Linnaeus (Linnaeus, 1753) was the first to name the physic nut *J. curcas* L. The genus name Jatropha derives from the Greek word jatr´os (doctor) and troph´e (food), which implies its medicinal uses (Kumar and Sharma, 2008). Table 5 presents vernacular names of *J. curcas*.

Table 5 Common/vernacular names of *Jatropha curcas* (Source: Schultze-Motel, 1986; Münch, 1986; Divakara et al., 2010).

Language/country	Common name
Angola	Mupuluka
Arab	Dand barrî, habel meluk
Brazil	Mundubi-assu
Chinese	Yu-lu-tzu
Costa Rica	Coquillo, template
Côte d'Ivoire	Bagani
Dutch	Purgeernoot
English	Purging nut
French	Pourghère, pignon d'Inde
German	Purgiernuß, Brechnuß

Table 5 continued

Guatemala	Pinón
Hindi (India)	Ratanjyot, bagbherenda, jangli arandi, safed arand, bagaranda
Indonesia	Jarak budeg
Italian	Fagiola d'India
Mexico	Piñoncillo
Nepal	Kadam
Nigeria	Butuje
Peru	Piñol
Philippines	Túbang-bákod, Tuba-tuba
Portuguese	Turgueira
Puerto Rico	Tártago
Sanskrit	Kanananaeranda, parvataranda
Senegal	Tabanani
Tanzania	Makaen
Thailand	Sabudam
Togo	Kpoti

The *J. curcas* is monoecious, flowers are unisexual and occasionally hermaphrodite flowers occur, each inflorescence yields a bunch of approximately 10 or more ovoid fruits (Dehgan and Webster, 1979; Kumar and Sharma, 2008). Flowering occurs during the wet season and two flowering peaks are often seen in summer and autumn (Kumar and Sharma, 2008). The seeds of *J. curcas* form within seed pods (Fig. 5) (King et al., 2009). Each seed pod typically contains three seeds (Fig. 5C) (King et al., 2009). The seeds mature about 3–4 months after flowering. The plant can be easily propagated from seeds or cuttings. It will grow under a wide range of rainfall regimes from 250 to over 1200 mm per annum (Katwal and Soni, 2003; Kumar and Sharma, 2008). The trees are deciduous, shedding the leaves in dry season. One major trait associated with the plant is its hardiness and sustainability in warm and arid climates, prefers well-drained alkaline soil (pH 6-9) for its growth (Kumar and Sharma, 2008). The plant does not compete with conventional food crops which make it an ideal choice on those vast areas of land that are underutilized. It is a small perennial tree or large shrub, which normally reaches a height of 3 – 5 m, but can attain 8 - 10 m under favorable conditions (Gaur, 2009). A seed yield of 5 – 8 mt/ha has been reported (Gübitz et al., 1998) and is comparable to a yield of 5.5 mt/ha in commercial soybean production. Description of *Jatropha platyphylla* is presented in *Chapter 9*.
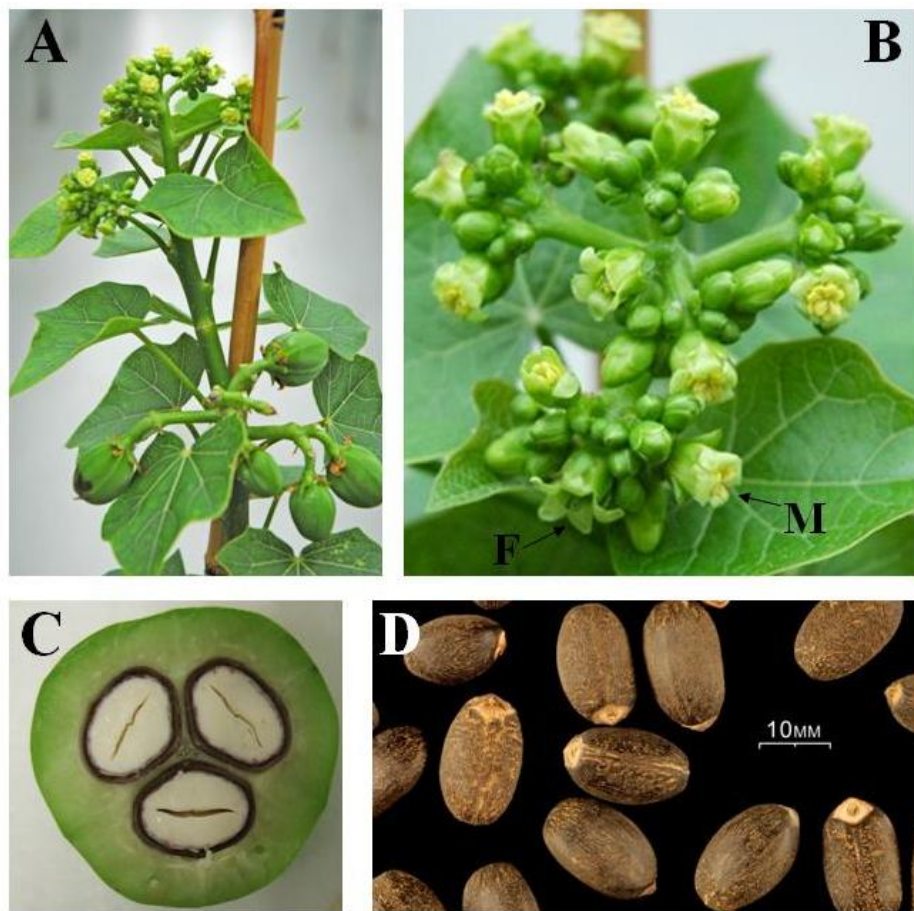
Figure 5: Images of *J. curcas.* **(A)** Young *J. curcas* plant with both flowers and developing seed pods. **(B)** *J. curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F). **(C)** Cross-section of a *J. curcas* seed pod containing three developing seeds. **(D)** Mature seeds of *J. curcas*. (Source: King et al., 2009).

1.4.2 Applications of Jatropha

The International Jatropha Organization has projected that in 2017 there will be ~ 33 million hectares of land cultivated worldwide with *J. curcas,* producing 160 mt of seeds and 95% of the total production will be in Asia (Siang, 2009). Jatropha seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar et al., 2007; Makkar and Becker, 2009). The cultivation of

Jatropha species as a source of oil for biodiesel production will in turn produce a number of by-products and co-products. The utilization of by-products and co-products may increase the overall value of the Jatropha biodiesel production chain. However, the presence of toxic components limits the utilization of many unprocessed *Jatropha*-based products.

1.4.2.1 Production of Biodiesel

Jatropha curcas seed contain ~ 35% crude oil (Makkar and Becker, 2009; King et al., 2009). The *Jatropha curcas* oil has good feed stock quality for biodiesel production and the produced biodiesel meets the European Union (EN14214) and United States of America standards (ATSM D6751) (Makkar and Becker, 2009; King et al., 2009). Jatropha oil fatty acid composition includes 14–16% palmitate (16:0), 5–8% stearate (18:0), 34-46% oleic acid (18:1), 29-44% linoleic acid (18:2) and a trace of longer chain saturated fatty acids (Foidl et al., 1996; Gubitz et al., 1999; King et al., 2009). The cetane number value is perhaps the most important factor for biodiesel. The actual cetane value of *J. curcas* biodiesel has been determined by a number of groups, and has so far been within the range of 50–57 (Foidl et al., 1996; Kumar et al., 2003; Sarin et al., 2007).

1.4.2.2 Ethnomedicine value

Jatropha can be used for the treatment of a variety of ailments due to its purgative effect. The role of plant products in ethnomedicine mainly includes extracts (organic or aqueous). The latex of the jatropha plant is believed to have an alkaloid (jatrophone) which has anti-carcinogenic properties (Devappa et al., 2011). The leaves and saps of *J. curcas* are used to control parasites (Fagbenro-Beyioku et al., 1998; Devappa et al., 2010b). In Trinidad and Tobago, the leaf extracts of *J. curcas/gossipiifolia* have been used to clean sores (Lans, 2007; Devappa et al., 2010b) and in Tanzania, the leaf extracts are used to treat skin rashes and oral candidiasis (Kisangau et al., 2007; Devappa et al., 2010b). In West Africa, the leaves of *J. curcas* are used to treat fever, mouth infections, jaundice, guinea-worm sores, and joint rheumatism. The roots of the plant are also used as an antidote to treat snake-bite. Several researchers have also isolated and characterized the substances responsible for wound healing and anti-inflammatory effects (Nath and Dutta, 1997; Staubmann et al., 1997; Kumar and Sharma, 2008). Seed oil from jatropha has also been used to treat eczema and skin diseases (Heller, 1996; Kumar and

Sharma, 2008). The root decoction is used as a mouthwash for bleeding gums, toothache, eczema, ringworm, and scabies and to treat dysentery and venereal diseases like gonorrhoea (Irvin, 1961; Oliver-Bever, 1986; Devappa et al., 2010b). However, many researchers caution the application of these extracts as a medicine prior to successful scientific research.

1.4.2.3 Phytochemicals and its agro-pharmaceutical importance

Jatropha is a source of phytochemicals with varying biological activities both *in vitro* and *in vivo*. The plant contains alkaloids, lignans, cyclic peptides and terpenes (Devappa et al., 2010b; 2011). Among the terpenes, diterpenes from various species of Jatropha (~ 68 types) have shown a wide range of biological activities, for example, phorbol esters which produce multiple biological effects. Among these are tumor-promoting, irritant, cytotoxic, anti-inflammatory, antitumor, molluscicidal, insecticidal, and fungicidal activities (Makkar and Becker, 2009; Devappa et al., 2010a,b; 2011).

Phorbol esters (a methanol extract of Jatropha oil) exhibits a strong insecticidal effect against *Busseola fusca* and *Sesamia calamistis* larvae (Makkar et al., 2007; Makkar and Becker, 2009; Devappa et al., 2010b; 2011) and *Lipaphis erysimi* (Solsoloy, 1995; Makkar and Becker, 2009; Devappa et al., 2010b). Phorbol ester also has pesticidal effects against *Sitophilus zeamays* and *Callosobruchus chinesis*, deterring their oviposition on sprayed corn and mungbean seeds (Solsoloy and Solsoloy, 1997; Makkar and Becker, 2009). Extracts from *J. curcas* L. were found to be toxic to snails transmitting *Schistosoma mansoni* and *S. haematobium* (Rug and Ruppel, 2000; Makkar and Becker, 2009). When, compared to aqueous extracts, methanol extracts showed the highest activity against all tested organisms with lethal concentration $(LC)_{100}$ values of 25 ppm for cercariae and the snail *Biomphalaria glabrata*; and 1 ppm for the snails *Bulinus truncatus* and *B. natalensis* (Makkar and Becker, 2009). Phorbol esters at a level of 1 ppm in water also killed all snails of the *Physa* species, which are also known to be intermediary hosts of schistosomes responsible for causing the deadly disease schistosomiasis (Makkar and Becker, 2009). Phorbol esters, when extracted from oil, could have applications as biopesticides in organic as well as conventional agriculture. In addition, they could also be used to control diseases such as schistosomiasis (Makkar et al., 2007; Goel et al., 2007; Makkar and Becker, 2009).

Antiflammatory compounds such as the flavonoids apigenin and its glycosides vitexin and isovitexin, the sterols stigmasterol, β-D-sitosterol and

its β-D-glucoside (Chhabra et al., 1990; Makkar and Becker, 2009) are known to be present in jatropha leaves. The Jatropha latex has a proteolytic enzyme, curcain, which has been demonstrated to have wound-healing properties (Nath and Dutta, 1997). A novel cyclic octapeptide, curcacycline (Gly-Leu-Leu-Gly-Thr-Val-Leu-Leu), present in Jatropha latex has been shown to inhibit the classical pathway activity of human complement and the proliferation of human T cells (Van den Berg et al., 1995; Makkar and Becker, 2009; Devappa et al., 2010a). Antimicrobial activity has been reported in oil, leaves and roots (Zeng et al., 2004; Eshilokun et al., 2007; Alyelaagbe et al., 2007; Onuh et al., 2008; Makkar and Becker, 2009).

Curcin present in Jatropha seeds and exhibited antitumor and anti-fungal effects (Lin et al., 2003a,b; Wei et al., 2004; Makkar and Becker, 2009). From Jatropha seeds an enzyme (β-1,3-glucanase) was isolated which acts as antifungal effects against *Rhizoctonia solani* Kuha. and *Gibberelle zeae* Schw. (Wei et al., 2005; Makkar and Becker, 2009). A compound (12-deoxyphorbol-13-phenylacetate) synthesized from phorbol ester which acts as anti-HIV by inhibiting the HIV entry into target cells (Wender et al., 2008; Makkar and Becker, 2009).

In addition, functional proteins (e.g., betaine aldehyde dehydrogenase and aquaporins) help the plant in drought resistance (Makkar and Becker, 2009; Devappa et al., 2010a,b, 2011). Pharmaceutically beneficial protein such as curcain exhibit wound healing properties. The plant is also a souuce of several biologically active cyclic peptides for example, mahafacyclin, pohlianin, chevalierin and curcacyclin with antimalarial properties, jatrophidin with antifungal activity and labaditin and biobollien with immunomodulatory effects (Makkar and Becker, 2009; Devappa et al., 2010a,b, 2011).

Jatropha seeds are good sources of phytate. Several beneficial effects of phytate including cancer prevention, hypercholesterolemic effects, reduction in iron-induced oxidative injury and reversal of initiation of colorectal tumorigenesis, and prevention of lipid peroxidation have been reported (Singh et al., 2003; Kumar et al., 2010).

1.4.2.4 Co-products for energy generation and their utilization

Jatropha wood and husks/shells (if available in large amounts) could be used for fuel purpose. The seed shell of Jatropha has 45–47% lignin and has a high energy value (19.5 MJ/kg) (Makkar and Becker, 2009). Related to the gross energy content, 2.1 kg of shells is equivalent to 1 kg of fossil oil. The

husk (dried fruit encapsulate) of Jatropha also has a high energy content (15.6 MJ/kg) (Makkar et al., 1998; Makkar and Becker, 2009) and, hence, both these materials could be used for generating energy through burning (Makkar and Becker, 2009). The portion of the fruit husk is in the range of 37–40%, and the shell portion of the seed is approximately 35% on dry matter basis (Makkar and Becker, 2009). Recently, Jatropha derived biodiesel has also been mixed with jet fuel and used as an aviation fuel (Gaur, 2009). The shells can also be used for making plywood.

1.4.2.5 By-products and its utilization

The oil has been used for decades for the production of high-value soap in Mali and other African countries (Makkar and Becker, 2009). The glycerine obtained (during biodiesel production) as a result of the trans-esterification process is added to soap in some countries. However, the production costs are high and the quality of soap is poor. But in developing countries, soap production has been very conducive where this business generates employment (Makkar and Becker, 2009). Oil from seeds has also been used as lubricant and as leather softener.

The pressed cake obtained after oil extraction represents a large amount of biomass potential. Jatropha generates approximately 1000 kg of seed cake per hectare. Currently, the seed cakes are used to produce biogas by fermentation as it produces more energy than cattle dung. Mahanta et al. (2008) also investigated seed cake (solid-state fermentation with *Pseudomonas aeruginosa*) as a substrate for the industrial production of enzymes such as proteases and lipases. Recently, Joshi and Khare (2011) reported that deoiled *J. curcas* seed cake was used as a substrate for the production of xylanase from thermophilic fungus *Scytalidium thermophilum* by solid-state fermentation. Devappa et al. (2009) also found that phorbol esters are degraded in soil, in six to nine days depending on the moisture and temperature of soil. This indicates the nitrogen rich Jatropha seed cake obtained after oil extraction could be used as a fertilizer and also could be applied to fields as mulch (Vyas and Singh, 2007; Sharma et al., 2008; Carels, 2009). The briquettes produced from seed cake could be used for domestic or industrial fuel. One kilogram of briquettes combusts completely in 35 min at 525–780 ^{o}C (Singh et al., 2008; Vyas and Singh, 2007; Carels, 2009).

1.4.3 Comparative physical and chemical characteristics of toxic and nontoxic *Jatropha curcas* genotypes

The seeds of *J. curcas* (toxic and non-toxic genotypes) are elliptical (Figure 5D) (Makkar et al., 2011). The seed, shell and kernel masses are similar for both the genotypes (Table 6). The seed has been reported to be rich in crude protein, lipids, neutral detergent fibers, and ash and these values are similar for the two genotypes (Table 6). The sugar or starch contents and the minerals composition (except sodium) of the toxic and non-toxic genotypes are almost identical is presented in Table 6. Nutritional quality of kernel meal used to assess by the profile of amino acids in proteins. The amino acid composition of the toxic and non-toxic genotypes is almost identical (Table 7). The levels of essential amino acids (except lysine) are similar with the FAO reference protein for a growing child of 2–5 years of age (Makkar and Becker, 2009) (Table 7). The amino acid composition of Jatropha kernel meal meal and soybean meal is similar (except lysine and the sulphur containing amino acids such as cystine and methionine); lysine is lower and the sulphur containing amino acids are higher in the Jatropha kernel meal compared to soybean meal. Essential amino acids content in the Jatropha kernel meals are higher than or similar to those of castor bean meal (Makkar et al., 1998; Makkar and Becker, 2009). Jatropha kernel meal contains low level of non-protein nitrogen (9.0% of the total nitrogen) which indicates that high level (91%) of true protein (Makkar et al., 1998; Makkar and Becker, 2009). When non-toxic genotype Jatropha kernel meal fed to fish and rat exhibited high growth and good protein utilization which suggest that quality of protein in Jatropha kernel meal is good (Makkar and Becker, 1999; Makkar and Becker, 2009).

Table 6: Physical and chemical parameters of *Jatropha curcas* (toxic and non-toxic genotypes) (Source: Makkar and Becker, 2009; Makkar et al., 2010)

	Jatropha curcas	
	Toxic	**Non-toxic**
Seed (g)	0.80 ± 0.08	0.73 ± 0.09
Shell (g)	0.31 ± 0.05	0.26 ± 0.03
Kernel (g)	0.49 ± 0.0.7	0.47 ± 0.07
Proximate composition (g kg^{-1} on dry matter) of kernel		
Crude protein	266 ± 11.2	268 ± 12.4
Oil	574 ± 5.0	575 ± 6.9
Ash	40 ± 6.7	45 ± 5.6
Gross calorific value	37.5 ± 055	37.8 ± 0.44

(MJ/kg)		
Defatted detoxified kernel meal (g per kg on dry matter basis)		
Crude protein	637 ± 11	624 ± 26
Crude lipid	11.4 ± 0.52	12.1 ± 0.41
Gross energy (MJ/kg)	18.7 ± 1.12	18.5 ± 1.04
Crude ash	94 ± 10.1	91 ± 10.4
Neutral detergent fibre	182	180
Total sugar	7.7-10.3	10.2
Starch	9.4-11.2	10.6
Minerals in defatted kernel meal (mg kg^{-1} on dry matter)*		
Boron	14 - 15.0	23.1- 25.6
Calcium	8995 - 9769	6660 - 7077
Copper	48 - 52	40 - 44
Iron	304 - 344	251 - 278
Potassium	19882 - 21064	21381 - 22878
Magnasium	17947- 19452	14432 - 15715
Maganase	69 - 74	53 - 57
Sodium	26652- 27190	219 - 226
Phosphorus	21171 - 22676	17533 - 18815
Zinc	105 - 114	80 - 89

* Unpublished data

Table 7: Amino acid composition (g/16 g nitrogen) of kernel meals of *Jatropha curcas* (toxic and non-toxic genotypes), soybean meal and FAO reference protein (Source: Makkar and Becker, 2009; Makkar et al., 2010)

	Jatropha curcas		Soybean meal	FAO reference protein (2-5 year old children)
Amino acids	Toxic	Non-toxic		
Methionine	1.56-1.91	1.38-1.76	1.32	2.50*
Cystine	1.77-2.24	1.58-1.81	1.38	
Valine	4.35-5.19	3.79-5.30	4.50	3.50
Isoleucine	3.93-4.53	3.08-4.85	4.16	2.80
Leucine	6.55-6.94	5.92-7.50	7.58	6.60
Phenylalanine	4.08-4.34	3.93-4.89	5.16	6.30**
Tyrosine	2.45-2.99	2.62-3.78	3.35	
Histidine	2.81-3.30	2.65-3.08	3.06	1.90
Lysine	3.63-4.28	3.40-3.49	6.18	5.80
Threonine	3.33-3.96	3.15-3.59	3.78	3.40
Tryptophan	1.31	ND	1.36	1.10
Non-essential				

Serine	4.67-4.80	4.59-4.91	5.18	--
Arginine	11.8-12.2	11.4-12.90	7.64	--
Glutamic acid	14.68-16.7	15.91-16.50	19.92	--
Aspartic acid	9.49-11.8	9.92-11.7	14.14	--
Proline	4.13-4.96	3.80-4.21	5.99	--
Glycine	4.40-4.92	4.18-4.61	4.52	--
Alanine	4.36-5.21	4.26-4.94	4.54	--

* Methionine plus cystine; ** Phenylalanine plus tyrosine; ND: not detected

Toxic and non-toxic genotypes jatropha kernel meal (heated; 121 ^{o}C, 66% moisture, 30 min) have similar digestibility and metabolisable energy (used *in vitro* gas method), however these meal have lower digestibility and metabolisable energy compared to soybean meal (Menke et al., 1979; Makkar and Becker, 2009; Table 8). The pepsin plus trypsin digestibility of Jatropha kernel meal protein is similar to heated soybean meal whereas, the *in vitro* rumen digestibility of non-toxic genotype Jatropha kernel meal was lower (~ 50%) compared to soybean meal which revealed that this meal has significant amount of undegradable protein in rumen, that could be used in post-ruminally and these results revealed that Jatropha kernel meal can be used as good quality of protein source in animal nutrition (Makkar and Becker, 2009). Moreover, similar application of Jatropha kernel meal from the toxic genotype could also be expected provided it has been detoxified.

Table 8: Pepsin plus trypsin digestibilities, available lysine, digestible organic matter, metabolisable energy and rumen-degradable nitrogen of heat-treated kernel meal (Source: Makkar and Becker, 2009; Makkar et al., 2010)

	Jatropha curcas		Soybean meal
	Toxic	**Non-toxic**	
Pepsin plus trypsin digestibility (% of total nitrogen)	89	90	91
Available lysine (mg/100 mg sample)	3.10	3.16	-
Available lysine (g/16 g N)*	4.87	5.06	-
Digestible organic matter (%)	78	77.3	87.9
Metabolisable energy (MJ/kg)	10.9	10.7	13.3
24 h *in vitro* rumen-degradable nitrogen (% of total nitrogen)	43.3	28.9	80.9

* Calculated using crude protein content in the sample

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Recently, in our laboratory a new non-toxic variety of Jatropha species (*Jatropha platyphylla*) has been identified. The physiochemical and nutritional properties have been discussed in *Chapter 9*.

1.4.4 Constraints: toxic component and antinutrients in *Jatropha curcas*

Makkar and Becker (1997) reported that the main toxic principle in *J. curcas* seeds, oil, and cake are the diterpene derivatives of a tigliane skeleton classified as phorbol esters. There are two known genotypes of *J. curcas*, toxic and nontoxic variety. A number of antinutrients are present in defatted kernel meal obtained from *J. curcas* genotypes (toxic and nontoxic) and these are listed in Table 9. In this study the antinutrients in defatted kernel meals were studied and are described in the next subsections.

1.4.4.1 Phorbol esters

Phorbol esters are naturally-occurring compounds which are widely distributed in the plant species of the Euphorbiaceae and Thymelaeceae. They are tetracyclic diterpenoids of phorbol type and esters of tigliane diterpenes (Evans, 1986; Devappa et al., 2010b, 2011). Six phorbol esters (Jatropha factors C1–C6) have been characterized from *Jatropha curcas* seed oil (Haas et al., 2002; Devappa et al., 2010b, 2011) and designated as C1 (A), C2 (B), C3 (C), epimers C4 (D) and C5 (E) and C6 (F), with the molecular formula $C_{44}H_{54}O_8Na$ (Mol wt. 733.37) (Figure 6). The phorbol esters are lipophilic, present mainly in oil or kernel and are not affected by heat treatment. The concentration of phorbol esters varies from 1 to 3 mg/g kernel meal and from 2 to 4 mg/g oil in different portions of *J. curcas* (Makkar et al., 1997; Makkar and Becker, 2009; Devappa et al., 2010b, 2011). Table 10 shows the phorbol ester content of different parts of toxic *Jatropha curcas.*

Table 9 Levels of toxic and antinutritional factors in unheated kernel meals of *Jatropha curcas* (toxic and non-toxic genotypes) (Source: Makkar and Becker, 2009)

Component	*Jatropha curcas*	
	Toxic	Non-toxic
Phorbol esters (mg/g kernel)[*]	2.79	ND
Total phenols (% tannic acid equivalent)	0.36	0.22
Tannins (% tannic acid equivalent)	0.04	0.02
Condensed tannins (% leucocyanidin equivalent)	ND	ND
Phytates (% dry matter)	9.40	8.90
Saponins (% diosgenin equivalent)	2.60	3.40
Trypsin inhibitor (mg trypsin inhibited per g sample)	21.3	26.5
Lectin activity (1/mg of meal that produced haemagglutination per ml of assay medium)	51–102	51–102
Glucosinolates	ND	ND
Cyanogens	ND	ND
Amylase inhibitor	ND	ND
Non-starch polysaccharides (NSP) (% in dry matter)		
Rhamnose	0.2	0.2
Fucose	0.1	0.1
Arabinose	2.5	2.7
Xylose	1.2	1.4
Mannose	0.3	0.3
Galactose	1.2	1.2
Glucose	4.7	4.7
Glucuronic acid	0.9	0
Galacturonic acid	2.6	3
Total NSP	12.7	13.6

[*] As phorbol-12-myristate 13-acetate equivalent. ND: Not detected
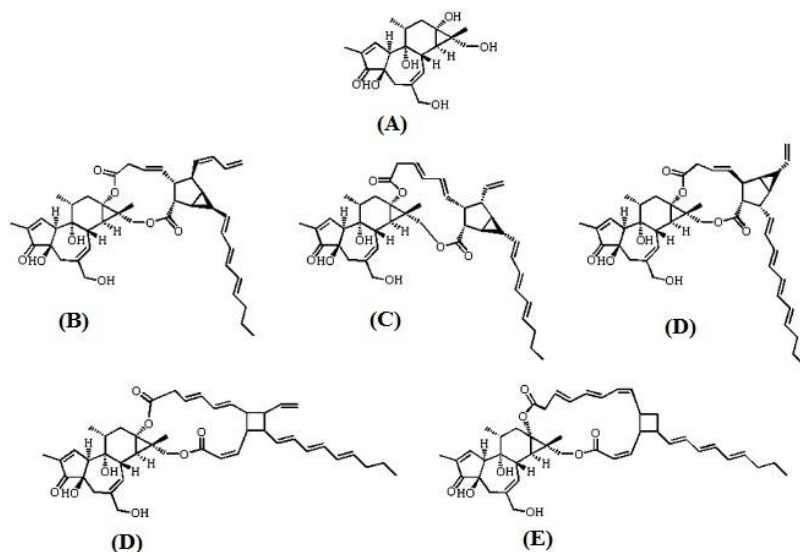
Figure 6: Structures of six phorbol esters (A, B, C, D, E and F) in *J. curcas* (Haas et al., 2002).

Phorbol esters, diterpenes of phorbol type, cause severe toxic symptoms in livestock. The phorbol esters are reported to mimic the action of diacylglycerol, an activator of protein kinase C which regulates different signal transduction pathways (Devappa et al., 2010a,b, 2011). The phorbol esters affects a number of processes including phospholipid and protein synthesis, enzyme activities, DNA synthesis, phosphorylation of proteins, cell differentiation and gene expression. It is considered to be a co-carcinogen and has purgative and skin-irritant effects (Goel et al., 2007; Devappa et al., 2010a,b, 2011).

Table 10: Phorbol esters in different parts of the toxic *Jatropha curcas* plant (Source: Makkar and Becker, 2009)

Parts	Phorbol esters (mg/g dry matter)[§]
Kernel	2.00–6.00
Leaves	1.83–2.75
Stems	0.78–0.99
Flower	1.39–1.83
Buds	1.18–2.10
Roots	0.55
Latex	not detected
Bark (outer brown skin)	0.39
Bark (inner green skin)	3.08
Wood	0.09

[§] As phorbol-12-myristate 13-acetate equivalent

Since last decade many studies have demonstrated that *J. curcas* exhibits toxicity in a broad range of living organism (from microorganisms to higher animals) (Devappa et al., 2010a,b, 2011). Feeding of *Jatropha. curcas* seeds, fruits or leaves caused toxicity depending on the dose and the animal species tested. Raw or defatted seeds when force-fed to fish, chicks, pigs, goat, mice and rats caused severe toxicity symptoms and led to death (Adam, 1974; Adam and Magzoub, 1975; Liberalino et al., 1988; Chivandi et al., 2000; Gadir et al., 2003; Devappa et al., 2011). The oil of *Jatropha curcas* (dosage of 2 g per kg body weight) eacute toxicity by restrains the birth of pups in rats (Odusote et al., 2002; Devappa et al., 2011). The *J. curcas* oil extracts (methanol:water, 9:1) exhibited skin toxicity to rabbit, mice and rats and the common symptoms appeared such as topical application were erythema, edema, necrosis, scaling and thickening of the skins (Gandhi et al., 1995; Devappa et al., 2011). The *Jatropha curcas* fruit extracts (methanol, petroleum ether and dichloromethane) caused pregnancy terminating effect in rats by fetal resorption (Goonesekera et al., 1995; Devappa et al., 2011). The LD_{50} in mice was found to be 27.3 mg per kg of body weight (Li et al., 2010; Devappa et al., 2011). The force-feeding of jatropha meal containing phorbol ester exhibited toxicity in mice, rats, and goats (Goel et al., 2007; Devappa et al., 2011). Purified phorbol esters feeding in fish and mice exhibited toxic effects and the major organs were affected such as intestine, liver, and kidney (Becker and Makkar, 1998; Li et al., 2010). In all of these animal studies, the major organs affected were intestine, liver, and kidney. Animals exhibited diarrhoea and hemorrhagic eyes and an autopsy showed inflammation of the gastro-intestinal tract.

1.4.4.2 Curcin

Curcin (toxalbumin), a plant toxin, belongs to a group of proteins called ribosome-inactivating proteins (RIPs), which inactivate ribosomes and finally halt protein synthesis. Based on physical properties RIP are classified into

I.   Type 1 RIP, which are single-chain (approximately 30 kD) proteins. They have enzymatic activity and inhibit cell-free protein synthesis in vitro, but are relatively nontoxic to cells and animals.

II. Type 2 RIP, which are heterodimeric proteins (approximately 60 kD) consisting of A chain (similar to type 1 RIP) attached to sugar-binding β chain (lectin) by a disulfide bond.

Type 2 RIP is highly toxic compared to type 1 RIP, which are relatively nontoxic *in vivo* due to the absence of the sugar-binding chain (Devappa et al., 2010a,b, 2011). The lectin chain is necessary to bind galactosyl moieties of glycoproteins and/or glycolipids found on the surface of eukaryotic cells and mediates retrograde transport of the A chain to the cytosol (Devappa et al., 2010a,b, 2011). The A chain, having enzyme activity (*N*-glycosidation), removes a specific adenine at A_{4324} in rat 28S rRNA (Endo et al., 1987; Endo and Tsurugi, 1987; Devappa et al., 2010a,b, 2011). In jatropha, purified curcin (Type 1 RIP) inhibited cell-free translation in the reticulocyte lysate system with an inhibitory concentration$_{50}$ (95% confidence limits) of 0.19 (0.11-0.27) nmol/L (Lin et al., 2003a,b; Devappa et al., 2010a,b, 2011). Curcin has cell-free translation inhibitory activity higher than most RIPs, such as saporin (0.5 nmol/L), luffin A (1 nmol/L), luffin B (4 nmol/L), and trichosanthin (0.32 nmol/L) (Barbieri et al., 1993; Devappa et al., 2010a,b, 2011). When raw curcin fluid (200 µg) was fed to fish (Gambusia) then within 99 h leads to death about 94.4% of fish, and the LD_{100} for mice was at 1.6 mg/kg of body weight on subcutaneous injection after 9 days (Hua-mei et al., 2007; Devappa et al., 2010a). In snail and schistosome larva the *J. curcas* seed extract (water extract) exhibited a low poisoning effect. In the water extract solutions, snail heart beating stopped at 48 h and schistosome larva stopped moving within 2 h. The LD_{50} values were 1 and 5 g/kg, respectively, could be associated with saponin and curcin (Hua-mei et al., 2007; Devappa et al., 2010a). Curcin (5 µg/ml), withdrawn hyphal growth and spore formation in the bacteria *Pyriclarimoryzae* Cav., *P. funerea*, and *Sclerotinia sclerotiorum* (Lib.) de bary, thus revealing the potential of being a biological bacteriocide and pesticide(Hua-mei et al., 2007; Devappa et al., 2010a).

1.4.4.3 Trypsin Inhibitors

Trypsin inhibitors are crystalline globular proteins that reduce the activity of trypsin and chymotrypsin which are pancreatic enzymes involving in protein digestion (Liener and Kakade, 1980; Hertrampf and Piedad-Pascual, 2000; Agbo, 2008). Trypsin inhibitors have a wide distribution in plants and are present in most legume seeds and cereals (Norton, 1991; Agbo, 2008). Trypsin inhibitors form irreversible complexes with trypsin inhibiting its activity (Liener and Kakade, 1980; Agbo, 2008). The level of trypsin inhibitor activity in the *J. curcas* kernel meal (toxic and non-toxic genotypes) and raw soybean meal was found to be similar, ranging from 18.4 to 27.3 mg trypsin inhibited per g (Makkar et al., 1997; Makkar and Becker, 2009). Common

carp fed *Jatropha curcas* (non-toxic genotype) heat-treated (45 min, 121ºC, 66% moisture) kernel meal (containing 1.3 mg trypsin inhibited per g) and unheated kernel meal (24.8 mg trypsin inhibited per g) based two diets and observed that no difference in growth performance and nutrient utilization which reveals that common carp are not so sensitive towards trypsin inhibitor (Makkar and Becker, 1999). However, feeding of unheated *J. curcas* kernel meal to monogastrics animals (poultry and pigs) and fish (except common carp) could produce unfavorable effects because of high content of trypsin inhibitors in *J. curcas* kernel meal (almost equal content in raw soybean meal) (Makkar and Becker, 1999). Usually, trypsin inhibitor is prone to heat and could be inactivated by heat treatment. However, black bean and kidney bean trypsin inhibitor (Bowman-Birk inhibitors) is heat stable (Friedman and Brandon, 2001; Devappa et al., 2010a). Trypsin inhibitors in jatropha kernel meal would be completely removed by heat treatment (121 ºC for 30 min) (Aderibigbe et al., 1997; Aregheore et al., 2003; Makkar and Becker, 2009).

1.4.4.4 Lectin

Lectins are carbohydrate-binding proteins (glycoprotein) and are ubiquitous in nature. Lectins possess the ability to bind reversibly and specifically to carbohydrates and glyco-conjugates which is responsible for their numerous physiological effects. For example, lectins bind avidly with intestinal glycoproteins on the epithelial surface and interfere with nutrient absorption. Lectins easily evade digestion and then enter in to the intestine where in they possibly will bind with the epithelium. Some lectins may cause disruption of membrane integrity and the initiation of a cascade of immune and autoimmune events that ultimately lead to cell death. It also interrupt lipid, carbohydrate, and protein metabolism, help enlargement and/or atrophy of internal organs, and amend the biochemical, hormonal and immunological conditions. High consumption of lectins distinctly threatens the growth and health of animals (Vasconcelos and Oliveira, 2004). The *J. curcas* kernel meal contains lectins at levels of 102 and 51 (1/mg of meal that produced hemagglutination per ml of assay medium) for toxic and nontoxic genotyopes respectively, levels that are of similar order of magnitude as in soybean meal (Makkar et al., 2007). The lectin activity of both genotype (toxic and non-toxic) meals has almost identical (Table 9). Jatropha lectins is heat labile and could be inactivated by heat treatment. However, black bean and kidney bean lectin is not prone to heat. Lectins in

the jatropha kernel meal could be completely inactivated by heat treatment (121 ᵒC for 30 min) (Aderibigbe et al., 1997; Aregheore et al., 2003; Makkar and Becker, 2009).

1.4.4.5 Cyanogenic Glycosides, Glucosinolates, and Amylase Inhibitors

These antinutrients were not detected in *J. curcas* and *Jatropha platyphylla* kernel meal or seeds or isolates (Makkar et al., 1997; Makkar and Becker, 2009 Makkar et al., 2011).

1.4.4.6 Saponins

Saponins are steroid or triterpene glycoside compounds present in a variety of plants. In plants, saponins may serve as anti-feedants or help in protecting the plant against microbes and fungi. Saponins often have a bitter taste, and thus, when present in high concentrations reduce plant palatability in livestock and aquafeed (Sen et al., 1998). In general saponins have high toxicity to fish when applied externally (Roy et al., 1990) and preparations of saponin extracts from tea seed cake have been used to eradicate predacious fish in ponds (Chen and Chen, 1997). The majority of work on saponins has concentrated on *Quillaja* saponin (derived from the *Quillaja saponaria* tree) because of its use in vaccine adjuvants (Oda et al., 2003). Bureau et al. (1998) concluded that 1,500 and 3,000 mg of Quallaja saponins/kg diet added to salmonid feed already containing 32% of soy protein concentrate caused significant intestinal damage and higher saponin concentrations depressed growth of chinook salmon and rainbow trout. These results require site-by-site comparison of purified soya saponins and Quillaja saponins, to provide conclusive evidence with diets where no soybean protein concentrate is included, as it may be argued that soybean protein concentrate saponins had an additive negative impact on diet acceptance and utilization by salmonids. Twibell and Wilson (2004) followed this finding with studies on channel catfish and were able to decrease the growth depressing effect of soybean meal (55% of the diet) by adding a cholesterol supplement to the formulation. They also found that up to 2,600 mg of purified soyasaponins (97% of soyasaponin) had no effect on feed intake of channel catfish. Saponins are present in kernel meal of both genotypes of *J. curcas* at levels of between 1.8 and 3.4% (as diosgenin equivalent); however, these saponins did not possess haemolytic activity. In addition, the levels of saponins present in toxic and nontoxic varieties of *J. curcas* are almost similar (Makkar and

Becker, 2009). These observations suggest that *J. curcas* saponins are harmless (Makkar et al., 1997, 1998; Makkar and Becker, 2009).

1.4.4.7 Tannins

Tannins are polyphenols found in various parts of many species of plants. Tannins are phenolic substances associated with toxic and antinutritional effects including reduced food/feed intake and growth, and impaired nutrient absorption (Butler et al., 1986). Tannins possess multiple phenolic hydroxyl groups leading to formation of complexes primarily with proteins and to a lesser extent with metal ions, amino acids, and polysaccharides. *Jatropha curcas* kernel meal contains negligible amounts of total phenols (0.2−0.4%) and tannins (0.02−0.04%). The condensed tannins were not detected in the *Jatropha* kernel meal (Makkar et al., 1998; Makkar and Becker, 2009), and tannin content in the bark was found to be low (outer dark bark, tannins 0.7% and condensed tannins 0.2%; inner green bark, tannins 3.1% and condensed tannins 1.7%; tannins as tannic acid equivalent and condensed tannins as leucocyanidin equivalent) (Makkar and Becker, 2009).

1.4.4.8 Phytates

Phytic acid (known as inositol hexakisphosphate or phytate when in the salt form) is the principle storage form of phosphorus in most plant seeds. The phytate content of *Jatropha curcas* kernel meal from both toxic and nontoxic genotypes is almost identical ranging from 7.2 to 10.1% (Makkar et al., 1997). Phytate is known to decrease the absorption of minerals, particularly calcium, zinc and iron. Therefore, the addition of phytase enzyme should be considered for feeds containing kernel meal from toxic, detoxified and non-toxic Jatropha genotype, to mitigate the adverse effects of phytate. Dietary role of phytate and phytase in fish is discussed in the *next section*.

1.4.4.9 Nonstarch polysachharides

The level of non-starch polysaccharides (NSP) is *Jatropha curcas* of both genotypes are similar (Table 9). The NSP content of soybean meal, rapeseed meal, cottonseed cake, linseed meal, coconut cake, palm cake and sunflower cake are 15.5, 17.8, 16.4, 19.3, 25.0, 36.8, 39.3 and 19.3% respectively (Knudsen, 1997)**.** The level of NSP in Jatropha meal is slightly higher than

that in soybean meal and lower than in other conventional protein-rich feed resources. They do not appear to have any adverse effects on common carp (*Cyprinus carpio*) or Nile tilapia when 75% of fishmeal protein in the diet is replaced by heated meal from the non-toxic genotype of Jatropha. The growth of both these species of fish was as good as for the fish fed a 100% fishmeal protein diet. Non-starch Polysaccharides and their role in fish nutrition are described in the *next section.*

For the effective utilization of kernel meal the removal of antinutrients and toxic principles is necessary. Antinutrients such as trypsin inhibitors and lectin can be deactivated by autoclaving and the adverse the effects of phytate can be mitigated by supplementation of phytase enzymes. However, the main toxic compounds are the phorbol esters which are heat stable and for which there are no readily available deactvating enzymes. Other strategies must therefore be applied for their removal.

1.5 Different approaches studied for detoxification of *Jatropha curcas*

In the past two decades, several approaches (chemical and organic solvents) were tried for detoxifying defatted cake/kernel meal and oil but without success. A few studies aimed at detoxifying Jatropha oil and meals by reducing phorbol ester concentration have been reported. Haas and Mittelbach (2000) reported that traditional oil refining methods like degumming, de-acidification, bleaching, and deodorization can decrease the phorbol esters content of the seed by about 50% (Haas and Mittelbach, 2000). They concluded that treatment with alkali hydroxides during acidification as well as bleaching with traditional bleaching earth caused the biggest decrease in the amount of phorbol esters in the oil. On the other hand, degumming and deodorization had little effect. Furthermore, Martınez-Herrera et al. (2006) studied the effect of various treatments such as hydrothermal processing techniques, solvent extraction, solvent extraction plus treatment with $NaHCO_3$ and ionizing radiation to inactivate the anti-nutritional factors in Jatropha kernel meal. Trypsin inhibitors were easily inactivated with moist heating at 121 ^{o}C for 25 min while phytate levels were slightly decreased by irradiation at 10 kGy. Saponin contents were reduced by ethanol extraction and irradiation. Extraction with ethanol, followed by treatment with 0.07% $NaHCO_3$ considerably reduced lectin activity. The same treatment also decreased the phorbol ester content by 97.9%. Makkar and Becker (1997) have reported that ethanol (80%) or methanol (92%) [1:5 w/v] reduced both the saponins and phorbol esters by 95% after four

extractions. Chivandi, et al. (2004) reported that laboratory scale petroleum ether extraction reduced the phorbol ester content in the *Jatropha curcas* seeds by 67.69% [6.5 mg/g in the raw kernels to 2.10 mg/g], double solvent extraction followed by moist heat treatment reduced phorbol esters by 70.77% to 1.90 mg/g. Double solvent extraction accompanied with wet extrusion, re-extraction with hexane and moist-heat treatment diminished phorbol ester content to 0.80 mg/g, a decrease of 87.69%. Rakshit and Bhagya (2007) have reported that up to 90% of the phorbol esters can be removed by treating the meal with 20 g/L of calcium hydroxide {Ca(OH)2}. A process is thus needed that efficiently extracts the oil and detoxifies the meal. Gaur (2009) developed a process that obtains high yields of jatropha oil and detoxifies the defatted (oil free) jatropha meal. The principle of solid-liquid extraction was utilized to detoxify the meal. Various organic solvents were used for the extraction. Extraction of ground Jatropha seed kernels in a Soxhlet apparatus involving a sequential combination of hexanes, followed by methanol proved highly efficient in detoxifying the meal. The phorbol ester content was reduced by 99.63% from 6.05 mg/g in untreated meal to about 0.06 mg/g in solvent-treated meal.

Many studies have been done on the detoxification of Jatropha seed meal and its utilization in animal feed. Chivandi et al. (2006); detoxified defatted *Jatropha curcas* meal (JCM) using 95% ethanol at 35°C to remove most of the highly lipo-soluble phorbol esters in the kernels. The ethanol-extracted meal was heated with pressurised steam at 90°C for 30 minutes to distil off the ethanol after which the meal was sun-dried and the ethanol recovered by condensation. The re-extracted meal was autoclaved at 121°C for 30 minutes to inactivate the heat labile antinutrients. This "detoxified" JCM was then fed to pigs for 8 weeks. Haematological and biochemical parameters were measured and it was concluded that dietary detoxified JCM had severe adverse effects on pigs. This indicates that the detoxification procedure had failed to completely remove and or neutralise the toxic anti-nutritional factors in the JCM. Some of the toxicity observed could be ascribed to the residual phorbol esters in the JCM. In the study of Belewu et al. (2010), autoclaved (121 °C, 15psi for 30 min) *Jatropha curcas* kernel cake was treated with the fungi (*Aspergillus niger* and *Trichoderma longibrachiatum)* and fed to West African dwarf goats for 70 days. The phorbol ester content was not reported either in the treated or untreated *Jatropha curcas* kernel cake. The growth and nutrient utilization was lower in *Jatropha curcas* fed groups compared to the controls which implied that the *Jatropha curcas* cake was not detoxified and could not be used as a component of animal feed.

The solid state fermentation (SSF) of seed cake using white-rot fungi, *Bjerkandera adusta and Phlebia rufa* decreased phorbol ester content by 91 and 97% respectively under optimized laboratory conditions (28 °C for 30 days) (de Barros et al., 2011). Similarly SSF using *Pseudomonas aeruginosa* PseA strain decreased phorbol esters to undetectable level within nine days under optimized conditions (30 °C, pH 7.0 and relative humidity 65%) (Joshi et al., 2011).

Most of the above reported detoxifications procedures were either unsuccessful, resource intensive or economically not viable. Therefore, we have developed a new method in our laboratory to detoxify Jatropha kernel meal and protein isolate (Makkar and Becker, 2010). This detoxification method is based on extraction of phorbol esters using organic solvents (alkaline methanol) and inactivation of trypsin inhibitors and lectin by heat treatment.

1.6 Aims

The aim of the present work was to elucidate the potential for safe use of Jatropha derived feed ingredients [detoxified Jatropha kernel meal (DJKM), heated *Jatropha platyphylla* kernel meal (H-JPKM) and detoxified Jatropha protein isolate (DJPI)] in commercially important finfish (common carp, *Cyprinus carpio* L.; rainbow trout, *Onchorynchus mykiss*; Nile tilapia, *Oreochromis niloticus* L.) and white leg shrimp (*Litopenaeus vannamei*) species.

The specific objectives were:
- To study the effects on growth, feed and nutrient utilisation including retention of fat and protein of increasing dietary levels of DJKM and soybean meal in common carp, rainbow trout and white leg shrimp.
- To study the effect on macronutrient digestibility and digestive enzyme activities on increasing dietary levels of DJKM and soybean meal in common carp and rainbow trout.
- To study the effects on general indicators of fish health (physiological, biochemical and haematological parameters), and gut and liver health (histological parameters) on increasing the dietary levels of DJKM and soybean meal in common carp and rainbow trout.
- To study the effects on energy budget and metabolic efficiency in fish, when DJKM meal and soybean meal in common carp.

- To study the effects on the expression of growth hormone and insulin-like growth factor-1 encoding genes on increasing dietary levels of DJKM and soybean meal.
- To study the physical properties and chemical constituents including toxic and antinutritional factors of *Jatropha platyphylla* seed.
- To study the effects on growth, nutrient utilisation, haematology and energy budget, on replacing fish meal protein with H-JPKM and soybean meal in Nile tilapia.
- To study the nutritive value of *Jatropha curcas* protein isolate vis-a-vis soy protein isolate in common carp.
- To isolate phytate from *Jatropha curcas* kernel meal and study its effects on growth and health of Nile tilapia.

Chapter 1

Section 2

Phytate and phytase in fish nutrition: A Review

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Phytate and phytase in fish nutrition: A Review

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Abstract

Phytate formed during maturation of plant seeds and grains is a common constituent of plant-derived fish feed. Phytate-bound P is not available to gastric or agastric fish. A major concern about the presence of phytate in the aquafeed is its negative effect on growth performance, nutrient and energy utilization, and mineral uptake. Bound phytate-P, can be effectively converted to available-P by phytase. During the last decade, phytase has been used by aqua feed industries to enhance the growth performance, nutrient utilization and bioavailability of macro and micro minerals in fish and also to reduce the P pollution into the aquatic environment. Phytase activity is highly dependent on the pH of the fish gut. Unlike mammals, fish are either gastric or agastric, and hence, the action of dietary phytase varies from species to species. In comparison to poultry and swine production, the use of phytase in fish feed is still in an unproven stage. This review discusses effects of phytate on fish, dephytinisation processes, phytase and pathway for phytate degradation, phytase production systems, mode of phytase application, bioefficacy of phytase, effects of phytase on growth performance, nutrient utilization and aquatic environment pollution, and optimum dosage of phytase in fish diets.

Introduction

In nature phytate is present in all seeds and possibly all cells of plants. It serves as a store of cations, of high-energy phosphoryl groups and is a naturally occurring compound formed during maturation of plant seeds and grains. A large body of evidence show that minerals are less available from feed of plant origin than from animal-based feed. Aquaculture is one of the fastest growing food sectors (FAO, 2008). The aquaculture feed industry relies on the fishmeal, which is the most preferred protein source for fish feed owing to excellent amino acid and fatty acid profile. Limited supply, high cost and stagnant production level restrict its use for sustainable farming (Baruah et al., 2004; New and Wijkstrom, 2002). The replacement of fishmeal with extensively available plant or grain by-products is getting increasing attention for the development of low-cost fish feed (Carter and Hauler, 2000; Galtin et al., 2007).

The main source of plant protein in terrestrial and aquatic feed includes soybean meal, corn (gluten), sunflower meal, canola/rapeseed meal, peas, and lupins. Soybean meal represents the highest proportion of plant protein in fish diets owing to high yield, relatively high crude protein content and easy and round the year availability. Nutritionists for many years have investigated the ways to utilise proteins of plants origin, because they are cheaper, readily available, and easily accessible than animal protein sources. However, one of the major constraints that limit use of plant proteins in animal feed is the presence of anti-nutritional factors, phytate being one of them. It is free form of inositol hexakisphosphate (IP6) and a polyanionic molecule with six phosphate groups that can strongly chelate with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts. This adversely affects the absorption and digestion of these minerals in fish (Papatryphon et al., 1999). The natural source of phosphorus (P) in the form of phosphate rocks is not renewable, which may lead to P crisis in future (Mullaney et al., 2000).

Lately there has been continuous pressure on farmer and nutritionist to effectively use more and more plant based phosphorus. From 50 to 80% of the total P content in plant seeds is stored in the form of phytate (Ravindran et al., 1995). P in this form is generally not bioavailable to monogastric animals (humans, dogs, pigs, birds) and also to agastric animals because they lack the intestinal digestive enzyme, phytase, required to separate P from the phytate molecule (Jackson et al., 1996). As a consequence of low digestbility of phytate by fish, most of the phytate-P ends up being excreted

into the water and may cause algal bloom pollution (Baruah et al., 2004). Moreover phytate can also integrate with cation groups on protein, amino acids, starch and lipids in feedstuff reducing the digestibility of these nutrients in fish, poultry and pig.

The ideal approach to maximise the nutritive value of plant-based diet is through hydrolysis of undigestable phytate by use of exogenous phytase enzyme. Feil (2001) mentioned that, because of versatile properties of phytate and phytase, interest is not limited to animal nutrition and but also extends to human nutrition and medical science. Also, interest in this area has been increasing at a fast pace in aquaculture (Debnath et al., 2005b; Gabaudan et al., 2006; Baruah et al., 2007a,b) because of increased need to improve protein utilisation of plant-sourced feed ingredients and to reduce dependence on fishmeal diet.

The potential for commercial exploitation of microbial phytase in fish feed has been reviewed by Cao et al. (2007). This review presents properties of phytase and their comparison from different sources, effects of phytase on P utilization in fish and on aquatic environment pollution, and factors affecting phytase efficacy. In our review, in addition to discussing reports that dealt with aspects reported in Cao et al. (2007) and have emanated during the last three years, a synthesis of information on the effects of phytate, dephytinisation methods, pathway of phytate degradation, phytase production systems and mode of application of phytase and their effects are presented. An attempt has also been made to suggest an optimum dose of phytase based on the available literature of different age groups of fish of different species.

Phytate

Phytic acid is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol (Figure 1). Phytic acid (known as inositol hexakisphosphate (IP6), or phytate when in salt form) is the principal storage form of P in many plant tissues. Inositol penta- (IP5), tetra- (IP4), and triphosphate (IP3) are also called phytates. Molecular formula: $C_6H_{18}O_{24}P_6$ and molecular mass: 660.04 g mol^{-1}.
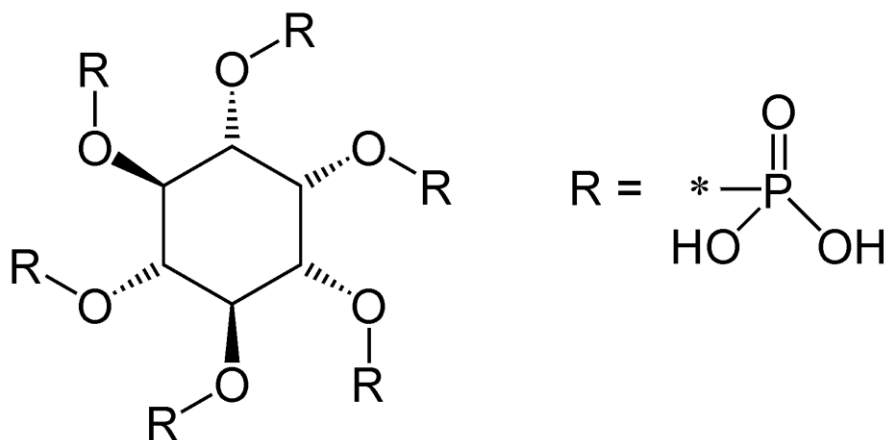
Figure 1 Chemical structure of phytic acid (Kumar et al., 2010)

Phytate is the storage form of P bound to inositol in the fiber of raw whole cereal grains, oil seeds and nuts. In animal nutrition, phytate has been described as 'both an anti-nutritional factor and an indigestible nutrient' (Swick and Ivey, 1992). However, the presence of phytate in human diets may have some positive consequences besides reducing the availability of calcium (Ca), zinc (Zn) and other trace minerals. The propitious concern includes anti-diabetic and anti-carcinogenic effet in humans (Kumar et al., 2010).

Phytate is found in potentially usable plant derived ingredients of fish feed such as soyabean, Jatropha kernel meal from non-toxic genotype and detoxified Jatropha kernel meal from the toxic genotype, rice, wheat, barley, maize, groundnut, sesame and rapeseed (Makkar et al., 1998; Makkar and Becker, 2009). Soybean meal, rapeseed meal, and sesame meal contain from 50 to 80% of total phorphorus in phytate form (Tyagi et al., 1998). The remaining P is represented by soluble inorganic phosphate and cellular P (phosphorus bound in nucleic acids, phosphorylated proteins, phospholipids, phospho-sugar). Phytate isolated from plants belongs to the group of organic phosphates and is a mixture of calcium-magnesium salt of inositol hexaphosphoric acid, called as phytin (Baruah et al., 2005; Debnath et al., 2005b).

Quantitative aspects of phytate

The concentration of phytate and phytase in the feedstuffs varies considerably. Phytate constitute between 0.7 and 2% of most cereal grains and oilseeds (Adeola and Sands, 2003). In general plant-derived fish feed

ingredients such as soybean meal, rapeseed meal, and sesame meal contain 1.0–1.5, 5.0–7.5 and 2.4 % phytate respectively (Francis et al., 2001). It has been estimated that about 14.4 million tonnes of phytate-P is produced annually from worldwide production of seeds and fruits. This amount of P is equivalent to 65% of annual sales of P as fertilisers (Lott et al., 2000). The activity of endogenous phytase is comparatively higher in cereals and cereal by-products than in legume seeds (Eeckout and De Paepe, 1994). Moreover, large variations in phytase activity among feedstuffs have been reported which depend on the genetic and environmental factors (Steiner et al., 2007). The native phytase activity, total P, phytate-P content and P bioavailabilty of some common feedstuffs are given in Table 1. Under physiological conditions in the gut, phytate chelates with positively charged cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}, and thus reduces the bio-availability of these minerals for animal (Erdman, 1979).

Table 1 Total phosphorus, phytate phosphorus and endogenous phytase activity in common feed ingredients used in fish diet (Sources: Selle et al., 2003; Godoy et al., 2005).

Ingredients	Phytase activity (FTU kg^{-1})	Total P (g/kg)	Phytate P (g/kg)	Proportion of phytate-P in total P (%)
Maize	25	2.40	2.05	85.4
Maize gluten	45	5.00	4.20	84
Corn	24	2.50	1.70	73
Gross defatted corn germ and bran	41	6.60	4.2	64
Fine defatted corn germ and bran	56	12.10	7.80	65
Hominy meal	100	7.35	6.65	90.5
Rice bran	129 (70-190)	17.51	15.83	90.2
Rice	112	1.2	0.8	65
Rice broken	20	0.85	0.40	47.1
Rice polishing	134	15.7	11.3	72
Wheat bran	2173 (1700-3090)	10.96	8.36	76.3
Wheat by-products	2173 (1700-3090	8.02	7.00	87.3
Wheat	503 (255-840)	3.08	2.20	74.9
Sorghum	35 (10-125)	2.92	2.41	82.7

Table 1 continued

Barley	348 (130-595)	2.73	1.86	67.3
Oats	38	2.43	2.10	86.4
Oats, dehulled	50	2.25	1.15	51.1
Groundnut meal	-	6.00	4.6	77
Palm oil meal	34	5.10	2.90	57
Soybeans, whole	40	5.55	3.08	55.5
Soybean meal	42 (10-95)	6.66	4.53	68.3
Coconut meal	37	4.30	2.40	56
Cotton seed whole	<10	6.05	4.25	70.2
Cotton seed meal	11 (5-50)	11.36	9.11	80.5
Sunflower meal	<10	9.05	7.48	82.8
Rapeseed meal	-	11.8	7	59
Canola meal	5 (5-35)	8.76	6.69	76.4
Peas	58	3.45	1.67	48.4
Faba beans	-	4.01	2.43	60.0
Lupins				
L. albus, whole	<10	4.47	2.49	55.7
L. angustifolius, whole	<10	3.10	1.60	51.6
L. angustifolius, dehulled	<10	3.80	1.89	49.7

Role of phytate

The effect of phytate on growth depends primarily on its amount in the diet and on the presence or absence of a distinct stomach (Hossain and Jauncey, 1993; Usmani and Jafri, 2002). Inclusion of 0.5 or 1% phytate in purified diets for the agastric common carp (*Cyprinus carpio*) caused a significant reduction in growth and feed efficiency (Hossain and Jauncey, 1993). Specific growth rate of rohu, (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) was significantly decreased when phyate was included > 1% of total diet (Usmani and Jafri, 2002). Channel catfish fed a diet containing 2.2% phyate had significantly reduced weight gain, feed efficiency and Zn content in the vertebrae compared to fish fed a diet containing 1.1% phytate (Satoh et al., 1989). Feeding Atlantic salmon (*Salmo salar*) with high dietary phytate (18 g phytate kg^{-1}) in the form of concentrated soyabean meal led to many folds drops in growth performance, and protein and minerals (P, Ca, Mg and Zn) bioavailability and utilization (Storebakken et al., 1998).

Decline in growth and mineral absorption have been demonstrated in striped bass (*Morone saxatih)* when fed with plant feedstuff containing high phytate (1.31%) (Papatryphon et al., 1999). In contrast, interestingly, Cheng and Guillaume (1984) reported a beneficial effect of 1% phytate (added as sodium phytate) on growth and exoskeleton development in shrimp *Penaeus japonicus*. Similarly, McClain and Gatlin (1988) reported improved growth and reduced Zn bioavailability in blue tilapia *Oreochromis aureus* fed 1.5% phytate. The authors did not explain these unexpected results. Many researchers (Papatryphon et al., 1999; Hossain and Jauncey, 1993) also reported reduction in plasma levels of Ca and Zn but not of Fe, Cu or Mg in common carp when feed supplemented with high phytate ($\geq$ 1%). Decrease in Ca and Zn availability was attributed to higher interaction of phytate with these ions than with Mg, Fe or Cu. Phytate chelate with di- and trivalent mineral ions such as Ca^{2+} (Fredlund et al., 1997), Mg^{2+} (Denstadli et al., 2006), Zn^{2+} (Fredlund et al., 1997), Cu^{2+} and Fe^{3+} preventing their availability for fish growth (Duffus and Duffus, 1991). In presence of phytate and added calcium, absorption of other mineral was depressed due to the formation of insoluble complexes (Sandberg et al., 1993). For example, calcium-bound phytate shows affinity with Zn and forms co-precipitates. The mineral binding strength becomes progressively lower as the solubility increases when phosphate groups are removed from the inositol hexaphosphate. Phytate may reduce reabsorption of endogenous Zn as well as affect availability of dietary Zn in fish and pigs (Hardy, 1998). In a study using rainbow trout (*Oncorhynchus mykiss*) as the test fish, feeds were selected for their low phytate content (Ketola and Harland, 1993) to minimize stream pollution by phosphates. Supplementation of a high phytate diets (2.58%) with low Zn feed (Zn at 0.05 g/kg) depressed chinook salmon growth, feed and protein conversion ratio and thyroid function, increased mortality, promoted cataract formation and induced anomalies in pyloric cecal structure, partly due to diminished Zn bioavailability to fish (Richardson et al., 1985). High dietary phytate ($\geq$ 1% of phytate) with low Zn in diet resulted in hypertrophication, cytoplasmic vacuolation of pyloric caecae, cataract and low zinc content in vertebrae of juvenile Chinook salmon (Francis et al., 2001). A significant depression of Zn levels in carcass of rainbow trout (*Oncorhynchus mykiss*) juvenile was observed when fed with phytate containing rapeseed protein concentrate (inositol phosphate 5 and 6 µmol/g were 19 and 2.5 µmol/g respectively in the diet) (Teskeredzic et al., 1995).

Phytate depresses protein and amino acid digestibility and utilisation efficiency in fish and other higher animals. Interactions of phytate with proteins depend on pH (Cheryan, 1980) as the phytate molecule is polyanionic. In acidic environments such as Nile tilapia stomach (pH 1 to 2), half of the phosphate moiety is negatively charged. This favours binding to soluble proteins at amino-groups on lysine, imidazole groups on histidine and guanidyl groups on arginine. In alkaline environment such as Nile tilapia intestine, (pH 8.5-8.8), ternary complexes are favoured. Both complexes are resistant to proteolytic digestion (Riche and Garling, 2004).

The formation of complex has been described as a bi-phasic reaction (Rajendran and Prakash, 1993). The reaction is characterised by initial rapid step where phytate binds protein via strong electrostatic attractions and changes the conformation of protein. This is followed by slower protein–protein interactions forming aggregation of protein and when the protein–phytate complexes exceed a critical size, these culminate into precipitation. Protein–phytate complex formation between α-globulin and sodium phytate has been shown to be maximal at pH 2.3 and dependant upon phytate to protein ratios (Rajendran and Prakash, 1993). Moreover, the extent of protein–phytate complex formation is probably also governed by various factors including pH, the source and solubility of protein and dietary levels of calcium and magnesium (Kemme et al., 1999). For example protein–phytate complexes have been well documented in wheat (Hill and Tyler, 1954) but are less likely to occur in maize (O'Dell and de Boland, 1976). The tendency of protein to be bound by phytate usually differs owing to the accessibility of basic amino acid residues to phytate (Champagne, 1988). *In vitro* studies have shown that phytate–protein complexes are less likely to be attacked by proteolytic enzymes (Ravindran et al., 1995) and even digestive enzymes like pepsin, amylopsin and amylase are inhibited by phytate. Rainbow trout when fed with purified diets containing 0.5% phytate, suffered reduction in protein digestibility and about 10% reduction in growth and feed conversion (Spinelli et al., 1983). Formation of sparingly digestible phytate–protein complexes was found to be the main reason for growth depression in rainbow trout.

Phytate is a strong chelator and forms complexes with lipid and derivatives along with other nutrients (Vohra and Satyanarayan, 2003). The complex of Ca/Mg–phytate and lipids is referred as 'lipophytins' and is the major constraint for energy utilisation derived from lipid source (Leeson, 1993). Lipophytins may also lead to the formation of metallic soaps in gut lumen of poultry (Leeson 1993). However there is a paucity of evidence

supporting the existence of lipid–phytate complexes in fish. Usmani and Jafri (2002) observed lower fat content in carcass of *Cirrhinus mrigala* when fed with high dietary phytate as compared to dephytated diet.

When phytate reacts with minerals and other nutrients, the formed complexes are insoluble in the upper small intestine (where maximum mineral absorption normally occurs) and it is highly unlikely that they provide absorbable essential elements. Thus, chemical interactions of phytate in the upper gastrointestinal tract are of particular concern. The form in which many minerals occur in foods is largely unknown, as is also the form in which they occur in the gut. Therefore, predicting the specifc interactions of phytate in the gastrointestinal tract and the nutritional implications of these interactions is difficult.

Processing conditions for removal of phytate (dephytinisation)

Removal or degradation of phytate would increase the bioavailability of many cations and the nutritional value of the meal. Several strategies to reduce phytate have, therefore, been considered.

Milling and Cooking

Milling of cereals removed phytate (Bohn et al., 2008), but this treatment also removed a major part of the minerals and dietary fibres and therefore cannot be a solution to the problem. Rosenbaum and Baker (1969) have reported that the cooking processes decreased both water and acid-extractable phytate-P in legumes. Reddy et al. (1981) did not find any breakdown of phytate-P during cooking of black gram seeds and cotyledons. They also observed losses of total P and phytate-P due to leaching into the cooking water. Heat treatments had minor effects on dephytinisation (Pontoppidan et al., 2007). A small effect usually observed using heat treatment is due to leaching of minerals into the boiling water.

Germination

Urbano et al. (2000) reported that during seed germination, phytate is utilized as a source of inorganic phosphate for plant growth and development. The liberation of phosphate from phytate occurs by enzyme hydrolysis. Germination also reduces phytate level in seeds or grains. Disappearance of phytate during germination depends on the native phytase activity and phytase induced during germination. Walker (1974)

observed a rapid rise in phytase activity in bush beans after 2 days of germination. Germination reduced phytate content by 60% and 40% in chickpea and soybean respectively (Chitra et al., 1996; Masud et al., 2007).

Fermentation

Fermentation process reduces phytate content in the fermented product (Lim and Tate, 1973). Part of phytate reduction in this process is due to the action of endogenous phytase and phytase induced through sprouting (Kumar et al., 1978) but exogenous microbial sources may provide additional phytase activity during the fermentation. Cereals and legumes fermentation appreciably reduces phytate content owing to endogenous phytase of cereals and that of added yeast and other useful microorganisms (Hirabayashi et al., 1998). Phytate was reduced by about one-third in soybeans as a result of fermentation with mold, *R. oligosporus*. The decrease in phytate was accompanied by an increase in inorganic P (Sudarmadji and Markakis, 1977). The reduction in phytate was due to the action of the enzyme, phytase produced by mold during fermentation.

Genetic alteration (Transgenesis and Mutation)

Non-lethal mutants of corn (Raboy and Gerbasi, 1996), barley and rice (Raboy, 1997) have been developed that contain only a fraction of the phytate that normal seeds contain, but contain normal levels of total P. As a result, the level of available P to fish is greatly increased (Sugiura et al., 1998a). Thee 'low-phytate' varieties of barley and corn have been used as a component in low polluting feed for fish (Jabeen et al., 2004).

Beside these, pronounced expression of phytase activity in transgenic alfalfa has been illustrated successfully by incorporating exogenous gene encoding for phytase into the genome of alfalfa plants. Thereby enabling its use in livestock, poultry, and fish feeds to eliminate the need for phosphorous supplementation in feed (Austin-phillips et al., 1999). Alfalfa meal is used for herbiovorous and omnivorous fish feed like grass carp and Nile tilapia (Ali et al., 2003).

Moistening, autolysis and other methods

Soaking of plant ingredients in aqueous solutions can remove up to two-thirds of the phytate by the action of endogenous phytase activity; however, it also results in loss of minerals, water-extractable proteins and vitamins

(Hurrell, 2004). It has been shown that phytate hydrolysis during soaking is greatly influenced by temperature and pH (Fredlund et al., 1997; Greiner and Konietzny, 1998, 1999). The optimal temperature range for the intrinsic plant phytases during soaking was found out to be 45 and 65 °C and the optimum pH between 5.0 and 6.0 (Greiner et al., 1998,1999). Chang et al., (1977) reported that at 50 °C and 60 °C, the hydrolysis of phytate from the beans was 31.0% and 49% respectively. Approximately 3 parts of the total phytate were hydrolysed and 1 part diffused into the water during 10 h of incubation of beans at 60 °C. The autolysis of phytate in beans was slow at both 35° and 55 °C. Tabekhia and Luhl (1980) demonstrated decrease in phytate of 7.7, 8.1, 13.2 and 19.1% for black-eyed beans, red kidney beans, mung beans, and pink beans respectively on soaking for 12 h at 24 °C in tap water. Iyer et al. (1980) observed that phytate content of beans (pinto, Great Northern, and red kidney beans) was appreciably reduced (52.7, 69.6, and 51.7% respectively) when soaked in distilled water for 18 h at room temperature.

The most successful dephytinisations so far involve endogenous enzymatic activity during germination, but this is a species dependent phenomenon where some plants are more sensitive to the treatment than others. Wheat, barley and rye all have high phytase activity in the grain (Table 1), whereas maize, millet and sorghum have low initial phytase activity that increases rapidly after germination (Egli et al., 2002). Reducing the level of phytate in plant products through genetic alteration may be more effective than reducing phytate by post-harvest treatments.

Above mentioned processing methods could reduce phytate to a certain extent but may also cause loss of minerals and other nutrients. Taking these constraints of traditional methods into account, addition of exogenous phytase to fish feed appears to be the best way to utilize phytate.

Phytases

Definition

Phytase, chemically known as *myo*inositol (1,2,3,4,5,6)- hexaphosphate phosphohdrolase, catalyses the hydrolysis of phytate rendering P available for absorption. The enzyme sequesters orthophosphate groups from the inositol ring of phytate to produce free inorganic P along with a chain of lower phosphoric esters (inositol pentaphosphate to inositol monophosphate) as intermediates (Baruah et al., 2004; Debnath et al., 2005) (Figure 2). This consequently decreases the chelation capacity of phytate for

different cations. Activity of phytase is expressed as FYT, FTU, PU or U, and all these mean the same. One unit of phytase is defined as the quantity of enzyme that liberates 1 micromol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5.5 and 37°C (Simons et al., 1990). This definition provides a useful measure of phytase activity and represents a simple benchmark measurement under well-defined assay conditions such as pH, temperature, duration, mineral content, agitation, etc.

Figure 2 Action of phytase
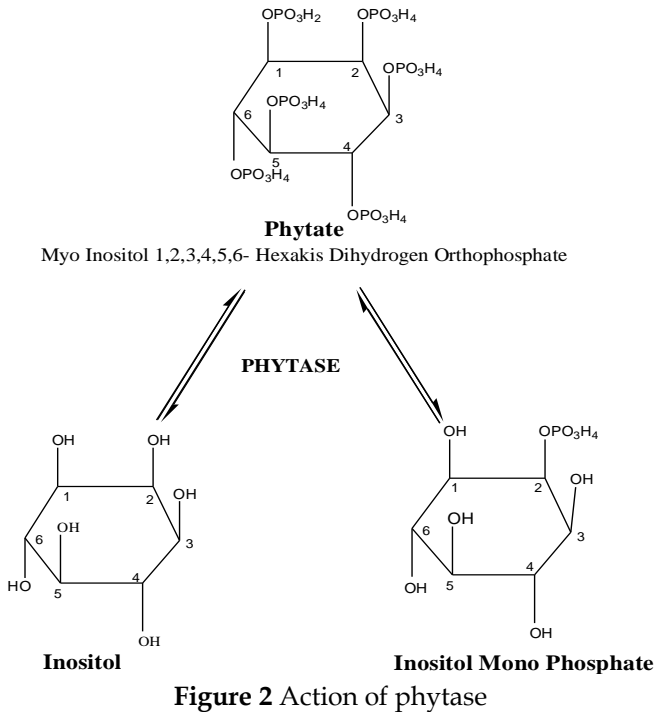
History

Phytase activity was first detected many decades ago in rice bran (Suzuki et al., 1907). Warden and Schaible (1962) were the first to show that exogenous phytase enhances phytate-P utilisation and bone mineralization in broiler chicks. However, before 1990s, the application of phytase has mainly been confined to poultry and swine to improve utilization of plant P. The first commercial phytase, Natuphos® was produced from *Aspergillus niger* and was released in market in 1991 (Selle and Ravindran, 2007). Following the prologue of commercial phytase, more emphasis were given to evaluating the effects of supplemental phytase on nutrient utilization and growth of common aquaculture species such as rainbow trout (*Oncorhynchus mykiss*)

(Forster et al., 1999), common carp (*Cyprinus carpio* L.) (Schaefer and Koppe, 1995), channel catfish (*Ictalurus punctatus*) (Li and Robinson, 1997), African catfish (*Clarias gariepinus)* (VanWeerd et al., 1999), salmon (*Salmo salar*) (Storebakken et al., 2000), stripped bass (*Morone saxatilis*) (Papatryphon et al., 1999), and Nile tilapia (*Oreochromis niloticus*) (Liebert and Portz, 2005).

Classification

Depending on site of hydrolysis

International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB) suggested that phytase feed enzymes fall into two categories depending on the site where the hydrolysis of the phytate molecule is initiated. These are 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). They are also known as myo-inositol hexakisphosphate 3-phosphohydrolase and myo-inositol hexakisphosphate 6-phosphohydrolase respectively. Former liberates the P moiety at position C3, whereas latter initiates the release of P at position C6 of the *myo*-inositol hexaphosphate ring (Cao et al., 2007). Phytate-degrading enzymes from microorganisms are considered to be 3-phytases, whereas seeds of higher plants are said to contain 6-phytases (Nayini and Markakis, 1986). Recently it was discovered that phytase isolated from *A. niger* shows 3-phytase activity whilst *Peniophora lycii* and *E. coli* contain 6-phytase (Greiner and Konietzny, 2006; Selle et al., 2007).

While according to Bohn *et al.,* (2008), phytase has been categorized into 3 groups: 3-phytase (EC 3.1.3.8), 5-phytase (EC 3.1.3.72) and 4/6 phytase (EC 3.1.3.26). Most of the 3-phytases are structurally homologous to β-propeller phosphatase, or histidine acid phosphatases, and are generally found in fungi and bacteria. Till date only a single 5-phytase (in lily pollen) has been detected and it has the conformation of histidine acid phosphatases (Mehta et al., 2006). 4/6-Phytases act on the carbon atom next to C5 of the inositol ring, most active in weak acidic environments (pH 4~6) with a temperature optimum in the range of 40 to 60 °C (Bohn et al., 2008).

Depending on optimal pH

The phytate-degrading enzymes can also be divided into two types based upon their optimal pH. These are acid phytase and alkaline phytase. Former shows the optimum activity at pH about 5.0 while the latter at pH near to 8.0 (Baruah et al., 2007b). Most of the microbial phytate-degrading enzymes

belong to acid type with exception from *Bacillus* group, which are alkaline phytase (Selle et al., 2007). However, it must be taken into account that microbial phytases of different sources can differ in their pH dependent bioefficacy (Onyango et al., 2005). Conversely most of the plant phytase tend to have a pH optimum at 5 (Wodzinski and Ullah, 1996). A list of characterised plants and animal phytases is presented shown in Table 2.

Sources of phytase

Phytase are widely distributed among various life forms wherein microorganisms are the most potential ones followed by plants. Filamentous fungi such as *Aspergillus ficuum*, *Mucor piriformis* and *Cladosporium* species (Stefan et al., 2005) are major sources of microbial phytases. These phytases are stable even at pH values above 8.0 and below 3.0 (Greiner and Konietzny, 2006). Plant phytases are heat labile and their activity is reduced or even eliminated in steam-pelleted diets (Jongbloed and Kemme, 1990); whereas, most of the corresponding microbial enzymes retain significant activity even after prolonged thermal exposure (Pointillart, 1988). Moreover, trials on pigs revealed that bio-efficacy of phytases from wheat and rye was only 40% compared to that from *Aspergillus niger* (Zimmermann et al., 2002). Higher effectiveness of microbial phytase is attributed to acidic pH of the stomach that is more conducive to microbial than plant phytase activity. Moreover, wheat and rye phytase is inactivated at pH 2.5 and is more susceptible to pepsin degradation than *A. niger* phytase (Phillippy, 1999). Phytase activity of animals is insignificant in contrast to plant and microbial counterparts (Weremko et al., 1997). In general there are four possible sources of phytate degrading enzyme. Phytase generated by the small intestinal mucosa, gut associated microfloral phytase particularly in large intestine, plant phytase and microbial phytase.

Table 2 Properties of phytases from different sources. (Sources: Greiner and Konietzny, 2006; Bohn et al., 2008)

Phytase source	pH optimum	Temperture optimum (°C)	Specific activity (U/mg protein) at 37 ^{0}C	Molecular weight (kDa)
Buttercup squash	4.8	48		67
Canola seed	4.5-5	50		70
Faba beans	5	50	636	65

Table 2 continued

Hazel seed	5			72
Legume seeds	8			
Lily pollen	8	55	0.2	88
Lupin seeds	5	50	539, 607, 498	57-64
Mung beans	7.5	57	2.4	160
Navy beans	5.3	50		
Peanut	5	55		22
Rapeseed	5.2	50		
Scallion leaves	5.5	51	500	
Soybean seeds	4.5-4.8, 4.5-5	55,58	2.4	119, 72-130
Sunflower	5.2	55		
Tomato roots	4.3	45	205	164
Typha latifolia pollen	8			
Barley	5;6	45, 55	117, 43	67
Maize seedling	5	55	2.3	71, 76
Maize root	5-5.1	35-40	5.7	
Oat	5	38	307	67
Rice	4,4,4.6	40		66,61
Rye	6	45	517	67
Spelt	6	45	262	68
Whole meal wheat	5.15	55		
Wheat bran	5			
Wheat bran	5.6,7.2			47
Wheat bran	6, 5.5	45,50	127, 242	68,66
Crude extract wheat	6	45		65
Rat intestine	7.0, 7.5-8.0			

Mucosal phytase derived from small intestine

Occurance of phytase activity in small intestine has been documented in fish. Tilapia *Oreochromis niloticus* x *O. aureus* (LaVorgna, 1998) are capable of releasing inorganic P from phytate and this phytase activity appears to be localized in the small intestinal brush border membrane (BBM) (Maenz and Classen, 1998). Therefore Nile tilapia can digest approximately 50% of

dietary phytate P. Research conducted by Ellestad et al. (2003) has confirmed the presence of intestinal BBM phytase activity in Nile tilapia and also observed some phytase activity in hybrid striped bass *Morone saxatilis* x *M. chrysops* and *M. chrysops* x *M. saxatilis* and common carp *Cyprinus carpio* also. Although intestinal phytase activity has been detected in several fish species, its application for phytate hydrolysis is not satisfactory for fish nutrition (Ellestad et al., 2003). Recently, Shi et al. (2008) isolated novel phytase gene, *appA* by degenerate polymerse chain reaction (PCR) and genomic library screening from *Buttiauxella sp. GC21*, a bacterium isolated from grass carp intestine. The phytase gene could be successfully expressed in *Escherichia coli* with high expression level. The successful purification and characterization of the enzyme may provide a basis for further large-scale production of phytase.

Gut microfloral phytase

Roy et al. (2008) isolated and enumerated the phytase-producing bacterial flora in the foregut and hindgut regions of the gastrointestinal tracts of ten culturable freshwater teleosts of different feeding habits, namely rohu (*Labeo rohita*), catla (*Catla catla*), mrigal (*Cirrhinus mrigala*), bata (*Labeo bata*), kalbasu (*Labeo calbasu*), Nile tilapia (*Oreochromis niloticus*), climbing perch (*Anabas testudineus*), common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*). The bacterial isolates were screened on the basis of their enzyme-producing ability. In case of the hindgut, maximum phytase-producing strains were present in grass carp and mrigal and minimum in rohu. Ellestad et al. (2002a) reported a very low intestinal phytase activity in hybrid striped bass. The same authors, in another study (Ellestad et al., 2002b), compared the phytase activity in the intestinal brush border of three teleost fish, carnivorous hybrid striped bass, omnivorous Nile tilapia and common carp. This study suggests that tilapia could digest more phytate while hybrid striped bass and common carp are not able to digest more than 1 or 2% of phytate P present in the diet. Forster et al. (1999) reported that Rainbow trout is able to hydrolyse less than 5% of dietary phytate P.

To date, there are only few studies, which claim the inclusion of gut microfloral derived phytase in fish feeds.

Intrinsic plant phytase

Native phytase activities are higher in cereals and cereal by-products, whereas lower activities have been reported for legume seeds (Eeckout and De Paepe, 1994) (Table 1). Such sources of phytases are of little relevance in fish diet, as the thermal process during feed manufacture would completely destroy the indigenous phytase enzymatic activity.

Exogenous microbial phytase

A wide spectrum of microbial phytase products for fish is now commercially available. The most commonly used microbial phytases are derived from fungi *(A. niger)* and bacteria *(E. coli)*. The catalytic properties of different microbial phytase sources have been reviewed by Greiner and Konietzny (2006), and are as shown in Table 3.

Pathway for phytase mediated phytate degradation

In theory, enzymatic hydrolysis of phytate generates a series of lower *myo-*inositol phosphates esters, via a progression of step-wise dephosphorylation reactions and ultimately leads to the production of free myo-inositol along with six inorganic P (Baruah et al., 2004; Selle et al., 2007). In this case phytase generates 282 g inorganic P from each kg of dietary phytate (Selle et al., 2007). A general illustration has been presented in Figure 2. However, phytases of different origins have different dephosphorylation pathways (Kaur et al., 2007). Among yeasts, the pathway of dephosphorylation of phytate by purified phytase of *C. krusei* WZ-001 was described by Quan et al. (2003). The complete catalytic reaction sequence is shown in Figure 3. As such, the route of enzymatic dephosphorylation by phytases of *P. rhodanensis* was investigated and elucidated up to IP3 level (Adelt et al., 2003), whereas (Greiner et al., 2001) described the complete hydrolysis sequence of phytate by *S. cerevisiae*.

Table 3 Phytases from different microbial sources and their catalytic properties. (Source: Greiner and Konietzny, 2006).

Phytase source	Phytase activity (U/mg) (37 ^{o}C)	pH optimum	Temperature optimum (^{o}C)
Aspergillus caespitosus	-	5.5	80
Aspergillus fumigatus	23–28	5.0–6.0	60
Aspergillus niger	50–103	5.0–5.5	55–58
Aspergillus oryzae	11	5.5	50

Aspergillus terreus	142–196	5.0–5.5	70
Penicillium simplicissimum	3	4	55
E. nidulans	29-33	6.5	
M. thermophila	42	5.5	
S. castellii	418	4.4	77
Cladosporium	909	3.5	40
K. pneumoniae	224, 297	5.5, 5.5	50, 60
K. aerogenes		4.5, 5.2	68
Peniophora lycii	1080	5.5	58
Thermomyces lanuginosus	110	6	65
Bacillus amyloliquefaciens	20	7.0–8.0	70
Bacillus subtilis	9.0–15	6.5–7.5	55–60
Citrobacter braakii	3457	4	50
Escherichia coli	811–1800	4.5	55–60
Klebsiella terrigena	205	5	58
Lactobacillus sanfranciscensis	-	4	50
Pantoea agglomerans	23	4.5	60
Pseudomonas syringae	769	5.5	40
Candida krusei	1210	4.6	40
Pichia anomala	-	4	60

Phytase production systems

Phytase is mainly manufactured by two methods, solid state fermentation (SSF) and submerged fermentation (SmF). SSF is a modern process where microorganisms are grown on solid materials without the presence of free liquid. It has the potential to produce large quantities of enzymes. While SmF is the traditional method used for the production of microbially derived enzymes. Enzymes derived from this method do not possess the properties of enzymes derived from SSF.

Key advantages of SSF produced phytase over SmF are: high concentration of enzyme activity; higher heat stability and many side acting enzymes. Given the complex nature of feed ingredients, phytase with side acting enzymes is always beneficial, e.g. to deal with non-starch polysaccharides. Nutrizyme P forte (2500 FTU/kg) and Nutrizyme 2p forte (5000 FTU/kg), commercially available products are synthesized using SSF technology. These release P from phytate effectively and also provide benefits of side acting enzymes such as xylanase, cellulase and pectinase. Commercially produced microbial phytases have been shown in Table 4.

Besides, the culture conditions, type of strain, nature of substrate and availability of nutrients should be taken into consideration for selecting a particular production technique, as they are the critical factors affecting the yield (Vats and Banerjee, 2004).

C. krusei	*P. rhodanensis*			*S. cerevisiae*	*Lupin phytase*
$Myo\text{-}InsP_6$	$Myo\text{-}InsP_6$			$Myo\text{-}InsP_6$	$Myo\text{-}InsP_6$
Ins $(1,2,4,5,6)P_5$ Ins $(1,2,3,5,6)P_5$	Ins $(1,2,4,5,6)P_5$		Ins $(1,2,3,4,5)P_5$	Ins $(1,2,4,5,6)P_5$	D-Ins $(1,2,4,5,6)P_5$
Ins $(1,2,5,6)P_4$	Ins $(1,2,5,6)P_4$	Ins $(1,2,4,5)P_4$	Ins $(2,3,4,5)P_4$	Ins $(1,2,5,6)P_4$	D-Ins $(1,2,5,6)P_4$
Ins $(1,2,5)P_3$	Ins $(1,2,6)P_3$		Ins $(2,4,5)P_3$	Ins $(1,2,6)P_3$	D-Ins $(1,2,6)P_3$
Ins $(1,2)P_2$				Ins $(1,2)P_2$	D-Ins $(1,2)P_2$
Ins $(2)P_1$				Ins $(2)P_1$	Ins $(2)P_1$
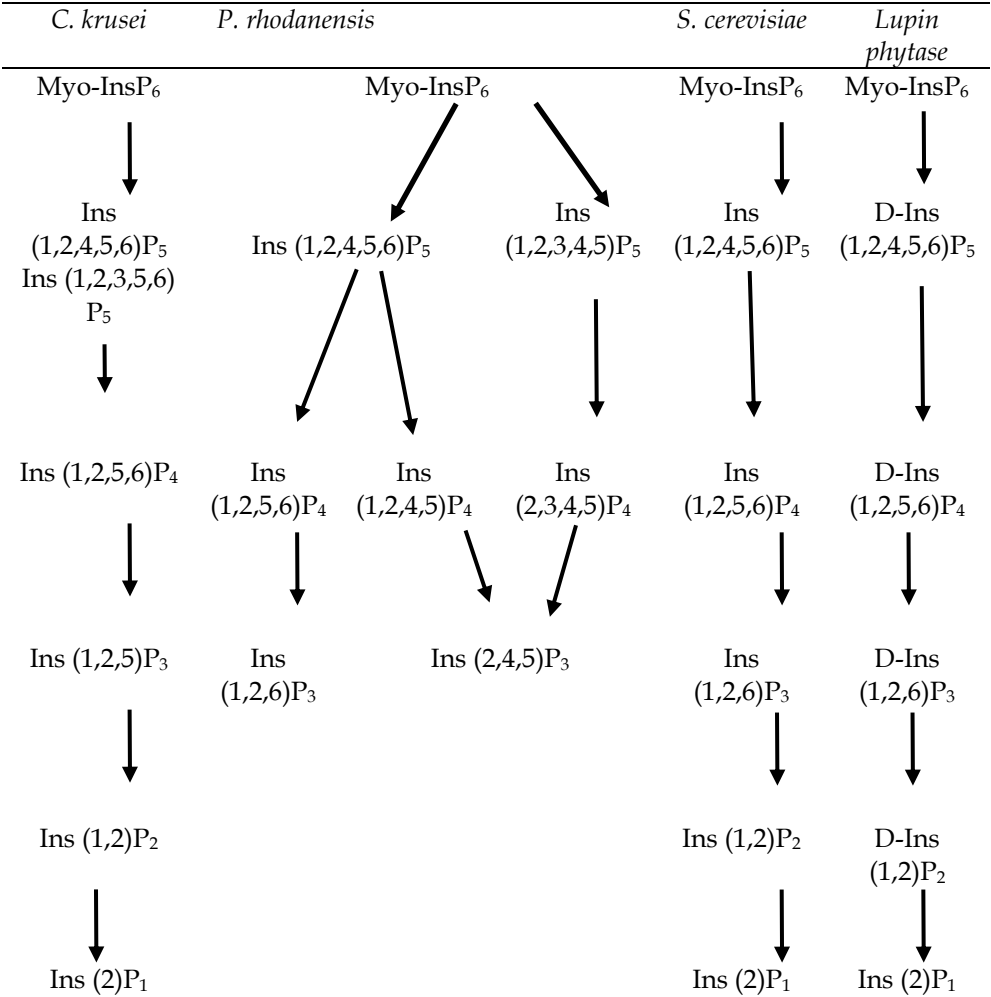
Figure 3 Pathways of phyate degradation by different types of yeast and lupin phytases (Source: Kaur et al., 2007)

Table 4 Commercially available microbial phytases **(**Sources: Hou 2001; Stefan et al., 2005; Cao et al., 2007)

Company	Country	Phytase source	Production strain	Trademark
AB Enzymes	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*A. oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*A. niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*P. lycii*	*A. oryzae*	Bio-Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*Penicillium simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*A. awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme® Roxazyme®

Factors influencing the bio-efficacy of phytase

Substrate and enzyme

Efficacy of microbial phytase is governed directly or indirectly by numerous interactive factors. These may include dietary substrate levels, fish species, the inclusion rate and source of phytase. Cao et al. (2007) reviewed that phytase dose at a level of 250–2000 FTU/kg feed is usually considered optimum for many fish species. Furthermore, different phytase sources might lead to different effects on growth parameters and nutrient deposition, as is evident from the work of Liebert and Portz (2005). They compared nutrient and P utilization using two different sources of microbial phytase in Nile tilapia diet. Two different sources of microbial phytase, SP1002 and Ronozyme®P were compared based on growth response, body composition, nutrient deposition and nutrient utilization. Superior performance data (growth response, body composition, nutrient deposition and nutrient utilization) were obtained using phytase SP1002. It was also found that supplementation of phytase SP1002 at a level of 750 FTU/kg diet was sufficient to improve growth, feed conversion, protein deposition, while supplementation of at least 1000 FTU/kg of Ronozyme®P resulted in intermediate growth. Parallel comparative and dose response studies were also done by Li and Robinson (1997), Robinson et al. (2002) in channel

catfish, Furuya et al. (2001) in Nile tilapia, Yoo et al. (2005) in Korean rockfish and Debnath et al. (2005a) in pangus catfish. It was conclded that optimum dose of phyatse for these fish species is 500 – 1000 FTU/kg feed. Cheng et al. (2004) reported that the optimum level of phytase supplementation in rainbow trout diets was approximately 500 FTU/kg diet. Vielma et al. (2004) repoted two trials: Trial 1 (a semi-purifed diet containing 50% soybean meal with phytase levels of 0, 500, 1000, 2000 and 4000 FTU/kg), and Trial 2 (commercial-type extruded feeds containing 36% soybean meal with phytase either 0 or 2000 FTU/kg feed). In both trials phytase decreased phytate content of faeces from 35 to 5 mg and from 34 to 14 mg phytate per g faecal dry matter; and apparent digestibility coefficient of P improved from 23% to 83% and from 35% to 54% in Trials 1 and 2 respectively, whereas, Zn digestibility significantly increased in Trial 1, but not in Trial 2.

pH value

As described earlier that the optimum microbial phytase activity occurs at two pH ranges, pH 5.0-5.5 and pH 2.5; activity at pH 5.0-5.5 being higher than at pH 2.5 (Simons et al., 1990; Kemme et al., 1999). The efficacy of phytase differs in agastric and gastric fish. The pH in digestive tract of agastric fish like common carp is around 6.5-8.4 while gastric fish has much lower pH (Ji, 1999). The large deviation in values of pH between optimal phytase activity and digestive tract of agastric fish, is responsible for the poor efficacy of phytase in stomachless fish. Gastrointestinal acidity in gastric fish like trout, catfish and other carnivorous fish is favourable for efficient hydrolysis of phytate by the microbial phytase in these species. Enhancement of   phytase utilisation by agastric fish is carried out by addition of acidifier such as citric acid, fumaric acid, lactic acid and formic acid in diet (Baruah et al., 2005). These are known to lower the dietary acidity and in turn reduce the pH of digestive tract and ultimately amplify the activity of exogenous phytase. The same author reported a significant synergestic interaction between citric acid and microbial phytase on growth performance, nutrient digestibility, bone mineralization and protein efficiency ratio in *L. rohita* juveniles (Baruah et al., 2007a,b).

Temperature

Phytase enzymes are thermolabile and have optimal temperature range of 45–60°C (Lei and Stahl, 2000). For this reason dietary phytase may get

denatured during extrusion process. The high temperature phase of extrusion partially leads to the destruction of intrinsic phytase in feed ingredients and thereby significantly reduces the availability of P, Zn, Cu and other nutrients in plant based feed (Cheng and Hardy, 2003). Pretreatment of feedstuff with phytase prior to extrusion could be a good choice to avoid the heating denaturation of phytase enzyme (Cain and Garling, 1995; Nwanna, 2007). Beside this, spraying the processed feed with liquid suspension of phytase could be another option to solve the temperature dependent problem (Vielma et al., 2004). In general it is carried out by mixing the enzyme concentrate with a stabilizer (such as $MgSO_4$) and then spraying the solution on processed feed. In this endeavour, the inclusion of heat-resistant enzymes would circumvent the aforementioned problem. Thermostability of phytase is considered to be a desirable feature in the animal feed as feed pelleting involves a brief exposure to 80–85 ^{0}C for a few seconds and eventually the activity of enzyme need to be retained. Phytases of *Schwanniomyces castellii*, *Arxula adeninivorans* and various *Pichia* spp. were found to be active in the range of 75–80 ^{0}C (Vohra and Satyanarayana, 2002).

Feed additives

It is speculated that dietary addition of organic acids and microbial phytase may have a synergistic effect on mineral availability in stomachless fish. It is supported from the evidence that acidity of the (gastro) intestinal tract influences the activity of phytase, optimum being at lower pH value. Therefore reduction of intestinal pH through dietary addition of organic acids increased the solubility of phytate–P and thus improved P absorption (Vielma and Lall, 1997). In addition organic acids can also bind various cations along the intestine and act as chelating agent (Ravindran and Kornegay, 1993), accounted for increased intestinal absorption of minerals (Sugiura et al., 1998a). Besides, dietary acidification these also stimulate the epithelial cell proliferation in the gastrointestinal mucosa (Sakata et al., 1995), thereby increasing the absorption of minerals. Another advantage of dietary acidification is the inhibition of intestinal bacteria competing with the host for available nutrients and reduction of toxic bacterial metabolites such as ammonia and amine. This is shown to improve the gut health of the animals (Ravindran and Kornegay, 1993). However, Baruah et al. (2007b) reported that addition of 3% citric acid activated microbial phytase in feed of rohu. Apparent absorption of Zn, its content in whole body and plasma, was

significantly (P< 0.05) higher in citric acid and microbial phytase fed groups. Interaction between organic acid and phytase was found for increased absorption of Na, P, K, Mn, Mg, Fe and N in whole body and plasma (Baruah et al., 2007b). In a previous study Baruah et al. (2005) concluded that prominent interaction of microbial phytase and citric acid enhanced bone mineralization of rohu juveniles. Moreover, Sugiura et al. (2001) also found a significant increase in the apparent absorption of Mg and P by the addition of 5% citric acid in the diet of rainbow trout.

The inclusion of Vitamin D_3 (cholecalciferol) and hydroxylated D_3 compounds may also complement the efficacy of phytase. Vitamin D analogs may indirectly improve utilization of phytate P digestion by increasing absorption of the hydrolyzed P but the interaction of vitamin D and phytase has not been clearly elucidated (Lei et al., 1994). However, it has been found that elevated dietary vitamin D_3 levels can alleviate some of the unfavorable effects of dietary calcium while phytase activity of the intestine and phosphatase activity of mucous membranes are not affected. Calcium absorption can be promoted by vitamin D_3, thus the negative effect of Ca on phytate utilization could be eliminated indirectly by reducing the formation of phytate- calcium (Qian et al., 1997). Vielma et al. (1998) reported increment in weight gain of rainbow trout when cholecalciferol at low dietary level was supplemented with phytase at 1500 FTU/kg soy protein concentrate based diet. Subsequent studies in broiler revealed that the inclusion of 1,25-dihyroxycholecalciferol in diets enhanced phytate-P utilisation (Driver et al., 2005). It has been shown that other feed additives like Zn may have deleterious effects on phytase efficacy. For example, Zn levels reduce the P-releasing efficacy of phytase in young pigs and chickens (Augspurger et al., 2004). It is likely that the formation of Zn–phytate mineral complexes, which are not readily hydrolysed by phytase, is responsible for this suppression of efficacy. However different microbial phytases require different metal ions for their activity. Most phytate-degrading enzymes characterized so far are greatly inhibited by Cu and Zn^{2+}. Kim et al. (1998a,b) reported that EDTA, Cd^{2+}, and Mn^{2+} strongly inhibit phytase of *Bacillus* sp. DS11 whereas Hg^{2+}, Mg^{2+}, Ba^{2+}, and Cu^{2+} at 5 mM inhibit it moderately. In the reaction mixtures containing 5 mM Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+} and Hg^{2+}, phytase activity of *Selenomonas ruminantium* was strongly inhibited (Yanke et al., 1999). The sensitivity pattern was similar to that of *E. coli* and *K. terrigena* phytase (Greiner et al., 1997). The well known fact that plant protein sources are generally deficient in lysine and methionine (Watanabe, 2002) provide a room for the inclusion of these

amino acid along with phytase to improve overall nutritive value of plant based diet. In this context Biswas et al. (2007a) studied the interactive study of phytase and lysine on *Penaeus monodon* juveniles and concluded that supplementation of both lysine and phytase in soybean-based diet not only enhances the nitrogen and P utilisation but also significantly changes the fatty acid profile of the tissue. Total n-3 fatty acid was reduced significantly (P<0.05) whereas total n-6 fatty acids content increased due to the dietary inclusion of lysine or phytase or both.

Calcium to phosphorus ratio

Ca and P are necessary to maintain an optimal bone development (Cruz and Tsang, 1992), more of both minerals being required during growing stages. The percentage of Ca in the whole, fresh (wet) body of finfish ranges from 0.5% to 1% with a ratio of Ca to P of 0.7 to 1.6 (NRC, 1993). In diet, it is necessary to take into account the Ca/P ratio, because it has important consequences for bone development. However, when this ratio increases harmful effects can appear. It is known that several aberrations in bone mineral homeostasis and bone metabolism associated with higher Ca:P ratio. However, dietary Ca levels and Ca:P ratios are also crucial to phytase efficacy as reviewed by Lei and Stahl (2000). Qian et al., (1996a) reported that reducing Ca:P ratios from 2.0 to 1.2:1 in phytase supplemented diets (700 and 1050 FTU/kg) of pig, increased phytase efficacy by the order of 16%. Liu et al. (2000) observed that reducing Ca:P ratios from 1.5 to 1.0:1 in phytase (500 FTU/ kg) supplemented low P diets increased P absorption in the small intestine of pigs. Similarly Qian et al. (1997) found that increasing Ca (5.61–10.20 g /kg) and Ca:P ratios (1.1–2.0:1) depressed weight gain of broilers. Additionally, increasing Ca:P ratio depressed *E.coli* derived phytase action in the pig diet and thus significantly depressed weight gain and feed efficiency (Adeola et al. 2006) They reported an increase in weight gain by 17.6% by reducing Ca: P ratios from 1.8 to 1.2:1. The probable reason for maintaining low Ca and Ca:P ratio in feed is due to high dietary Ca or a high ratio of Ca:P that interferes with P absorption and reduces the effectiveness of phytase activity. However only cursory attention has been paid towards optimisation of Ca: P ratio in fish feed. However, it can be hypothesized that high concentration of Ca in fish feed will chelate with phytate forming insoluble complex or compete with phytase for binding site at the myoinositol ring and thus block the site of phytase mediated substrate hydrolysis (Qian et al., 1996b) or increase the pH value to inhibit the activity

of phytase (Cao et al., 2007). Moreover, Cao et al. (2007) recommended Ca:P ratios in fish meal in the range of 1.1–1.4:1 at which phytase execute high efficiency.

Mode of application of phyatse

Microbial Phytase are suplemented in fish feed as powder, granulate or liquids forms. The preferable method of phytase application in fish diets is by coating a liquid form of the enzyme after extrusion and drying which prevents activity loss during pellet formation (Verlhac et al., 2007). On-line spraying device is simple to install on the existing production lines and it is not an expensive device. On the other hand, the use of enzyme in liquid form in post-extrusion allows much flexibility in formulation. The possibility of pre-treatment of raw materials has been investigated in many studies. It was found that rainbow trout fed a soybean meal based diet pre-treated with phytase, resulted in increase in P availability and absorption (Cain and Garling, 1995; Sugiura et al., 2001). Many researchers (Teskeredzic et al., 1995; Storebakken et al., 1998; Van Weerd et al., 1999; Cheng and Hardy, 2003; Riche and Garling, 2004; Yoo et al., 2005) have reported positive effect of phytase pre-treatment on P availability. Masumoto et al. (2001) found no significant difference in P availability between fish fed the soybean meal based diet supplemented with phytase and the diet containing phytase pre-treated soybean meal in Japanese flounder. Likewise, Yoo et al. (2005) reported no remarkable difference in P digestibility between soybean meals pre-treated with phytase (1000 FTU/kg) when included in diet and the phytase supplemented diet (1000 FTU/kg) in Korean rockfish. Denstadli et al. (2007) compared on-line phytase pre-treatment of plant feed ingredients and phytase coating in diets of Atlantic salmon. Pre-treatment of wheat and soy protein concentrate for a period of 1 h in the pre-conditioner (at 45°C) led to significantly higher P digestibility in comparison with the phytase supplemented diet. However, before the introduction of heat-stable phytase product forms, phytases were generally added postpelleting to prevent inactivation due to high pelleting temperatures, but the advent of heat stable phytase from *A. niger* (Han and Lei, 1999; Xiong et al. 2005), *A. fumigatus* (Peng et al., 2002) and *E. coli* (Garrett et al., 2004) has made direct enzyme addition possible through the conditioning/pelleting process.

Dietary phosphorus requirement in fish

Phosphorus is a critical element for fish and other livestocks. It plays a major role in the structure and function of living cells. It is an integral component of adenosine triphosphate (ATP), nucleic acids, nucleotides, phospholipids, proteins, and a key component of many coenzymes. These compounds function in energy releasing cellular reaction, cellular division and growth, in the transport and metabolism of fats, and in the absorption and utilization of carbohydrates, fatty acids, and proteins. Thus P is an essential nutrient for growth, skeletal development and reproduction in fish (Asgard and Shearer, 1997). However fish can absorb dissolved P and other minerals from the water across the gill membrane and in the case of marine fish, through the digestive tract. The concentration of P in fresh water and sea water is low and therefore fish require relatively high dietary source of P to meet their requirement for growth (NRC, 1993). Dietary P requirement of most aquaculture fish species varies from 0.4 to 0.9% of diet (NRC, 1993; Kaushik, 2005). Japanese eel and Korean rockfish have a low P requirement of around 0.3% of diet. Some species such as haddock has a much higher requirement, reaching nearly 1% of diet. Plant protein ingredients contain significantly less P than fishmeal and the partial replacement of fishmeal by plant feed ingredients could help reducing the P discharge. Phosphorus deficiencies induce skeletal deformities such as curved spines and soft bones in Atlantic salmon, cephalic deformities in common carp, scoliosis in haddock and halibut (Lall and Lewis-McCrea, 2007).

It is unlikely that differences in P availability contribute to the large differences in reported requirements. The effect of fish size on the dietary requirements for essential elements has not been specifically examined, although measurements made on Atlantic salmon (Shearer et al., 1994) and rainbow trout (Shearer, 1984) indicate that body concentrations of P change considerably with fish size. The mechanistic model presented by Shearer (1995) suggests that the dietary P requirement increases from first feeding to the juvenile stage, and then decreases in adults. The range of fish sizes used in P requirement studies has varied considerably and may have affected the reported requirements (Table 5).

Table 5 Phosphorus requirement in different fish species

Fish species	Initial fish weight (g)	Feed efficiency	P requirement (g/kg diet)	P source	References
Nile Tilapia	0.4	0.6	4.6	NaP	Haylor et al. (1988)
Blue Tilapia	0.8	0.52	5	NaP	Robinson et al. (1987)
Nile Tilapia	35	0.6	5.3	CaP	Furuya et al. (2001)
Common	5	0.8	6 - 7	K2P +	Ogino and Takeda

				NaP	(1976)
carp					
Sunshine bass	10-20	0.6	5	KP	Brown et al. (1993)
Guppy	Fry	0.3	5.3–12.3	KP	Shim and Ho (1989)
Red drum	1.3	0.5	8.6	NaP	Davis and Robinson (1987)
Channel catfish	6	1.1	4.0	NaP	Wilson et al. (1988)
Channel catfish	6	0.99	8	CaP	*Table 5 continued*
Channel catfish	1.8	-	8	NaP	Lovell (1978)
Rainbow trout	50	1.1	2.4–5.9	NaP	Rodehutscord (1996)
Rainbow trout	35	1.0	3.4–5.4	NaP	Ketola and Richmond (1994)
Rainbow trout	1.2	1.1	7-8	K_2P + NaP	Ogino and Takeda (1978)
Atlantic salmon	1.4	1.45	10-11	CaP_2	Asgard and Shearer (1997)
Chum Salmon	1.5	0.95	5-6	NaP	Watanabe et al. (1980)
Atlantic salmon	6.5	0.5	6	CaP	Ketola (1975)
Atlantic salmon	57	0.9	6	NaP + KP	Lall and Bishop (1977)

NaP: Sodium phosphate monobasic, KP: Potassium phosphate monobasic, K_2P: Potassium phosphate dibasic, CaP_2: Calcium phosphate monobasic, CaP: Calcium phosphate dibasic

Feed efficiency: Body mass gain (g)/Feed fed

Calculation of available phosphorus in diets

An accurate estimation of P content in feed ingredients is essential during diet formulation to meet nutritional requirements and to minimise P waste output from aquaculture operations Verlhac et al. (2007). Hua and Bureau (2006) have developed a model to estimate available P content of salmonid fish feed based on levels of different types of P such as bone P, phytate, organic P, Ca monobasic, Na/K inorganic P supplement and Ca dibasic supplement.

The proposed model is:

P= 0.68 bone-P + 0.84 organic P + 0.89 Ca monobasic, Na/K Pi + 0.64 Ca dibasic + 0.51 phytase/phytate – 0.02 (phytase/phytate)² - 0.03 (bone P)² - 0.14 bone P* Ca monobasic, Na/K Pi (P<0.0001, R²=0.96).

Phosphorus availability from different feed ingredients

Phosphorus availability from different ingredients fed to fish is depicted in Table 6. Various studies have clearly shown that the availability of P from plant based meal is lower as compared to fishmeal. Phoshporus digestibility varies in relation to the form provided in the diet. Kaushik (2005) stated that the monobasic phosphate form has a comparable digestibility irrespective of fish species; the dibasic form renders lower bioavailability in common carp as compared to channel catfish and rainbow trout, the tribasic calcium form of phosphate is very poorly digested by common carp (13%) and higher by rainbow trout (64%). Fishmeal P mainly comes from bone that is available in the hydroxyapatite form. Riche and Brown (1996) reported that availability of P from fishmeal is greater for rainbow trout than for common carp. The reason for this could be that the common carp has a poor ability to hydrolyse calcium phosphate since it does not possess stomach and therefore cannot secrete gastric acid in their digestive system. Phosphorus availability in common carp has been shown to increase by addition of citric acid (Sugiura et al., 1998b). Other factors such as growth stages might influence P absorption. Satoh et al. (2002) have elucidated that small fish have a very poor capacity to absorb P from plant protein source and this capacity improves as fish grows.

Benefits of phytase application in aquaculture

Enhancement in phosphorus bioavailability

Many researchers have reported a positive effect of phytase supplementation on total P availability in fish (Tables 6 and 7). Soybean meal based diet added with 500 and 1000 FTU/kg of phytase could free 20% and 40% of phytate-P in common carp and 60% and 80% in crucian carp *Carassius carassius* respectively (Schafer and Koppe, 1995). Addition of 8000 FTU/kg of microbial phytase resulted in higher P bioavailability in channel catfish (Jackson et al., 1996; Yan and Reigh, 2002). A momentous increase in P availability of rainbow trout when reared with dietary phytase has been reported (Riche and Brown, 1996). In the same species the phytase inclusion at the rate of 1500 FTU/kg feed improved P availability as indicated by higher apparent availability of P, bone ash, plasma and body P concentrations (Vielma et al., 1998). Furthermore, Sugiura et al. (2001) and Vielma et al. (2004) demonstrated that dietary microbial phytase in soybean meal-based diets in rainbow trout significantly improved ADC of P. Sajjadi and Carter (2004) reported that addition of 2000 FTU/kg feed (canola meal

based diet) in Atlantic salmon significantly increased P digestibility and retention. Similar results were reported by Hughes and Soares (1998) in striped bass *Morone saxatilis* fed with plant based diets supplemented with phytase at 2400 FTU/kg. Combination of enzymes including phytase (200 FTU/kg feed) in Japanese sea bass, had no significant effect on specific growth rate and feed conversion ratio but P retention was significantly increased (Ai et al., 2007). On the other hand, Masumoto et al. (2001) found that phytase improved P digestibility in Japanese flounder fed a high soybean meal diet. With another diet (soy protein concentrate based diet) in the same experiment, they observed that phytase improved P retention in comparison with a non-supplemented diet as well as in comparison with inorganic P supplemented diet. In Nile tilapia (*Oreochromis niloticus*) dietary phytase at a level of 1000 FTU/kg diet significantly increased the levels and ADC of P (Cao et al., 2008). This result is consistent with the assessment conducted by Furuya et al. (2001) on the same species where phytase supplementation between 500 and 1500 FTU/ kg improved P availability and 750 FTU/kg resulted in the similar performance as with inorganic P addition (NaH_2PO_4; 15 g/kg diet) (Liebert and Portz, 2005). Likewise, the inclusion of exogenous phytase in Nile tilapia diet could act as a replacer for supplemented P in diet (Phromkunthong et al., 2006).

In agastric fish like common carp *Cyprinus carpio*, the supplementation of phytase in diet increased plasma P concentration against phytase free diet (Nwanna and Schwarz, 2007). In the same fish species, Mai et al. (2002) observed that phytase had higher but non-significant effect on levels of plasma inorganic P compared with the fish fed diet without phytase. Potential of phytase application in feed of rohu, a agastic fish has also been investigated by Baruah et al. (2007a); maximum apparent digestibility of P and crude protein was recorded when phytase-supplemented diets contained 750 FTU phytase/kg diets. It was seen that exogenous phytase was substantially efficient in enhancing the bioavailability of P and thus reducing the amount of faecal-P. Thereby, inclusion of phytase in aquafeed tends to reduce the phosphate load from fish wastes and thus eventually prevents phosphate induced algal bloom contamination. Any reduction in P excreted by fish and other animals is of benefit to both the environment and sustainable production.

Table 6 Effects of phytase on total phosphorus (P) availability from plant protein-based diets in different fish species

References	Plant protein sources in fish diet	P availability (%) without phytase	Phytase dose FTU/kg diet	P availability (%) with phytase
Rainbow trout (*Oncorhynchus mykiss*)				
Lanari et al., 1998	(Soybean meal 55%, soy protein 25%); (Soybean meal 33%)	25; 58.6	1000; 1000	57; 68.1
Vielma et al., 1998	Soy protein concentrate 50%	44.5	1500	69.7
Forster et al., 1999	(Canola protein concentrate with high Ca level); (Canola protein concentrate); (Canola protein concentrate); (Canola protein concentrate)	37.4; 33.0; 4.8; -0.8	(500, 1500, 4500); (1500); (500, 1500, 4500); (1500);	(42.7, 40.1, 51.0); 45.9; (20.6, 26.8, 45.4); 30.3
Sugiura et al., 2001	(Soybean meal 30%); (Soybean meal 50%); (Soybean meal 30%); (Soybean meal 50%)	56; 27; (-14); (-19)	1000; (500, 1000, 2000, 4000); 1000; (500, 1000, 2000, 4000)	62; (54, 68, 82, 90); 90; (33, 56, 79, 91)
Cheng et al., 2004	(Soy protein concentrate 50%); (Soybean meal 50%);(Soybean meal 50%); (Soy protein concentrate 50%); (Soybean meal 50%)	39.7 ; 39.9; 31.5 ; 6.3; 0.5	(500, 1000, 2000, 4000); (500, 1000, 2000, 4000); (500, 1000, 2000, 4000); (500, 1000, 2000, 4000); (500, 1000, 2000, 4000)	(94.97, 96.16, 96.3, 96.6); (80.8, 88, 91.9, 95); (70.1, 78.7, 78.9, 85.2); (60.7, 75.4, 70.4, 57.2); (52.4, 59.8, 78.3, 67.4)
Cheng and Hardy, 2004	(Soybean meal 15%, distiller's dried grain with solubles 15%);(Soybean meal 15%, dried grain with solubles 15%)	80.1; 22.2	(300, 600, 900, 1200); (300, 600, 900, 1200)	(87.0, 89.1, 86.3, 87.5); (17.8, 64.1, 53.8, 96.9)
Vielma et al., 2004	(Soybean meal 50%); (Soybean meal 36%); (Soybean meal 55%)	28.3; 35.1; 61.8	(500, 1000, 2000, 4000); 2000; (500 1000)	(64.9, 78.6, 81.5, 84.2); 54.2; (71.7, 72.4)
Atlantic salmon (*Salmo salar*)				
Sajjadi and Carter, 2004	Canola meal based diet	63.8 ; 68.3	2000; 2000 + Pi	74.06; 69.1

Table 6 continued

Denstadli et al., 2007	Soybean meal 43%, Wheat 12%	1) 31.2 2) 31.4 3) 36.7 4) 38.1	1878	1) 37.2 2) 33.0 3) 38.6 4) 38.9
Red sea bream (*Pagrus major*)				
Biswas et al., 2007b	Soybean meal 30%; Soybean meal 30%	25.1; 54.3	(1000, 2000, 3000, 4000); (1000, 2000, 3000, 4000)	(31.8, 36.0, 32.6, 30.7); (76.7, 86.9, 77.2, 79.1)
Japanese flounder (*Paralichthys olivaceus*)				
Masumoto et al., 2001	Soybean 67%; Soy protein concentrate 40%	8.9; 1.3	(Phytase + citric acid); Phytase	87.3; (47.5, 37.6)
Striped bass (*Morone saxatilis*)				
Hughes and Soares,1998	(Wheat middlings 26%, Soybean 27% Corn gluten 25%); (Wheat middlings 21%, soybean 37%, corn gluten 10%)	29.7; 61.2	(800, 1300, 2400)	82.8, 64.9, 92.5
Papatryphon et al., 1999	Soybean meal 41%, CGM 30%	45	500, 1000, 2000	56.2, 67.1, 77.6
Sea bass (*Dicentrarchus labrax*)				
Oliva-Teles et al., 1998	Soybean 67%	25.2	1000, 2000	79.8, 71.5
Japanese sea bass (*Lateolabrax japonicas*)				
Ai et al., 2007	Soybean meal 17%, rapeseed meal 10%, peanut meal 10%	26.0	200	36.3
Korean rockfish (*Sebastes schlegeli*)				
Yoo et al., 2005	Soybean meal 25.8%; Soybean meal 34%	57.6; 61.8	(1000, 2000); (1000, 2000)	(86.3, 87.0); (84.0 89.5)
Nile tilapia (*Oreochromis niloticus*)				

Table 6 continued

Liebert and Portz, 2005	Wheat 32.5% Soybean 23.5% Corn 22.5%,Wheat gluten 11.5%	11.8	500, 750, 1000, 1250	24.5, 27.6, 31.7, 31.6
Portz and Liebert, 2004	(Wheat 32.5% Soybean 23.5% Corn 22.5%, Wheat gluten 11.5%); (Wheat 32.5%, Soybean meal 23.5%, Corn 22.5)	49.1; 12.5	(500, 1000, 2000, 4000); (500, 1000, 2000, 4000)	(60.1, 71.4, 71.1, 73.9); (40.8; 50.0; 51.5; 52.8)
Red tilapia (*Oreochromis niloticus* Linn.)				
Phromkunthong et al., 2006	(Soybean meal 45%, Rice bran 18%, cassava 20%); (Soyabean meal 45%, Rice bran 18%, cassava 20%)	41.2; 27.4	(750, 750 + DCP); (750, 750 + DCP)	(62.9, 63.5); (43.1, 40.9)
Asian catfish Pangasius (*Pangasius pangasius*)				
Debnath et al., 2005	Soybean meal 45.0%	55.9	150, 250, 350, 500, 1000, 2000	61.9, 66.1, 72.7, 67.6, 66.9, 71.8
African catfish (*Clarias gariepinus*)				
Van Weerd et al., 1999	Soybean meal 68.5%	29.3	380, 750, 1000	40.5, 68.1, 67.6
Channel catfish	Soybean 46%, cottonseed 14%, corn 32%	31.2	1000, 3000	55.1, 62.5
Indian major carp (Rohu) (*Labeo rohita*)				
Baruah et al., 2007a	(Soybean meal 41%, Rice products 23%); (Soybean meal 41%, Rice products 23%)	61.6; 69.4	(500, 500 + CA); (500, 500 + CA)	(74.2, 82.7); (75.9 83.4)
Common carp (*Cyprinus carpio* L.)				
Schäfer et al., 1995	Soybean meal 52.5% ; Soybean meal 52.5%	32.0; 30.4	500; (500, 1000)	49.4; (42.3, 47.1)
Mai et al., 2002	Soybean 37.6%, wheat bran 18.7%	27	750, 1500, 2250	44, 51.4, 57.2

The individual characteristics (Plant protein source in fish diet, P availability (%) without phytase, Phytase dose Units/kg and P availability (%) with phytase) are separated with semicolon (;) and presented in sequence.

Table 7 Influence of phytase on total phosphorus (P) availability from feed ingredients in fish species

References	Fish feed ingredients (plant protein source)	P availability (%) without phytase	Phytase dose FTU/kg diet	P availability (%) with phytase
Rainbow trout (Oncorhynchus mykiss)				
Riche and Brown, 1996	Canola meal; Solvent extracted soybean meal; Full fat soybean; Peanut meal; Corn gluten meal; Cottonseed meal	4.8; (-13.4); 8.4; 22.1; 30.7; NA	3.8×10^6	46.2; 46.6; 64.4; 75.6; 76.8; 56.3
Cheng and Hardy, 2002	Canola meal; Barley; Wheat	12.2; 79.4; 61.6	500	41.8; 82.7; 64.6
Cheng and Hardy, 2003	Raw soybean; Expelled soybean; Extruded fullfat soybean	21.2; NA; 12.5	750; 200; (200, 400, 600, 800, 1000)	NA; 31.7; (81.3, 92.2, 89.7, 95.2, 93.9)
Vielma et al. (2006)	Rapeseed meal; Soybean meal; Corn gluten meal; Sunflower meal; Corn gluten meal; Lupin seed meal	(-1.0); 48.3; 61; (-0.9); 45.0; 65.2	750	53.8; 85.2; 118; 45.7; 72; 84.6
Verlhac (2007)	Soy protein concentrate; Pea meal; Faba bean meal;	29.9; 74.1; 47.8	750	46.9; 80.3; 69.9
Nile tilapia (Oreochromis niloticus)				
Phromkunthong et al. (2007)	Soybean meal; Palm kernel cake; Rice bran; Corn; Cassava	47.9; 25.5; 35.2; 23.6; 72.4	750	76.9; 50.4; 59.5; 58.3; 92.6
Sea bass (Dicentrarchus labrax)				
Papatryphon and Soares (2001)	Isolated soy protein; Soybean meal; Corn gluten meal; Wheat middlings	48; 59 ; 52; (-10)	1000	74; 87; 70; 11

The individual characteristics (Fish feed Ingredients, P availability (%) without phytase, Phytase dose FTU/kg, P availability (%) with phytase) are separated with semicolon (;) and listed in sequence.
NA: Not available

Enhancement of bioavailability of other nutrients and minerals

Supplementation of phytase can hydrolyse phytate and increase the concentration of minerals in plasma, bone and the whole body (Jackson et al., 1996; vanWeerd et al., 1999; Papatryphon and Soares, 2001; Debnath et al., 2005b; Liebert and Portz, 2005). Addition of phytase at a level of 1000 FTU/kg diet was sufficient to significantly increase Ca, Mg, and Mn content of bone in channel catfish; and addition of phytase at a level of 8000 FTU/kg feed significantly increased the bioavailability of naturally occurring Zn from feed (Yan and Reigh, 2002). Studies on rainbow trout showed that phytase supplementation increased the apparent absorption of Ca, Mg, Cu, Fe, Sr and Zn in low-ash soybean meal (Sugiura et al., 2001). Furthermore phytase supplementation in extruded soybeans increased ADC of Mg, Mn, and Zn significantly (Cheng and Hardy, 2003). Baruah et al. (2005) conducted an experiment on rohu fingerlings by supplementing the microbial phytase at the level of 0, 250, 500, 750, and 1000 FTU/kg diets. Phytase-supplemented groups in general recorded significantly ($P < 0.05$) higher percentage of bone ash and also higher concentration of bone Ca and P compared with the non-supplemented group. Bone ash and bone P content were found to be highest in dietary feed with phytase level of 750 FTU/kg diet, which did not differ significantly ($P > 0.05$) from those of fish on 500 FTU/kg diet. Bone Ca content was also highest with the inclusion of 750 FTU/kg diet. These results were similar to those observed for rohu (Baruah et al., 2005), common carp (Schäfer et al., 1995), and other fish species (Storebakken et al., 1998; Papatryphon et al., 1999; Yan and Reigh, 2002; Debnath et al., 2005b; Liebert and Portz, 2005). From these studies it can be concluded that bone ash and bone P are sensitive indicators of the P status in fish. This is because the P requirement for maximum bone mineralization is greater than maximum body weight gain. Insufficient P intake leads to the mobilization of P from the bone and transfer to soft tissues and metabolic processes (Baeverfjord et al., 1998). Increment in bone ash in fish fed phytase-supplemented diets is an indication that the mineral bioavailability was significantly increased by dietary manipulation (Baruah et al., 2005; Debnath et al., 2005b). Nwanna et al. (2007) conducted a feeding trial on common carp to evaluate the effect of wet-incubation of dietary plant feedstuffs with two different phytases, PtN (Natuphos®) and/or PtR (Ronozyme®), before pelleting, on mineral digestibility and mineral deposition in the fish. Pre-treatment of the feedstuffs with PtN and PtR at level of 4000 FTU/kg each, enhanced apparent digestibility of minerals and

their deposition in the fish. It increased mineral utilization, which resulted in increased growth and feed efficiency of the fish. Phytase supplementation also enhances digestibility of minerals which are bound to phytate. Apparent digestibility of Zn was significantly improved by addition of phytase to a semi-purified diet containing 50% soybean meal fed to rainbow trout while no dose dependent effect was observed (Cheng et al., 2004). Moreover, dietary phytase have been shown to increase the apparent availability of protein, ash, Ca, Cu, Mg, Fe, Sr and Zn in low ash diets while little effect was observed in high ash diets (Sugiura et al., 2001).

Supplementation of phytase (500 or 1000 FTU/kg feed) did not affect Zn availability in rainbow trout (Vielma et al., 2000). Cheng and Hardy (2004) reported that graded level of phytase inclusion in the rainbow trout diet did not affect body composition; whereas, it was effective in releasing most minerals and trace mineral. This indicates that supplementation of trace minerals in rainbow trout diets could be reduced when phytase is added in the diet. Masumoto et al. (2001) observed that P concentrations in whole body and plasma were higher in Japanese flounder fed a phytase supplemented. Phytase or monocalcium phosphate supplementation in carp diet increased body ash and P. Schäfer et al. (1995) observed that P excretion was lower by 30% on feeding a diets supplemented with phytase compared to a diet supplemented with monocalcium phosphate. A significant effect of phytase supplementation on whole body and bone mineralization was demonstrated in Asian catfish, pangus (*Pangasius pangasius*). A minimum dose of phytase at 250 and 500 FTU/kg feed increased the mineral absorption and bone ash contents in pangus (Debnath et al., 2005).

Nwanna et al. (2005) studied the effect of phytase in African catfish and reported that the effect of phytase at 8000 FTU/kg feed (raw soybean based diet) has no effect on growth performance, Mg and Zn but improved feed conversion and body P, Ca and Mn balance. Li et al. (2004) conducted an experiment on phytase supplemented to catfish diets and found that 250 FTU/kg increased feed intake, body weight gain, feed efficiency, and bone ash and P concentration.

Enhancement of protein and amino acid digestibility

Phytate can nonselectively bind to proteins and it has been shown to inhibit activities of enzymes including pepsin, trypsin and alpha-amylase (Liener, 1994) as well as to decrease protein digestibility. Dephytinization of dietary phytate by exogenous phytase accounts for increased protein utilisation in

common carp (Schafer et al., 1995), Atlantic salmon (Storebakken et al., 1998; Sugiura et al., 1998b), European seabass (Oliva-Teles et al., 1998), tilapia (Heindl, 2002) and pangus (Debnath et al., 2005b) by degrading the pre-formed phytate- protein complexes. Forster et al. (1999) assessed the potential of using dietary phytase to improve the nutritive value of canola protein concentrate diets for rainbow trout. Likewise, chemical and enzymatic processing of canola meal effectively reduced most of the anti-nutritional factors and enhanced canola meal digestibility in rainbow trout (Mwachireya et al., 1999). The digestibility and nutritional value of expeller and solvent-extracted Australian canola meals when included in the diets of juvenile red seabream (*Pagrus auratus*) was comparable to those of the fishmeal (Glencross et al., 2004).

In Crucian carp, phytase supplementation of 500 FTU/kg diet could improve digestibility of crude protein by 6.6% (Lie et al., 1999). Inclusion of soybean phytase in diet of Atlantic salmon improved protein utilisation parameters, ADC, and body levels of Ca, Mg and Zn and retention of P (Storebakken et al., 1998; Vielma et al., 1998, 2000). Moreover soyabean phytase in diet of flat fish, Greenback flounder (*Rhombosolea tapirina*) resulted in significant increase in nitrogen and mineral digestibility (Bransden and Carter, 1999). Phytase supplementation in expelled soybeans diet of rainbow trout increased ADC of amino acid significantly compared to raw soybeans but had no significant effect when added in extruded soyabean (Cheng and Hardy, 2003). However, spraying soybean meal-based diets with phytase improved protein digestibilities in rainbow trout (Vielma et al., 2004).

Protein digestibility in rainbow trout was significantly increased when fed a practical diet supplemented with 2000 FTU/kg and also when reared with soybean meal-based diets sprayed with phytase (Vielma et al., 2001, 2004). Although, protein digestibility was significantly influenced by phytase supplementation, protein retention efficiency was not enhanced in red sea bream fed soybean meal based diets supplemented with graded doses of phytase (Biswas et al., 2007b).

Phytase supplemented diet in Pangus increased apparent net protein utilisation (Debnath et al., 2005b) and apparent protein digestibility and were significantly ($P<0.01$) higher at a minimum supplement of 500 FTU/kg or higher in contrast to diet without phytase. Atlantic salmon was fed a diet based on canola meal with and without phytase and inorganic P supplementation. No effect on protein digestibility was observed (Sajjadi and Carter, 2004). However the impact of phytase on protein availability and utilisation in fish is somewhat contentious. There is discrepancy among

authors for the positive impact of phytase on protein and amino acid bioavailability. Research conducted on rainbow trout by Prendergast et al. (1994) and Teskeredzic et al. (1995) showed that pre-treatment of rapeseed protein concentrate with the enzyme phytase did not improve the protein utilisation by rainbow trout. Likewise, no positive effect of phytase on protein digestibility could be noted in rainbow trout (Lanari et al., 1998), Atlantic salmon (Storebakken et al., 1998) and striped bass (Papatryphon et al., 1999). Similarly Riche et al. (2001) reported that tilapia offered diet with and without phyatse showed no difference in protein utilisation, and also concluded that the available methionine and lysine decreased with increasing incorporation of phytase pretreated soybean meal. Phytase addition in poultry, pigs and swine diets also showed conflicting results as observed for fish. The probable reason for the neutral and/or negative interaction of phytase and amino acid is that removal of phytate may increase the efficiency of other anti-nutritional factors and protect amino acids from degradation, or decrease leaching of water soluble components (Cao et al., 2007). More research is needed to obtain a better insight into the mechanisms for the phytase-protein interaction and availability of proteins and amino acids.

Enhancement of growth performance

Supplementation of phytate-containing diets with phytase neutralises the negative effects of phytate and increases growth in fish. Positive impact of phytase on growth of fish has been reported by a number of authors: Jackson et al. (1996) in channel Catfish, VanWeerd et al. (1999) in African catfish, Papatryphon and Soares (2001) in striped seabass, Vielma et al. (2000) in rainbow trout, Debnath et al. (2005) in pangus catfish, Liebert and Portz (2005) in nile tilapia, Nwanna et al. (2005) in common carp and Baruah et al. (2007a) in rohu. These authors have demonstrated phytate hydrolysis in plant-based diets by phytase and improvement of fish growth and mineralization. Moreover, fish fed the diets containing $\geq$250 FTU phytase/kg feed consumed more feed and gained more weight in comparison to fish fed the basal diet containing no microbial phytase (Li and Robinson, 1997). Vielma et al. (2004) reported increase in weight gain from 243 to 459 % in rainbow trout fed soybean meal-based diets with phytase and P supplementation. Similar results have been reported for salmonids (Rodehutscord and Pfeffer, 1995; Riche and Brown, 1996; Sugiura et al., 2001). Studies on carp showed that incorporation of microbial phytase in basal diets (soyabean meal based diets) improved overall growth

performance in rohu fingerlings (Baruah et al., 2007a). Nwanna et al. (2007) reporetd higher growth performance of common carp fed a diet (incubated plant feed ingredients) containing phytase than another diet (without incubated plant feed ingredients) with and without phytase. This was possible because incubation process reduce phytate content (about 40%) of diet, improve P and mineral utilisation as compared to untreated diet. Liebert and Portz (2005) reported that the optimal growth of Nile tilapia is achieved by phytase supplementation at 750–1250 FTU/ kg in plant-based diets, whereas, Cao et al. (2008) observed that 1000 FTU/ kg feed gives better growth performance and feed conversion in the same fish species. Vielma et al. (1998) observed that the inclusion of phytase at 1500 FTU/kg feed in comparison to no inclusion of phytase improved the weight gain of rainbow trout. Specific growth rate and feed conversion ratio were significantly improved when trout were fed with phytase supplemented diet at 2000 FTU/ kg feed (containing 55% of soybean meal) (Vielma et al., 2001). Conversely, no substantial effect of phytase addition was observed on performance of large sized rainbow trout (initial body mass 250 g and final body mass about 2 kg) fed a diet supplemented with phytase at 1000 FTU/kg (Vielma et al., 2000).

Oliva-Teles et al. (1998) reported that there was no effect on growth performance, protein digestibility, energy retention on phytase supplementation in the diet of sea bass. Forster et al. (1999) and Sajjadi and Carter (2004) did not report any improvement on the growth of rainbow trout and Atlantic salmon when fed with canola protein concentrate incorporated with phytase. Similarly Masumoto et al. (2001) and (Yoo et al., 2005) reported no effect of dietary phytase on weight gain of Japanese flounder and Korean rockfish (*Sebastes schlegeli*). The discrepancy in above findings may be associated with differences in their diet composition and also with different rearing conditions (Baruah et al., 2007a). These contrasting results also suggest that dietary substrate levels of phytate are an important determinant of the magnitude of phytase responses. These reports confirmed that supplementing exogenous microbial phytase in feed ration exhort an enhancement in growth rate and performance which could be be attributed to various factors, in individual or/and combined form, namely better bio-availability of P (Rodehutscord and Pfeffer, 1995; Vielma et al., 2000; Baruah et al., 2007a) and minerals (Vielma et al., 2004, Debnath et al., 2005b), improved protein digestibility (Vielma et al., 2004; Liebert and Portz 2005, Debnath et al., 2005a; Baruah et al., 2007a) and increased absorption of nutrients owing to well functioning of the pyloric cecal region of the intestine (NRC, 1993).

Reduction in pollution from aquaculture operation

Discharge of high levels of soluble P from fish culture systems into open water environment stimulate phytoplankton growth, resulting in wide fluctuations in dissolved oxygen concentrations (Li et al., 2004). Many studies have reported a clear effect of phytase supplementation in reducing P excretion from fish. Ai et al. (2007) showed that the total P effluent was significantly lowered when fish reared with a diet supplemented with phytase (200 FTU/kg). Likewise, soybean meal based diets supplemented with phytase decreased the excretion of P from red sea bream and maximum reduction was reported at 2000 FTU/kg feed (Biswas et al., 2007b). Similar results were also observed for rainbow trout (Sugiura et al., 2001). Faecal waste of P in rainbow trout was reduced by phytase supplementation in soybean protein concentrate diet (Viema et al., 1998) and a significant decrease was noticed when practical diet supplemented with phytase at a level of 2000 FTU/kg (Vielma et al., 2001) was fed. Phosphorus concentration in faecal matter was reduced when trout were fed a diet with phytase supplemented at 500 and 1000 FTU/kg compared to non-supplemented feed (Verlhac et al., 2007).

Storebakken et al. (2000) observed that phytase treated soy protein concentrate based diet induced significantly lower excretion of P compared to when a fishmeal diet was fed to Atlantic salmon. P content of faeces was also reduced in Atlantic salmon fed a phytase supplemented diet (Sajjadi and Carter, 2004). In juvenile catfish, *Ictalurus punctatus*, Li and Robinson (1997) reported that microbial phytase supplementation in diets reduced the excretion of faecal P by about 60%. Many studies suggest potential environmental benefits to the extent of 30 to 40% reduction in P excretion (Omogbenigun et al., 2003).

Optimum dose of phytase in fish diet

In dose response studies, phytase addition between 250 and 1500 FTU/kg feed is usually considered as optimum in many fish species as shown in Table 8. Optimum dose of phytase level in Nile tilapia diet, is 750 –1000 FTU/kg feed (Liebert and Portz, 2005). Whereas, Furuya et al. (2001) observed that phytase supplementation between 500 and 1500 FTU/kg diet is required to maintain growth of Nile tilapia fed with plant-based diets. Many researchers (Jackson et al., 1996; Li and Robinson, 1997) observed that 250–500 FTU/kg diet to plant based diets of phytase is adequate to

effectively improve the growth performance, bioavailability of nutrient and phytate P of channel catfish. For stripped bass, the phytase supplementation of 1000 FTU/kg is adequate to maintain growth rate and health comparable to an inorganic-P supplemented diet (Papatryphon et al., 1999). Phytase supplementation of 500 FTU/kg in Crucian carp diet could improve growth rate, minerals utilization, phytate-P utilization (Lie et al., 1999). Baruah et al. (2007a) observed maximum apparent digestibility of P, crude protein and maximum growth when phytase-supplemented diets contained 750 FTU/kg in feed of rohu (agastic fish). The dietary microbial phytase supplementation at 500 FTU/kg diet improved growth in Pangus fingerlings (Debnath et al., 2005). Similarly, 1000 FTU/kg of phytase in the diet in Korean rockfish exhibited superior growth performance than without phytase diet (Yoo et al., (2005). These findings indicate that the optimal dose of phytsase varies with different fish species, diet formulations and phytase sources. Cao et al. (2007) mentioned that the optimum dose depends on many factors such as fish species, different phytase sources, diet formulation (amount of substrate for phytase) and selected response parameters. Thus, the dose of phytase addition in each fish diet should be adjusted based on fish species, sources of phytase, diet formulation, phytate content (Table 8).

There is no doubt that phytases have an array of applications in aqua industries; and there is a wide scope for enhancing worthiness of such enzymes by manipulating their properties such as enhancing heat resistance, increasing storage life, making it work at wide pH range and decreasing production price.

Table 8 Optimum dose of phytase with reference to body mass of fish and phytate level in diets of different fish species

Initial fish weight (g)	Phytate-P (%)	Phytate (%)*	Phytase (FTU/kg feed)	Fish species	References
13.5	0.26	0.98	750	Nile tilapia	Liebert and Portz, 2005
68.8	0.27	1.01	1000	Nile tilapia	Portz and Liebert, 2004
5.5	0.46	1.73	1000	Red tilapia	Phromkunthong and Gabaudan, 2006
-	0.49	1.84	1000	Common carp	Bai et al., 2003
12.6-13.7	0.39	1.46	750	Indian major carp (Rohu)	Baruah et al., 2007b
6.8	0.41	1.54	1000	Channel catfish	Jackson et al., 1996
44-60	0.19	0.71	750	African catfish (*Clarias garipinus*)	VanWeerd et al., 1999
2.0	0.2	0.75	500	Asian catfish (*P. pangasius*)	Debnath et al., 2005
24.5	0.35	1.31	1000	Striped bass	Papatryphon et al., 1999
18.0	0.28	1.05	500	Rainbow trout	Forster et al., 1999

*Phytate = Phytate P x 3.75

Conclusions

Phytases have been mainly, if not solely, used as an animal feed additive in diets largely of swine and poultry, and to some extent of fish in commercial production systems. In aquaculture the plant protein sources (meals or concentrates) are being increasingly used due to their relatively low cost and ample availability. Use of plant protein based diets in aquaculture is inevitable in the near future since fishmeal availability is deceasing and to sustain aquaculture industry alternative protein sources are required. Plant ingredients have limitations due to the presence of phytate and other antinutritional factor that restrict their inclusion in fish diets. Phytate-rich plant ingredients restrict the bioavailability of P along with other minerals. A great potential exists for using phytases in plant protein based diets, which can enhance the digestibility and bioavailability of P and trace elements, reduce the amount of inorganic-P supplement in the diet to maximize growth and bone mineralization, and markedly decrease P load to aquatic environment. Although some information is available on increase in nitrogen and energy utilization on addition of phytase, more research is required to conclusively state that phytase enhance protein and energy utilization. The optimum doses for phytase in fish diet to replace inorganic P are not yet known. Phytase supplementation in fish feed could also increase

bioavailability of nitrogen, leading to reduction in feed cost. Though the role of phytase supplementation has been well proven and documented for poultry and pig production, its efficient use in aqua feed requires further work. Because of the pH specificity of phytase, the addition of organic acid along with phytase, especially in agastric fish, like carp is of special interest, and needs further research. In addition, there is a need to enhance awareness among fish nutritionists and the fish feed manufacturing industry on the use of phytase as an effective and efficient approach in the formulation of cost effective, growth promoting and low polluting fish feeds based on plant protein sources, for profitable and sustainable aquaculture production.

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Chapter 1

Section 3

Non-starch Polysaccharides and their role in fish Nutrition - A Review

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Non-starch Polysaccharides and their role in fish Nutrition - A Review

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Abstract

The success and sustainability of aquaculture depends on minimizing the operational cost of feed that in general comprises 50 to 60% of the total cost in intensive farming. The major feed ingredient, fish meal, is expensive and there is increasing competition with other livestock industries for the available static supply of fish meal. Hence, the incorporation of plant-derived materials (cereals, legumes, oil seed cake, leaf meal) in fish feeds is getting increasing attention. One of the main constraints in the utilization of plant ingredients in aquaculture is the presence of indigestible carbohydrates, which consist primarily of non-starch polysaccharides (NSPs) and are often referred as antinutritional factors. These form a part of the cell wall structure of cereals and legumes. The presence of NSPs in the diet interferes with feed utilization and adversely affects performance of the animal. Supplementation of NSP-degrading enzymes in feed mitigates the adverse effects of NSPs. The effects of NSPs in pigs and poultry have been widely studied; however little information exists for fish. This review synthesizes the available information on fish and highlights the knowledge gaps. It is hoped that this review will provide a momentum to the research on the roles of NSPs in fish nutrition and physiology and on the efficient use of NSP-degrading enzymes. The effectiveness of these enzymes depends on the proper selection of the enzymes, which among many factors depends mainly on the nature or type of NSPs in fish feed. This review, therefore, also focuses on classification and structure of NSPs. The available information on fish, synthesized in this review, would also contribute to enhanced use of plant based protein sources in fish diets by the fish feed industry.

Keywords: Fish feed, Non-starch polysaacharide, Anti-nutritive effects, Nutrient metabolism, Immunostimulants

Contents

1. Introduction

The growing demand of fish and limited supply from wild capture are giving momentum to the development of aquaculture. The progress of culture based fisheries is determined mainly by the quality of feed delivered. Fish meal (FM) having high protein content and favourable amino acid profile is highly preferred by fish culturists. The total world FM production is about 5 to 6 million tonnes per annum, which account for 4 to 5% of total fish production of 144 million metric tonnes (FAO, 2008). In spite of being the most important protein source in commercial feeds, production of FM is restricted to certain part of world only; as a result it is becoming more expensive and slowly getting out of reach by many aquaculture practising countries. Furthermore, abundance of FM appears to be ending since the level of FM production is expected to remain stable over the next 10 years (Mazurkiewicz, 2009). As a consequence, the fisheries sector may have to undergo a recession phase in coming years. In order to provide sustainability, therefore, it is utmost important to lower the inclusion of FM in aquafeeds and replace it with plant-based sources. The higher availability and low cost of plant based feeds count for their advantage over FM. Although the carbohydrate component of grains and legumes may provide a cheap source of dietary energy for fish, it is poorly utilized compared to protein and lipid by most fish species (Allan et al., 2000). In addition, the quality and level of protein in, and palatability of, plant based protein sources are generally inferior to FM. However, the main limitation with the plant-derived materials such as legume seeds, soybean meal, different types of oilseed cake, canola or rapeseed meal, sunflower oil cake, root tuber meal is the presence of a wide range of antinutritional factors such as, protease inhibitors, non-digestible carbohydrates, lectins, saponins, phytates and possibly allergenic storage proteins (Francis et al., 2001). In addition to these factors that hamper digestion in fish (Storebakken et al., 1998; Refstie et al., 1999), non-starch polysaccharides (NSPs) play an important role. In general, NSPs are a complex group, composed predominantly of linked monomers of hexoses and pentoses, e.g. galactose, glucose, arabinose, xylose and mannose (van Barneveld, 1999). The NSP content in wheat and lupin may account for 25% and 50% of the total grain and seed respectively and acts as the primary energy storage carbohydrate in lupin (van Barneveld, 1999). However, in fish and other monogastric animals enzymes such as β-glucanases or β-xylanases that digest NSPs are scarce or non-existent (Kuz'mina, 1996). Consequently, the dietary NSPs remain indigestible and cannot be used as

an energy source. The additions of NSP containing feedstuffs to the diets of monogastric animals, for example, broiler and swine reduces the apparent digestibility of diet and have negative impacts on growth. However, only a limited number of such studies have been conducted in fish. Refstie et al. (1999) have demonstrated negative effects of NSPs on digestion and absorption of lipid in Atlantic salmon. Non-starch polysaccharides are also thought to be responsible for a slower rate of gastro-intestinal passage of NSP containing diets in fish (Storebakken et al., 1999). Feeding of salmonids with diets incorporated with NSP has been shown to reduce the availability of nutrients (Storebakken and Austreng, 1987).

At present approximately 2.0 billion tonnes of cereal grains and 140 million metric tonnes of legumes and oil seeds are produced world wide per year and approximately 230 million metric tonnes of fibrous materials are produced as a by-product. This wide availability of plant resources can very well be utilised as cheaper fish feed ingredients through proper management of the NSPs in these plant materials. Feed processing and utilisation of exogenous enzyme (β-glucanase and β-xylanases) have been used to decrease the negative effects of NSP and thus to improve the nutritive value of feed. Moreover, there is a contemporary trend to seek for feed ingredients which may contribute to better health by interferring with colonisation and microbial growth in the gut. In this regards NSPs such as β-glucans and mannose have been probed to have immunostimulanting activities (Kumar et al., 2005).

2. Classification of non-starch polysaccharide

The term NSP covers a large variety of polysaccharide molecules excluding α-glucans (starch). NSPs have been classified based on different criteria. In old days, the classification was based originally on the methodology used for extraction and isolation of polysaccharides. The residue remaining after a series of alkaline extractions of cell wall materials was called cellulose, and the fraction of this residue solubilised by alkali was named hemicellulose (Neukom, 1976). Another classification was based on the differences in solubility. This classification includes three categories of NSP namely crude fibre (CF), neutral detergent fibre (NDF) and acid detergent fibre (ADF). CF refers to the remnants of plant material after extraction with acid and alkali and includes variable portions of the insoluble NSP. NDF comprises the insoluble portion of the NSP plus lignin while ADF refers to a portion of insoluble NSP comprised largely, but not solely, of cellulose and lignin. However, this basis of categorization lacked precision with respect to both

chemical structures and biological functions and moreover the nutritional significance of values obtained using this method in monogastric nutrition therefore is doubtful.

As a consequence of ambiguity for NSP classification, Bailey (1973) proposed a clearer classification of NSP. Under this division NSP falls into three main groups namely cellulose, non-cellulosic polymers and pectic polysaccharides. Arabinoxylans, mixed-linked β-glucans, mannans, and xyloglucan come under the category of non-cellulosic polymers while polygalacturonic acids substituted with arabinan, galactan and arabinogalactan are included in the group of pectic polysaccharides (Table 1).

Table 1 Classification of non-starch polysaccharides

Catergory	Monomeric residue	Linkage	Sources
Cellulose	Glucose	β-(1→4)	Most cereals and legumes
Non-cellulosic polymers			
Arabinoxylans	Arabinose and Xylose	β-(1→4)-linked xylose units	Wheat, rye, barley, oat, rice, sorghum
Mixed-linked β-glucans	Glucose	β-(1→3) and β-(1→4)	Oat and barley
Mannans	Mannose	β-(1→4)	Coffee seed
Galactomannans	Galactose and mannans	β-(1→4)-linking mannan chains with α-(1→6)-linked galactosyl side groups	Locust bean gum and guar gum
Glucomannans	Glucose and mannans	β-(1→4)-linked mannan chain with interspersed glucose residues in the main chain	Sugar beet pulp, lilies, irises
Pectic polysaccharides			
Arabinans	Arabinose	α-(1→5)	Cereal co-products
Galactans	Galactose	β-(1→4)	Sugar bean meal, sugar-beet pulp
Arabinogalactans (Type I)	Arabinose and Galactose	β-(1→4) galactan backbone substituted with 5-linked and terminal arabinose residues	Grain legumes
Arabinogalactans (Type II)	Arabinose and Galactose	β-(1→3,6)-linked galactose polymers associated with 3- or 5-linked arabinose residue	Rapeseed cotyledon

2.1. Cellulose

Cellulose is a complex polysaccharide, consisting of 3,000 or more β-(1→4) linked D-glucose units. This bond generally makes cellulose ingestible for monogastric animals, due to lack of the enzyme cellulase in the digestive tract. Cellulose is the basic structural component of plant cell walls and comprises about 33% of all vegetable materials. It is the most abundant of all naturally occurring organic compounds, comprising over 50% of all the carbon in vegetation.

Cellulose quantity in whole grains can vary from species to species and is largely a consequence of the thickness of the husk and seed coat. The one which contain more cellulose tends to have thicker and stronger cell wall. Seed endosperms cells have only thin cell walls and in a well-filled grain the proportion of cellulose to starch, or other reserve polysaccharide, should be low (Brett and Waldron, 1996).

Cellulose is a straight chain polymer where no coiling or branching occurs, and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues. The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on the same or on a neighbour chain, holding the chains firmly together side-by-side (Figure 1). The chains can stack together to form larger microfibrils which make cellulose highly insoluble in water, but can swells in concentrated sodium hydroxide solutions. Through the use of hydrogen-bond breaking reagents such as N-methylmorpholino-N-oxide, cellulose can be brought into solution. Moreover, to be used as a dietary supplement, cellulose-rich maize bran can be converted to a cellulosic gel through thermal and shear treatments, followed by alkaline peroxidation and shearing (Fincher and Stone, 2004). Cellulose microfibrils may also associate with water and matrix polysaccharides such as the (1-3, 1-4)-β-D-glucans, heteroxylans (arabino-xylans) and glucomannans (Fincher and Stone, 1986a).

The hydrolysis of cellulose is limited in animals but can be degraded by some microbes such as bacteria and fungi (Xiao and Xu, 2002). Since fish lack cellulase in intestine, cellulose is indigestible in fish; and therefore it is of no nutritional value in formulated fish feeds. However, developments in fish nutrition and aquaculture technology have encouraged the use of cheaper feed ingredients; including those containing cellulose. Therefore, it is important to develop methods for the improvement of enzymatic hydrolysis of cellulose.
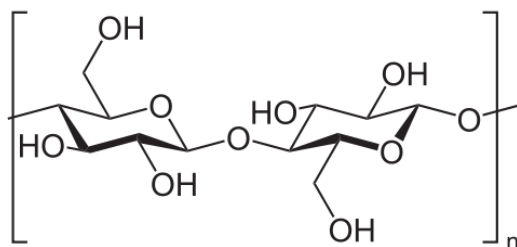
**Fig. 1.** Chemical structure of cellulose

2.2. Non-cellulosic polymers

2.2.1. Arabinoxylans

Arabinoxylans have been identified in a variety of tissues of the main cereals: wheat, rye, barley, oat, rice, sorghum (Fincher and Stone, 1986b). Although these polysaccharides are minor components of entire cereal grains, they constitute an important part of plant cell walls. Thin walls that surround the cells in the starchy endosperm and the aleurone layer in most cereals consist predominantly of arabinoxylans (60-70%); exceptions are endosperm cell walls of barley (20%) and rice (40%) (Fincher and Stone, 1986b). Non-endospermic tissues of wheat, particularly the pericarp and testa, also have very high arabinoxylan content (64%) (Selvendran and DuPont, 1980).

The structures of cereal arabinoxylans are composed predominantly of two pentoses, arabinose and xylose (Izydorczyk and Biliaderis, 1995). Their molecular structure consists of a linear backbone of β-(1-4)-linked xylose units to which substituents are attached through O-2 and O-3 atoms of the xylosyl residues (Perlin, 1951) (Figure 2). Arabinoxylans form highly viscous aqueous solution which may create problems when wheat is used in processes such as production of grain spirits. The degree of arabinose substitutions will influence the conformation adopted by arabinoxylans and the resulting viscosity of solutions.

Morever, when the arabinose residues are stripped off the xylan backbone (using oxalic acid), aggregation appears at a xylose/arabinose ratio of about four and precipitation occurs when this is increased above ten (Sternemalm et al., 2008). The loss of arabinose side chains also correlates with a loss in water binding capacity (Sternemalm et al., 2008). When this is absent the molecule binds less water and becomes less soluble. The degree of arabinose substitution has little influence on the overall semi-flexible conformation and hence the viscosity (Sternemalm et al., 2008). However, in

wheat flour, the distribution of the type of substitution is not random but the distribution of substituted (irrespective of of the substitution type) residues along the chain appears random (Dervilly-Pinel et al., 2004). The arabinose residues may also be linked to other groups attached such as glucuronic acid residues, ferulic acid crosslinks and acetyl groups (Sørensen et al., 2007).
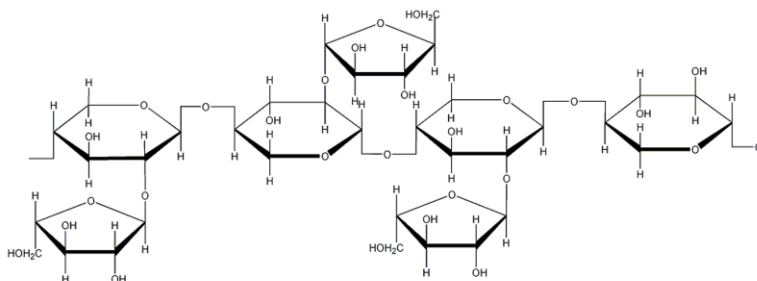
Fig. 2. Chemical structure of the arabinoxylan

Most of the arabinoxylans in cereal grains are insoluble in water because they are anchored in the cell walls by alkali-labile ester-like cross links (Mares and Stone, 1973). But the arabinoxylans not bound to the cell walls can form highly viscous solutions and they can absorb about ten times their weight of water. In the presence of oxidative agents, such as H_2O_2/peroxidase, arabinoxylans can rapidly develop a gel network as a result of the re-establishment of cross-links (Geissmann and Neukom, 1973). The formation of gel coincides with the disappearance of ferulic acid group of oxidative agent and fully cross-linked arabinoxylans hold up to 100 g of water per g polymer (Izydorczyk and Biliaderis, 1995).

Apart from covalent cross links, arabinoxylans may also form "junction zones" by intermolecular hydrogen bonding between unsubstituted regions of the xylan backbone (Fincher and Stone, 1986b). Not only can arabinoxylans establish covalent cross links, they may also form "junction zones" by intermolecular hydrogen bonding between unsubstituted regions of the xylan backbone (Fincher and Stone, 1986b; Sørensen et al., 2007). Such type of interaction of arabinoxylans may be of great importance in determining their conformational changes and solubility properties, and thus their anti-nutritional activities.

2.2.2. Mixed-linked β-glucans

The physical and physiological properties of β-glucans are of commercial and nutritional importance. Increasing interests in β-glucans during the last

two decades are largely due to their acceptance as functional, bio-active ingredients (Cui and Wood, 2000). Cereal β-glucans have been associated with the reduction of plasma cholesterol and a better control of postprandial serum glucose levels in humans and animals (Bhatty, 1999). The mixed-linked- β-glucans are unique to the Poales, the taxonomic order that includes cereal grasses. They are also known as cereal β–glucans located in the subaleurone and endospermic cell wall (Ebringerova´, 2006) where they associate with cellulose microfibrils during cell growth. Oat and barley contain 3 to 12% and more depending on the cultivar. However, the ubiquitous structural feature of these polysaccharides is well established. The structural features of β-glucans are important determinants of their physical properties and functionality, including their physiological responses when they are considered as ingredients in cereal-based foods and other formulated products. These features include ratios of linkages joining glucose units, presence and amount of long cellulose-like fragments and molecular size (Izydorczyk and Biliaderis, 2000). In general, it consists of a linear chain of glucose units joined by both β-(1→3) and β-(1→4) linkages (Bengtsson et al., 1990) (Figure 3).

It should be noted that the mixed-linked β-glucans and cellulose both are comprised of β-linked glucose units but there is little similarity in their physical properties. Cellulose consists only of (1→4)-β-linkages and is therefore stiff, highly crystalline and non-soluble. On the other hand, the β-(1→3)-linkages break up the regular structure of β-(1→4) chains of the β-glucan molecule and make it soluble and flexible (Anderson and Bridges, 1993). Therefore, the β-glucans are often isolated by aqueous extraction followed by precipitation with ammonium sulphate (McCleary, 1986).

The potential application of β-glucans as food hydrocolloids has been also proposed. In addition, β-glucans have been shown to form gel under certain conditions (Lazaridou et al., 2003) and can be utilized as thickening agents to modify the texture and appearance in gravies, salad dressings and ice cream formulations (Wood, 1986) or may be used as fat mimetics in the development of calorie reduced foods (Inglett, 1990).
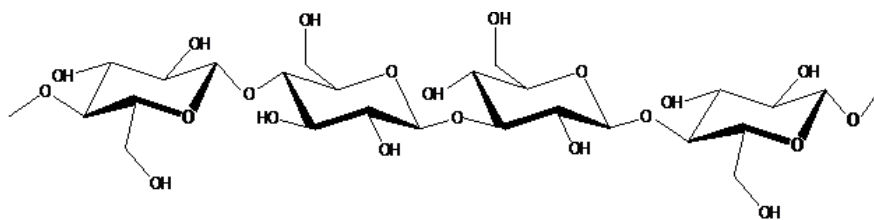

Fig. 3. Primary structure of β-D-glucans (Ebringerova´, 2006)

2.2.3. Mannans

Linear chains of β-(1→4) mannan are found in the cell walls of the coffee seed endosperm (Wolfrom et al., 1961). In most cases, these polysaccharides are highly insoluble in water and very dense. Accordingly, it has been suggested that the mannans forms the molecular basis for the hardness of the plant. Mannan has also been reported to be present in the cell walls of several siphonaceous green algae in the families *Acetabularia, Codium and Halicoryne* (Frei and Preston, 1968). Furthermore, mannan is also found in some red algae, such as *Porphyra umbilicalis* (Jones, 1950). In some of these algae, mannan is the main structural polymer and displays microfibrillar morphology (Chanzy et al., 1984).

The mannan-type hemicelluloses, can be divided into two groups: (i) galactomannans and (ii) glucomannans (Ebringerova´, 2006)

2.2.3.1. Galactomannans

Galactomannans are reserve polysaccharides in the seed endosperm of leguminous plants (Leguminosae). They are water soluble and can imbibe water, thus providing a water-holding function for the seed (Reid, 1985). They are composed of β-(1→4)-linked mannan chains with α-(1→6)-linked galactosyl side groups (McCleary, 1985) (Figure 4). Both the solubility and the viscosity of the galactomannans are influenced by the mannose to galactose ratio, which can vary from 1 to 5 (Reid, 1985). Furthermore, the distribution of the substituents can vary considerably, which also affects the physical properties of galactomannans (Daas et al., 2000). Two of the most well characterised galactomannans are found in locust bean gum and guar gum, isolated from the seeds of *Ceratonia siliqua* and *Cyanaposis tetragonolobus*, respectively (Rol, 1973).
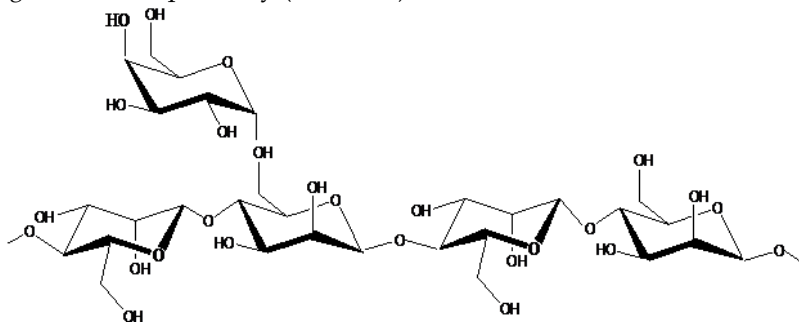

Fig. 4. Primary structure of Galactomannans (Ebringerova´, 2006)

2.2.3.2. Glucomannans

Glucomannans are present as a minor component in cereal grains (Fincher & Stone, 1986b) and act as storage polysaccharides in the seeds of certain annual plants, for example some lilies *(Liliaceae)* and irises *(Iridaceae)* (Meier and Reid, 1982). Glucomannans are also found in the bulbs, roots and tubers of several other types of plants. Many of these glucomannans are water soluble and are composed of a β-(1→4)-linked mannan chain with interspersed glucose residues in the main chain and are often acetylated (Figure 5). The mannose to glucose ratio ranges from 4 to 1 to below 1 to 1 (Meier and Reid, 1982). The structural and physicochemical properties of the storage glucomannan, known as 'konjac mannan' in food industry, have been reinvestigated (Nishinari et al., 1992). The results indicate that this polysaccharide, composed of glucose and mannose in the ratio 1 to 1.6, has the backbone slightly O-acetylated, and branched (about 8%) at position 6 of glucopyranose residues. The branches terminated by both glucopyranose and mannopyranose have been suggested as a new structural feature.
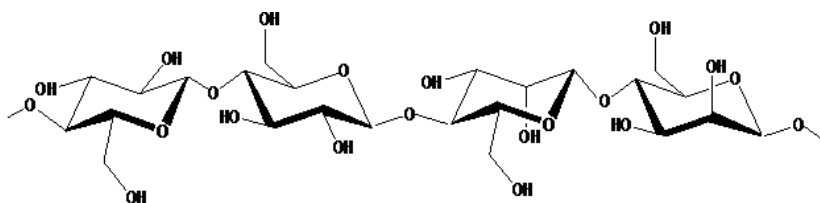
Fig. 5. Primary structure of Glucomannans (Ebringerova´, 2006)

2.3. Pectic polysaccharides
Pectin consist mainly of D-galacturonic acid (GalA) units (Thakur et al., 1997; Ridley et al., 2001) as the backbone, joined in chains by means of α -(1-4) glycosidic inkage. These uronic acids have carboxyl groups, which in the presence of divalent cations (usually calcium) has considerable effect on viscosity, solubility, and gelation formation (Thakur et al., 1997).

Arabinans, galactans and arabinogalactans

These three classes of polymers together constitute the neutral pectic substances. Pure arabinans and galactans are present in plant cell wall but in a very low amount. The arabinans are polymers of (1→5)-α-L-arabinose residues with some degree of branching through O-2, O-3 or both positions.

Galactans are polymers of β-1,4 linked linear polymers, which can present with small amount of 6-linked residues (Ghosh and Das, 1984).

The arabinogalactans occur in two distinct types in plant cell walls. Type I which is very common in grain legumes, is characterized by β-(1→4) galactan backbone substituted with 5-linked and terminal arabinose residues (Cheetham et al., 1993). The type II arabinogalactan is commonly found in rapeseed cotyledon (Siddiqui and Wood, 1972). Type II is characterised by β-(1→3,6)-linked galactose polymers associated with 3- or 5-linked arabinose residue. Unlike type I arabinogalactans, type II are not a structural component of the cell wall but are thought to be associated with extracellular space and with plasmalemma. However, a low molecular weight type II arabinogalactan associated with a hydroxyproline-rich peptide (arabinogalactan proteins, AGPs) has also been isolated from wheat flour (Fincher and Stone, 1974).

3. Non-starch polysaccharides

Non-starch polysaccharides are complex polysaccharides other than the starch. These polysaccharides are typically long polymeric carbohydrate chains containing up to several hundred thousand monomeric units. Non-starch polysaccharides can comprise up to 90% of the cell wall of plants (Selvendram and Du pont, 1980). The most abundant plant cell wall NSPs include cellulose, hemicellulose and pectins; while fructans, glucomannans and galactomannans belongs to the group of NSP that is not so abundant as cellulose, hemicellulose or pectins and serve as the storage polysaccharides within the plants. Mucilages, alginates, exudates gums, β-glucans and various modified polysaccharides are other constituents of the non-starch polysaccharides (Asp et al., 1992). The physiological impact of individual NSP is dependent on the sugar residues present and nature of the linkage present between these residues. However, NSPs differ from starch not only in the type of monomers present but also differ by the number and type of monomeric units linked together, the order in the chain and the types of linkages between the various monomers. Starch is composed entirely of glucose monomers, which are linked by α-glycosidic bonds while NSPs are composed of different kinds of monomers, which are linked predominantly by β-glycosidic bond. The difference in bonding structure has profound effects on digestibility, as different classes of enzymes are required for the hydrolysis of α- and β-glycosidic bond. The predominant starch digestive enzymes are α-amylase (1, 4-α-d-glucan glucanohydrolase), α-glucosidase (1, 4-α-glucosidase) and oligo-1-6-glucosidase. In combination, these

enzymes specifically hydrolyse the α-glycoside bonds of starch to yield glucose. The activity of these enzymes in fish intestine varies with fish species, and is based on the feeding habits. Activity of these enzymes is high in herbivorous, medium in omnivorous and low in carnivorous fish (Kuz'mina 1996; Krogdahl Hemre and Mommsen, 2005). On the other hand, the enzyme required to digest NSP such as β-glucanase and β-xylanases are very scarce or even absent among fish species (Kuz'mina, 1996).

Apart from NSPs, other unavailable carbohydrates in food are oligosaccharides, fructans, lignin and resistant starch. However, NSPs along with lignin and resistant starch are the major constituents of total dietary fibre. Lignin is a high molecular weight polymer composed of phenylpropane residues formed by the condensation of the aromatic alcohols, cinnamyl, guaiacyl and syringyl alcohols (Southgate, 1993). Resistant starch can be defined as that fraction of dietary starch, which escapes digestion in the small intestine. It is the sum of starch and degradation products of starch.

4. Non-starch polysaccharides in fish feed

The NSPs in the aquaculture feeds are present as an integrated part of the cell wall of plant ingredients and also in a purified soluble form such as guar gum to stabilize the pellet. In general NSPs are present in two wide catergories of crops namely cereals grains and legumes. Non-starch polysaccharides in cereal grains are composed predominantly of ß-glucans, arabinoxylans, and cellulose. Wheat, rye and triticale contain substantial amounts of both soluble and insoluble NSP while very low levels of NSPs are present in corn and sorghum. Grain legumes also contain considerable amounts of NSPs. Pectic polysaccharides are the main NSPs present in the cotyledon of legumes. Cellulose and xylans, besides being the major NSPs in cereal grains, are only found in the hulls or husks of most legumes.

Apart from cereals and legumes, NSPs are also present in other parts such as roots, tubers and leaves. These ingredients are used in many countries as possible feed resources. The NSPs content of ingredients used as aqua feeds has been presented in Table 2. Moreover, an extensive report on carbohydrate and lignin contents of plant materials used in animal feeding is already been published by Bach Knudsen (1997).

Table 2 Non-starch polysaccharide content (g/kg dry matter) of ingredients used in fish feed

Ingredients	Cellulose	Total NSP	Lignin	Soluble NSP[a]	Insoluble NSP
Cereals					
Maize	22	97	11	9	66
Wheat	20	119	19	25	74
Wheat bran	72	374	75	29	273
Rye	16	152	21	42	94
Barley-hulled	43	187	35	56	88
Barley-hulless	10	124	9	50	64
Corn gluten	75	351	_	34	242
Oats-hulled	82	232	66	40	110
Oats-hulless	14	116	32	54	49
Oat hull meal	196	505	148	13	295
Legumes / Seed meal					
Peas	53	180	12	52	76
Soyabean meal	62	217	16	63	92
Rapeseed meal	52	220	134	55	123
Lupins	131	405	_	134	139
Sunflower cake	123	315	_	57	136
Jatropha curcas kernel meal (non-toxic genotype)	56	136[b]	-	-	-
Detoxified *Jatropha curcas* kernel meal (toxic genotypes)	65-76	160[b]	-	-	-
Detoxified *Jatropha curcas* protein isolate (toxic genotypes)	-	105[b]	-	-	-
Miscellaneous					
Sugar beet pulp	195	779	35	407	177
Cottonseed cake	92	257	_	61	103
Cottonseed meal	90	283	_	66	127
Alfafa leaf meal	139	329	_	77	113

[a] Non-cellulosic polysaccharides. [b] Our unpublished data

5. Methods for non-starch polysaccharides quantification

It is difficult to develop an accurate analytical method for estimating NSPs content in feed. This is because of the complexity and diversity of the polysaccharides involved. In general gravimetric and monomeric component analytic approaches are used for quantifying NSPs. The gravimetric method of fiber analysis assumes that all residues are present in fiber, whereas component analysis quantifies the amount of constituent sugars present in a substrate and then, via summation determines the total NSP concentration.

5.1. Gravimetric analysis

Gravimetric method is the traditional way of fiber analysis, which involves chemical or enzymatic solubilization of dietary protein, starch and fat, followed by weighing of the insoluble residue. The crude fiber estimation is an example of gravimetric analysis. However, it is not an accurate estimation of total NSPs since the recovery of cellulose, hemicelluloses and lignin is low (Van Soest and McQueen, 1973). The refinement form of gravimetric crude fibre analysis is the detergent method of fiber analysis, which delineates the form of fiber present in feedstuffs. The two forms of detergent fiber are neutral detergent fiber (includes cellulose, hemicellulose and lignin) and acid detergent fiber (includes cellulose and lignin), the difference of these two fractions is an estimate of hemicellulose in a feed. Even though detergent methods of fiber analysis have many advantages over crude fiber estimation, both underestimate the amount of total fiber in a feed due to inability to recover pectins, mucilages, gums and β-glucans which are soluble components of fiber. Later, Asp et al. (1983) developed an enzyme based gravimetric method in which a sample is pretreated with enzymes for the digestion of starch and protein, followed by the recovery of soluble components via precipitation in ethanol and the insoluble components by filtration. This method was further modified by Jeraci et al. (1989), incorporating a urea enzymatic dialysis to assure the removal of essentially all starch. Moreover, during the same decade Theander and Aman (1982) developed an indirect method to analyze total dietary fiber (TDF) in food stuff by quantifying the amounts of uronic acids, sugars, klason lignin and starch and then calculating the TDF as the sum of the uronic acids, sugars and klason lignin minus the concentration of starch. In contrast, Prosky et al. (1984) developed a direct method of TDF quantification. These assays have been further expanded to allow quantification of both soluble and insoluble dietary fiber components (Theander et al., 1995) and refined to increase precision and decrease the complexity and time required (Lee et al., 1996).

5.2. Monomeric component analysis

Monomeric component analysis was first developed by Englyst and Cumming (1988). In this method all starch is hydrolyzed enzymatically and NSPs are measured as the sum of the constituent sugars released by acid hydrolysis. The individual sugars are subsequently quantified by Gas Chromatography (GC) or by High Performance Liquid Chromatography

(HPLC) (Englyst et al., 1992; Englyst et al., 1994). Moreover, a single value for total sugars may be obtained by colorimetric procedure that measures NSPs as reducing sugars (Englyst et al., 1994). The GC technique for dietary fiber analysis, preferred by many researchers, measures NSP as the sum of neutral sugars obtained by GC and uronic acids measured separately (Mongeau et al., 2001). In this procedure, the sugars are reduced to their alditols with alkaline sodium borohydride and acetylated with acetic anhydride in the presence of methylimidazole as catalyst. Since the uronic acid-containing polysaccharides are more difficult to hydrolyze and require treatment with concentrated acid at high temperature; they are measured separately by colorimetry (Scott, 1979). The HPLC method of dietary fiber analysis (Englyst et al., 1994) is most common and measures NSPs as the sum of neutral sugars and uronic acids directly by electrochemical detection.

It must be noted that the values obtained by GC or HPLC are typically lower as comparison to the gravimetric method of analysis because of the exclusion of lignin and resistant starch during chromatographic assay. Besides, the application of Near infrared reflectance (NIR) or transmission (NIT) spectroscopy for rapid estimates of non-starch polysaccharides are in vogue (Blakeney and Flinn, 2005). Near infrared reflectance spectroscopy provides fast, safe, and inexpensive analysis. It is, however, a comparative technique that relies on multivariate calibration of sample spectra and accurate reference analysis (Williams and Norris, 2001; Neas et al., 2002). It has the potential to be exploited as a rapid analytical method for nutritionally important components, including polysaccharides.

6. The anti-nutritive effect of non-starch polysaccharides

The enzymes for NSP digestion such as β-glucanases and β-xylanases are scarce or not present in fish (Kuz'mina, 1996). Consequently, the dietary NSPs remain undigested and therefore negatively affect the animal performance. The adverse effect is associated with the viscous nature of NSPs, their physiological and morphological effects on digestive tract, interaction with epithelium, mucus and microflora of gut (Table 3). Subsequently, it has been reported that soluble NSPs such as mixed linked β-glucans present predominantly in oats and barley, increase intestinal transit time, delay gastric emptying and glucose absorption, increase pancreatic secretion, and slows absorption. However, the insoluble NSPs like pentosans (arabinoxylans and xylans) decrease transit time, enhance water holding capacity and assist in faecal bulking in non-ruminant animals (Pluske, 2001). Thereby, soluble NSPs exert more undesirable effect than insoluble forms of NSPs because former can create a viscous milieu in the

gut of the animal unlike the latter one. The factors associated with the detrimental effects of NSPs are discussed below.

Table 3 Factors responsible for antinutritive effects of non-starch polysachharides

Factors	Effects		References
Changes in digesta viscosity	➤	Reduce mixing of digestive enzymes and substrates	Choct et al., 1996; Ikegami et al., 1990; Hossain et al., 2001; Amirkolaie et al., 2005; Leenhouwers et al., 2007a,b;
	➤	Hinder effective interaction of digestive enzyme at the intestinal mucosal surface	
	➤	Increase residence time of the digesta	
	➤	Increase intestinal volatile fatty acid (VFA) production	
	➤	Reduce absorption of minerals especially sodium ion	
	➤	Impair nutrient digestion and absorption	
	➤	Reduce animal performance	
Alteration in the gastric emptying and rate of passage	➤	Reduce rate of gastric emptying	Rainbird and Low, 1986; Potkins et al., 1991; Shimeno et al., 1992; Kaushik et al., 1995; Refstie et al., 1999; Bach Knudsen, 2001; Hossain et al., 2001; Leenhouwers et al., 2007a.
	➤	Increase rate of passage of stomach content	
	➤	Delay intestinal absorption of glucose	
	➤	Reduce plasma cholesterol and glucose levels	
Alteration of gut physiology	➤	Hinder endogenous secretion of water, proteins, electrolytes and lipids	Pettersson and Aman, 1989; Angkanaporn et al., 1994; Choct, 1997; Hossain et al., 2001
	➤	Enhance bile acid secretion, and significant loss of these acids in the faeces	
	➤	Hamper absorption of lipids and cholesterol in intestine	
	➤	Limit intestinal enzyme activity	
Alteration in the gut morphology	➤	Increase size and length of digestive organs	Baserga, 1985; Jin et al., 1994; Nabuurs, 1998; McDonald, 2001; Iji et al., 2001; Leenhouwers et al., 2006.
	➤	Reduce concentrations of DNA in jejunum, ileum, and liver indicating programmed cell death	
	➤	Augment concentrations of RNA in	

Table 3 continued

		the colon	
	➤	Reduce villi length	
	➤	Increase depth of intestinal crypts in jejunum and ileum	
	➤	Impair water absorption, can lead to dehydration	
	➤	Increase rate of turnover of intestinal mucosal cells	
Alteration in the native gut microflora	➤	Stimulate microbial fermentation in intestine.	Wood and Serfaty, 1992; Choct, 1997; Amirkolaie et al., 2006; Leenhouwers et al., 2007a,b.
	➤	Enhance volatile fatty acid such as acetic acid, propionic and butyric acids production	
	➤	Lower pH of intestinal tract; on long term may disturb the normal micrbiota prevailing in gut	
	➤	Influence bioavailability of dietary minerals	
	➤	Decrease oxygen tension, favour development of anaerobic microbiota	
Alteration in gut mucus layer	➤	Increase concentrations of lumenal mucin in stomach and small intestine	Satchithanandam et al., 1996

6.1. Modulation in digesta viscosity

The solubility and molecular weight of NSPs determine the viscosity. The solubility is not specific to the sugar composition or linkage present in NSPs but depends on the chemical structure and association of NSPs with the cell wall components. However, the physical effect of viscosity on digestion and absorption of nutrients appear to be similar regardless of the sources of NSPs. Moreover, the binding of NSPs with the intestinal brush border increases the thickness of the unstirred water layer adjacent to the mucosa, leading to impaired nutrient digestion and absorption (De Lange, 2000). Furthermore, increased endogenous intestinal secretion of water, nutrients and other electrolytes has been suggested to explain reduced nutrient digestion (Choct, 1997). High viscosity also increases residence time of the digesta and therefore increases intestinal volatile fatty acid (VFA) production. The resulting drastic changes in the gut ecosystem decrease nutrient digestion and eventually reduce performance (Choct et al., 1996). In rainbow trout (*Oncorhynchus mykiss*), reduced nutrient digestibility on inclusion of dietary soluble NSP (guar gum) was associated with an increase

in digesta viscosity (Storebakken, 1985). Similarly, inclusion of soyabean NSPs in the diet of Atlantic salmon resulted in a relatively high viscosity in the intestinal content that was reflected in the reduction of amino acid and lipid digestion (Refstie et al., 1999). Cumulative apparent absorption of amino acids, nitrogen, and sulphur was slowed down in Atlantic cod (*Gadus morhua*) by dietary bioprocessed soybean meal which is possibly due to high water binding capacity of this soy product (Refstie et al., 2006).

Tilapia *(Oreochromis niloticus)* fed with cereal grain NSPs led to augmentation in digesta viscosity and reduction in digesta dry matter. Moreover, the absorption of minerals especially sodium ion was significantly negatively correlated with digesta viscosity (Amirkolaie et al., 2005; Leenhouwers et al., 2007b). Similarly, inclusion of soluble NSPs in the diet of African catfish (*Clarias gariepinus*) induced large increases in digesta viscosity and thereby intestinal fermentation activity, digesta dry matter content and digestive organ weights and the nutrient digestibility was adversely affected beyond a certain threshold for viscosity (Leenhouwers et al., 2006; Leenhouwers et al., 2007a). In addition, supplementation of galactomannan-rich endosperm of Sesbania (*Sesbania aculeate*) seeds in the diet of common carp (*Cyprinus carpio*) resulted in increased viscosity in the intestinal content, thereby affecting the nutrient absorption and utilisation (Hossain et al., 2001). In general, the soluble NSPs are generally viscous in nature, and therefore they enhance the viscosity of diet as well as intestinal digesta. The magnitude of viscosity development in gut with response to dietary NSPs varies among animals and also depends on the source of NSP (Montagne et al., 2003). Digesta viscosity in the proximal intestine of African catfish and Nile tilapia was considerably higher in rye fed groups than in those fed maize and wheat (Leenhouwers et al., 2007a,b) diet.

The age of fish may be an important factor determining the sensitivity towards viscosity. The probable reason for this age related reactiveness is that a more developed intestinal microbiota in older animals is able to utilise NSPs more efficiently (Choct and Kocher, 2000; Refstie et al., 2006a) than in younger ones.

6.2. Alteration in gastric emptying and rate of passage

Dietary soluble NSPs increase the viscosity of digesta in monogastric animals and decreases the rate of passage whereas the insoluble NSPs such as cellulose and hemicellulose increase the passage rate (Johansen et al., 1996).

Dietary inclusions of soluble NSPs reduce the rate of gastric emptying in fish, which can delay the intestinal absorption of glucose (Bach Knudsen, 2001) and possibly of other nutrients. In African catfish, inclusion of viscous cereal grains in diet reduced the plasma cholesterol and glucose levels (Leenhouwers et al., 2007a). In common carp fed diets containing Sesbania endosperm, both muscle and plasma cholesterol levels were significantly lowered (Hossain et al., 2001). Also significant reduction in total cholesterol levels in blood plasma of rainbow trout, yellowtail and Atlantic salmon fed diets containing NSPs rich soybean meals has been reported by various authors (Shimeno et al., 1992; Kaushik et al., 1995; Refstie et al., 1999). A reduced blood cholesterol level is probably associated with binding or trapping of bile salts in the gut due to high viscosity, as also observed in rats fed with galactomannans from guar gum (Moundras et al., 1997).

6.3. Alteration of gut physiology, gut morphology, native gut microflora and mucus layer of gut

Apart from increasing the gut viscosity, the soluble NSPs elicit the anti-nutritive effect by modifying the gut functions. These hamper the endogenous secretion of water, proteins, electrolytes and lipids (Angkanaporn et al., 1994). Non-starch polysachharides can enhance bile acid secretion and subsequently result in significant loss of these acids in the faeces (Ikegami et al., 1990). This can result in increased hepatic synthesis of bile acids from cholesterol to re-establish the homeostasis, which may ultimately influence the absorption of lipids and cholesterol in the intestine, resulting in lower blood cholesterol levels (Hossain et al., 2001). These impacts could lead to remarkable changes in the dynamics of the gut physiology ensuing poor nutrient assimilation efficiency by the animal. Non-starch polysachharides may also influence lipid metabolism in the intestine through binding with bile salts, lipids and cholesterol (Vahouny et al., 1981). Hitherto, there are no reports on the direct inhibition of intestinal enzyme synthesis by NSPs but the activities of most enzymes may be reduced through coupling to NSPs or physical restriction of enzyme access to substrates (Pettersson and Aman, 1989). Digestive enzyme activity of broiler chicks responded to diets supplemented with commercial NSPs (Iji et al., 2001). The jejunal maltase and sucrase activities were highest in chicks that were fed the alginic acid (low viscous)-supplemented diet and lowest in chicks fed the gum xanthan (highly viscous) -supplemented diet. The activity of aminopeptidase N in the ileum was also stimulated by highly viscous NSP diet. However, the uptake of amino acid L-tryptophan into

brush-border membrane vesicles was unaffected by NSPs supplement. To our knowledge, no information is available on the direct effects of NSPs on fish gut physiology.

A number of authors have reported that dietary NSPs have a considerable impact on the gut anatomy and gut development. A prolonged consumption of soluble NSPs is associated with increased size and length of the digestive organs in pigs (McDonald, 2001), chickens (Iji et al., 2001) and fish (Leenhouwers et al., 2006) accompanied by a decrease in nutrient digestion. Pigs fed with high dietary fibre food for 14 days resulted in significant reduction in concentrations of DNA in jejunum, ileum, and liver indicating programmed cell death while concentrations of RNA in the colon was significantly greater (Jin et al., 1994). These authors also reported enlargement in the width of intestinal villi and increased depth of intestinal crypts in jejunum and ileum, and increased rate of cell proliferation and increased crypt depth in the large intestine. Since the crypts are the principal sites of cell proliferation in the intestinal mucosa (Baserga, 1985), an increase in the rate of crypt-cell proliferation along with the reduction in concentrations of DNA may increase the rate of turnover of intestinal mucosal cells. It could be an explanation for the reduction in villus height and crypt depth ratio observed in the small intestine of pigs subjected to feed supplemented with high fibre (Jin et al., 1994). Similarly, administration of an oral dose of cellulose to pre-weaning pigs reduced villus length by approximately 15% in the jejunum and ileum (Jin, 1992). Furthermore, McDonald et al. (2001) reported that addition of sodium carboxymethylcellulose (CMC) to a highly digestible cooked rice-based weaner diet (40 g/kg diet dry matter) for 13 days significantly increased the intestinal viscosity of digesta within the small and large intestine, which led to decreased villus length and increased crypt depth. Similar results have been reported by Hopwood et al. (2002).

The shortening of villi results in an impaired absorption because shortening results in an absolute loss of intestinal surface area and cells. Since the osmotic water absorption is governed by nutrient absorption, the decline in nutrient absorption caused by reduction of villi length eventually impairs the water absorption. Moreover, an increased crypt depth is associated with an increased water secretion into the intestinal lumen. This large fluid amount in intestinal lumen is generally not absorbed by partially developed large intestine of young animals which may give rise to clinical symptoms of dehydration (Nabuurs, 1998). In chicken, feeding with gum xanthan supplemented diet for 14 days resulted in deepening of the jejunal crypts and reduction in villi height and villi surface. Additionally the

viscous NSPs increased the weight of the small intestine. However, changes in the weight of visceral organs were generally due to variation in rate of cell proliferation, cell size or protein synthesis (Iji et al., 2001). This was further confirmed in this study by an increase in the protein: DNA ratio (cell size) of the jejunum and by a decline in the DNA content (cell population) of chickens rose on NSPs containing diets.

Conversely, Moore et al. (1988) found that villus shape or surface morphology in jejunum of growing pigs was not affected by high-fiber diet, although some loss of epithelial cells at the apex of the villi was observed. In rats, ingestion of pectin (25 g pectin/kg of elemental diet for 14 days) significantly increased villus height and crypt depth (Andoh et al., 1999). Supplementation of a low viscosity carboxymethylcellulose (CMC) (40 g CMC/kg air-dried diet) to a cooked rice-based diet increased the small intestinal villus and crypt depth in newly weaned pigs without altering the shape of villi (McDonald et al., 2001).The above studies highlight the effects of NSPs on gut morphology of monogastric animals. It is also evident that information on fish is lacking.

Mechanisms for modifying gut morphology: The consumption of NSPs rich diet alters the intestinal physiology and anatomy by its ability to increase digesta viscosities. The rate of crypt-cell proliferation, cell migration along the crypt–villus axis, and cell extrusion from the villous apex via apoptosis and cell sloughing control the dynamic process of small intestinal cell turnover. The presence of high digesta viscosity in lumen may increases the rate of villus cell losses, leading to villus atrophy, a phenomenon associated with an increased crypt-cell production, and generally with increased crypt depth (Montagne et al., 2003).

The delay of digesta passage in intestinal tract, as a result of increase in viscosity, may stimulate microbial fermentation in intestine. The fermentation of NSP produces volatile fatty acids (VFA) as end product. Administration of NSPs in diet of Tilapia and African catfish has been shown to increase VFA level in intestinal tract (Amirkolaie, Verreth, & Schrama, 2006; Leenhouwers et al., 2007a,b). Acetic acid is the prominent VFA produced in African catfish, which is in agreement with other studies on tilapia and marine herbivores (Clements and Choat, 1995; Kihara and Sakata, 1997; Amirkolaie et al., 2006). Besides acetic acid, propionic and butyric acids are also produced following microbial fermentation in fish but the concentration of these two acids varies among fish species. A low concentration of propionic and butyric acid in tilapia was observed (Amirkolaie et al., 2006) while a significant high amount of these acids was reported from marine herbivorous fish (Clements and Choat, 1995). The

production of these organic acids in intestinal tract may lower the pH of surrounding milieu, which on long term may disturb the normal microbiota prevailing in the gut. Besides, gastric acidity influences the bioavailability of dietary minerals (Wood and Serfaty, 1992) by regulating the chelation and complex formation of the element and by altering the transport mechanisms of minerals (Ravindran and Kornegay, 1993). Moreover, increase in the residence time of digesta in the intestine following intake of soluble NSPs may decrease oxygen tension and favour the development of anaerobic microbiota (Choct, 1997). Although it is not fully known whether a sudden change of gut ecology is detrimental to the efficiency of nutrient utilisation, the maintenance of health status relies mainly on normal endogenous microbiota. The normal microbiota confers many benefits to the intestinal physiology of the host. Some of these benefits include the metabolism of nutrients and organic substrate and the contribution to the phenomenon of colonization resistance. However, when the delicate balance of normal microbiota is upset, pathogens that arrive or that have already been present but in numbers too small to cause disease take the opportunity to multiply. Furthermore, the proliferation of some anaerobic organisms can lead to production of toxins and deconjugation of bile salts which are essential for the digestion of fat (Carré et al., 1995).

Mucus is the protective layer of the entire gastrointestinal tract, which is exposed to all chemical and physical forces of digestion. Several studies have correlated the dietary NSPs intake and mucin concentration in gastro-intestinal tract. In pigs, inclusion of pea fibre in a wheat diet tended to increase the output of mucins in the ileal digesta from 6.1 to 7.3 g per day for the diets supplemented with 0 and 240 g of pea fibre per day, respectively (Lien et al., 2001). Similarly ileal glucosamine and galactosamine excretion increased continuously with fiber intake (Reverter et al., 1999). When wheat bran (150 g/kg diet dry matter) added to a protein-free diet of pigs, the ileal output of galactosamine at the terminal ileum increased from 1.93 to 4.13 g per day (Fuller and Cadenhead, 1991). Feeding carboxy methyl cellulose (CMC) as a digesta viscosity inducing non fermentable polysaccharide to weaned piglets for 15 days significantly increased viscosity of ileal digesta and ileal mucin concentration (Piel et al., 2004). The mechanisms by which dietary NSPs modify mucin characteristics are not well understood. The physical scrape and proteolytic breakdown of mucus gels are the main factors releasing mucins in the gut lumen (Allen, 1981). Therefore, it could be hypothesized that the erosion of mucus layer may be due to an increase in the bulk of digesta, stretching the intestinal

mucosa and scraping mucin from the mucosa as they pass through the digestive tract.

It could be contemplated that effects reported above may also appear in fish but till date no such studies have been conducted in fish.

7. Effect of non-starch polysaccharides on nutrient metabolism

Feeding of NSPs negatively influences the metabolism and utilisation of dietary nutrients like glucose, lipid, amino acid and minerals. It is because of reduction in rate of gastric emptying leading to depression in nutrient absorption (Bach Knudsen, 2001). Moreover, Leenhouwers et al. (2006) explained that reduced digestibility of nutrients is related to partial distribution of digestive enzymes in a viscous solution and a lowered flow at the mucosal layer. Enhanced endogenous losses of nutrients and increase in the thickness of the unstirred water layer adjacent to the mucosa also lead to the diminution in nutrient digestion and absorption (De Lange, 2000).

7.1. Effect on glucose

The inclusion of NSPs in basal diet of monogastric animal including fish has been reported to delay the intestinal absorption of glucose. In African catfish it was demonstrated that feeding diets containing rye at a level of 400 g/kg diet (dry matter) decreased plasma glucose level (Leenhouwers et al., 2007a). In salmonid fish, inclusion of guar galactomannans and alginates as NSP sources in the diet reduced the availability of glucose when compared to NSP free diets (Storebakken, 1985; Storebakken and Austreng, 1987). Significantly lower intestinal maltase activity in Atlantic salmon has been reported on feeding defatted soybean meal containing NSPs at a level of 100 g/kg diet (dry matter) (Kraugerud et al., 2007). In fish, knowledge on the glucose digestibility in response to dietary NSPs is limited and only few studies have been conducted to elucidate the effect of NSP on carbohydrate metabolism and absorption. However, a considerable number of reports are available in pigs supporting a negative effect of dietary NSP on glucose level. In growing swine the use of guar gum in basal feed halved the rate of absorption of glucose in the jejunum (Rainbird et al., 1984). A reduction of 25% in the plasma glucose concentration in pigs fed with semipurified diets supplemented with 40 g/kg guar gum in the diet (dry matter) has been reported (Sambrook and Rainbird, 1985). Furthermore, Nunes and Malmlof (1992) demonstrated that feeding 60 g/kg guar gum in the diet to swine reduced glucose absorption by 32%. Furthermore, the inclusion of 60 g/kg

guar gum in semipurified diet of swine reduced the postprandial production of insulin by 30%, insulin like growth factor-1 (IGF-1) by 58%, gastric inhibitory polypeptide (GIP) by 55% and glucagon by 41% (Nunes and Malmlof, 1992). Lower blood glucose level was observed when trout were fed an experimental diet prepared by replacing 62.5% of fish meal protein by detoxified *Jatropha curcas* kernel meal (DJKM) which contained 16% NSP (Kumar et al., 2010).

7.2. Effect on protein

Inclusion of NSPs in the diet of fish has been well documented to reduce amino acid digestibility. The decrease in nitrogen utilisation efficiency following NSP rich diet is probably due to increase of N secretion either endogenously and/or through intestinal bacteria. Leenhouwers et al. (2006) reported that inclusion of soluble NSPs from guar gum at a level of 40 g/kg and 80 g/kg in the diet (dry matter) of African catfish diet significantly increased digesta viscosity in the proximal and distal intestine. This increase in viscosity was accompanied by a reduction in the apparent digestibility coefficient of protein. Storebakken (1985) reported reduced protein digestibilities in rainbow trout fed with 25–100 g guar gum per kg diet. African catfish reared with high viscous rye supplemented diet lowered the protein digestibility much more than those fed with low viscous wheat based diet (Leenhouwers et al., 2007a). However, it was in contrast with the findings of Leenhouwers et al. (2007b) with tilapia where reduction in protein digestibility was observed more in fish fed with low viscous wheat than high viscous rye based diet. This suggests that viscosity is not the only factor that explains the differences in protein digestibility in Nile tilapia. This species of fish is less sensitive to viscous dietary ingredients than African catfish, due to its more herbivorous feeding habit (Leenhouwers et al., 2007b). Similarly, salmon fed on soya diets with high levels of soluble NSPs resulted in reduced protein digestibilities (Refstie et al., 1999). Furthermore, Sesbania endosperm, a leguminous seed at $\geq$ 7.2% in diet of common carp (Hossain et al., 2001) and at $\geq$ 5.8% in diet of tilapia (Hossain, Focken, & Becker, 2003) substantially reduced the protein efficiency ratio and protein productive value. Other studies that included purified soluble NSPs in fish diets also found reduced protein digestibilities (Shiau et al., 1988).

7.3. Effect on lipid and cholesterol level

Increasing the NSP content in the diet of monogastric animals has been reported to decrease the utilisation of lipid. Increase in digesta viscosity caused by intake of NSP containing diet has been shown to affect emulsification negatively, and to reduce lipolysis (Pasquier et al., 1996). Non-starch polysachharides may entrap bile salts, thus reducing their efficiency in solubilizing fats and consequently impairing lipid absorption (Ebihara and Schneeman, 1989). In African catfish, the digestibility of fatty acids was reduced considerably when fed with wheat and rye respectively at a level of 400 g/kg of basal feed (dry matter) (Leenhouwers et al., 2007a). Feeding of common carp with Sesbania endosperm (containing about 75% of galactomannan) at levels of $\geq$7.2% significantly reduced both muscle and plasma cholesterol levels (Hossain et al., 2001). Similar effects were observed when tilapia was fed the Sesbania endosperm at levels of $\geq$ 5.8% (Hossain et al., 2003). Moreover, reduced total cholesterol levels in blood plasma of Atlantic salmon fed diets containing soybean meal have also been reported (Refstie et al., 1999), which could possibly be ascribed to NSPs present in soybean meal. Similarly, 62% of FM protein replacement by DJKM (detoxified jatropha kernel meal) resulted in lower lipid digestibility in trout, which could possibly be due to the presence of NSPs in DJKM (Kumar et al., 2010). The presence of NSPs in feeds has been hypothesized to reduce fat absorption in trout by disturbing micelle formation in the gastro intestinal tract (Øverland et al., 2009)

The hypocholesterolemia response on NSPs intake has also been reported in Atlantic salmon (*Salmo solar*) fed with soybean meal containing NSPs at a level of 100 g/kg diet (dry matter) (Kraugerud et al., 2007). This is probably associated with binding of cholesterol with bile salts in the gut.

Moreover Potter (1995) described another possible mechanism for hypocholesterolemia as an enhancement of bile acid excretion and consequently creation of an environment in which cholesterol is being 'pulled' from the body. In this state, hepatic cholesterol metabolism alters to provide cholesterol for enhanced bile acid synthesis.

7.4. Effect on mineral

Various components of NSPs interact with minerals and have been shown to decrease mineral absorption. Components of polysaccharides and lignin that interact with minerals include the carboxyl group of uronic acid, carboxyl and hydroxyl groups of phenolic compounds and the surface hydroxyl of cellulose (Torre et al., 1995). Moreover, NSP induced digesta viscosity has been shown to hinder mineral absorption (Van der Klis et al., 1995).

Absorption of Ca, Mg, Na and P were considerably lower when African catfish were fed with a diet supplemented with rye (Leenhouwers et al., 2007a), while only Na absorption was significantly reduced when fed to Nile tilapia (*Oreochromis niloticus*) (Leenhouwers et al., 2007b). Elevated faecal sodium excretion in Atlantic salmon as an effect of feeding soy products was pointed out by Storebakken et al. (1998). Likewise, in the same fish species a negative impact of defatted soyabean meal on K, Na, Zn and of native soy-NSP on Cu, Fe and K utilization were observed (Kraugerud et al., 2007). Furthermore, in rainbow trout Na excretion has been found to increase proportionally with dietary cellulose level (Øvrum and Storebakken, 2007). In contrast to above findings, no correlation was observed between NSP content in diet and sodium excretion in Atlantic salmon (Aslaksen et al., 2007).

Although dietary NSPs have mainly been reported to have negative effect on mineral utilization, epidemiological data suggests that intake of plant ingredient rich in NSPs can be a protective factor against the abnormalities caused by metal toxicity. Despite a number of studies conducted to elucidate the effects of dietary NSPs on mineral bioavailability, little attention has been devoted to the understanding of their chelating mechanism.

8. Effect on growth performance and body composition

Low-protein soy products have been shown to induce negative effects on the digestibility of nutrients in salmon, which was probably an effect of the viscosity caused by the NSP in soybean product (Refstie et al. 1999). Besides, in rainbow trout, tilapia and Atlantic salmon, an increased water content of the gut induced by the water-binding properties of dietary soluble NSP have been suggested as the reason for the reduced nutrient digestibilities and diminished growth performance (Storebakken, 1985; Shiau et al., 1988; Refstie et al., 1999). Rainbow trout fed a diet containing 10% guar gum, which contains high proportions of galactomannan, resulted in significant reduction of growth and dry matter and fat content in fish tissues. Moreover, in common carp and trout the replacement of 75% and 62.5% of FM protein by DJKM respectively decreased growth performance. This adverse effect was attributed to the presence of NSPs in DJKM (Kumar et al., 2008; Kumar et al., 2010). Dietary inclusion of Sesbania endosperm at levels of 7.2%, 10.8% and 14.4% in the feed of common carp resulted in reduced body weight gain that were 57%, 48% and 39%, respectively of the control diet (Hossain et al., 2001). Additionally, the inclusion of Sesbania endosperm

influenced whole body proximate composition of fish which significantly increased whole body moisture, reduced lipid and gross energy content (Hossain et al., 2001). Moreover, similar observations of higher whole body moisture and lower lipid content in common carp fed diets containing various levels of rape seed, mustard oil-cake, linseed and sesame meal have been made (Dabrowski and Kozlowska, 1981; Hossain and Jauncey, 1989). The study of Hossain et al. (2001) showed no significant difference in the hepatosomatic index of fish. It is in agreement with other studies on fish and poultry in which the effects of feeding soluble NSPs on relative liver weight were insignificant (Storebakken, 1985; Iji et al., 2001; Leenhouwers et al., 2006). Conversely, the study conducted on African catfish showed stimulating effects of guar gum on stomach and intestine weights (Leenhouwers et al., 2006). Feeding trial on tilapia showed that weight gains of fish fed diets containing 5.8%, 8.7% and 11.8% Sesbania endosperm were 82%, 73% and 64%, respectively compared to the control diet (Hossain et al., 2003) and also had significantly higher whole body moisture, lower lipid and lower gross energy contents. It was also noticed that the reduction in whole-body lipid was more pronounced with the diets containing higher levels of endosperm. It may be because of the thickening of the fluid layer closest to the mucosal wall, thereby preventing contact of digestive enzymes with the substrates and the formation of the micelles required for the lipid absorption (Wang et al., 1992). In contrast to the above findings, Leenhouwers et al. (2007a) reported no effect on the growth performance in African catfish with increase in intestinal viscosity. A previous study with African catfish also found that the guar gum induced changes in digesta characteristics were not accompanied by reduced fish performance (Leenhouwers et al., 2006). These differences could be attributed to factors such as differences in NSP concentration, NSP structure, concentration of other dietary components, fish species, and age of fish (Petersen et al., 1999; Leenhouwers et al., 2007a).

9. Non-starch polysaccharides and effect on gelatinization

Fish in general have a limited capacity for carbohydrate utilization and processing method such as gelatinization has been reported to improve the nutrient bioavailability to the fish. Gelatinization is thermal modification of raw dietary carbohydrates. During this process carbohydrate granules are modified in such a way that their susceptibility to enzymatic action increases (Kumar et al., 2006), making digestion more complete. Carbohydrate gelatinization and its impact on digestibility and growth have been well

documented in fish such as carp (Kumar et al., 2006) and rainbow trout (Podoskina et al., 1997). Wheat being the major source of starch also contains a low amount of NSPs. Arabinoxylan, the main NSP in wheat flour, is reported to hinder the gelatinization and retrogradation properties of wheat starch due to its water binding capacity and high viscosity (Shogren et al., 1987). Moreover, addition of an isolated NSP rich fraction to starch markedly affects starch gelatinization and pasting properties (Sasaki et al., 2000). Furthermore, Tester and Sommerville (2003) reported that soluble NSPs restrict the gelatinization of maize starch and wheat starch by reducing hydration of the amorphous regions and consequently limiting depression of the glass transition temperature within these regions. This suggests that the effect of NSPs on the gelatinization event, in general is mediated via reducing the volume fraction of water in the system and restricting water availability and mobility due to hydration process (Tester and Sommerville, 2003). The plant tissues containing starch are expected to contain NSPs, which could hinder the effect of processing on starch digestibility. Better understanding of the effects of different NSP types on gelatination would aid in the efficient utilization of plant carbohydrates in fish nutrition.

10. Interaction with antibiotics

The efficiency of NSP utilisation by fish is dependent on the nature of the microbial population residing in the gut. It, could, therefore, be speculated that the supplementation of antibiotics that alter the gut microflora would have an influence on the dietary fibre utilisation (Annison and Choct, 1991). A large body of evidence suggests that the anti-nutritive activity of NSPs in poultry is related to the gut microflora, as addition of antibiotics to diets increased nutritive value of diets (Langhout and Schutte, 1995). Moreover, in swine, supplementation of virginiamycin at a level of 11 ppm in diets containing 50% oats improved dry matter, energy, hemicellulose and cellulose digestibilities. Furthermore, the antibiotic supplementation also improved absorption and retention of minerals such as Ca, P, Mg, Cu, Fe, Zn and Mn (Ravindran and Kornegay, 1993). Similarly, 0.7% nebacitin in pig diet containing 10.5% fibre enhanced the apparent N digestibility by 12% and salinomycin at a level of 82 mg/kg of a diet (dry matter) containing 20% wheat bran increased P absorption (Moore et al., 1986). Only a very limited number of works has been done on the interactions of NSPs and antibiotics in fish. In Atlantic salmon, soyabean meal-induced enteritis was not ameliorated by adding the broad-spectrum antibiotic oxytetracycline to the

diet at 3g/kg diet (dry matter) (Bakke McKellep et al., 2007). In the distal intestinal mucosa of both soyabean meal and soyabean meal supplemented with oxytetracycline -fed fish, a significant increase in the proliferative compartment length as well as apparent increases in the number of cells undergoing cellular repair processes and apoptosis were observed as pathophysiological responses related to the soyabean meal induced enteritis (Bakke McKellep et al., 2007). In the same species, it was seen that soyabean meal supplemented with oxytetracycline resulted in reduced relative liver weight and did not modify the responses to dietary soyabean meal influencing gut morphology, hydrolysis of protein and starch, and absorption of amino acids, nitrogen and sulphur (Refstie et al., 2006b).

11. Purified non-starch polysaccharides as immunostimulants

Immunostimulants are chemical substances that activate the generalized immune response system of animals. Such substances are also known to render animals more resistant to infectious diseases and reduce the risk of disease outbreaks if administered prior to stress-causing situations. NSP such as ß-1,3-glucans acts as an immunostimulants and thus improve health, growth and general performance of many different animal groups, including farmed shrimp, fish and terresterial animals (Kumar et al., 2005). Supplementation of β-1,3 glucan in diets enhances the nonspecific cellular defence mechanisms by increasing the number of phagocytes and the bacterial killing activity of macrophages in rainbow trout, Atlantic salmon, catfish, and carp and also by the macrophage mediated superoxide anions production (Kumar et al., 2005). In an Indian major carp, *Labeo rohita*, yeast glucan (β-1-3 glucan) has been observed to enhance the phagocytic activity of leucocytes and stimulates generation of reactive oxygen species (ROS) in phagocytes (Ali et al., 1996). A number of studies have shown that β 1-3/1-6-glucans enhance the biological activity of shrimp hemocytes and to improve growth, survival rate and feed conversion efficiency under experimental conditions (Table 4). β-glucans have been successfully used to increase the resistance of shrimp *Penaeus japonicus* against vibriosis (Kumar et al., 2005). *Penaeus monodon* when fed with ß-glucan at 0.2% (w/w of the feed) significantly increased phenol oxidase, the number of hemocytes and the bacterial killing activity against vibriosis, white spot syndrome virus, *Vibrio damsela* and *V. harveyi* and also enhanced survival and immunity during brood stock rearing (Chang et al., 2003). *Macrobrachium rosenbergii* post larvae showed enhanced growth and resistance to *V. alginolyticus* by dietary administration of ß-glucan (Misra et al., 2004). However, the long term

administration of β-glucan in fish and shellfish can reduce the immune response to basal levels and also loose effect on those bacteria that are resistant to phagocytosis.

Table 4 Role of β-glucan in health and production of fish and crustaceans (adapted from Kumar et al., 2005)

Fish/crustacean	Route of administration	Advantage
Fish	Feed or injection	• Enhances production of antibodies against pathogens. • Acts in synergy with antibiotics. • Enhances resistance to bacterial disease and efficacy of vaccine.
Fish and shrimp	Injection and oral	• Acts as a true adjuvant and enhances antibody production
Halibut larvae	Immersion	• Increases survival rate
Coho salmon	Injection or oral	• Protects against *Aeromonas salmonicida*.
Shrimp	Feed	• Increasesr growth, reduces mortality, and increases feed utilisation
Fish and shell fish	Feed	• Acts in synergy with vitamin-C
Shrimp post larvae	Immersion	• Enhances the survival rate
Penaeus monodon	Feed	• Protects against White Spot Syndrome Virus infection • Increases survival rate
Macrobrachium rosenbergii larvae	Bath treatment (10 mg/L)	• Increases lysosomal activity • Protects against *Vibrio alginolyticus*
Carp	Injection (500 mg/kg)	• Enhances protection against *Aeromonas hydrophila*
Asian catfish	Feed	• Enhances immunity against *Aeromonas hydrophila*

Mannose units that comprise glucomannans, belong to the category of compounds that adhere to receptors used by pathogenic microbes as the first step of colonisation of the gut. Therefore, supplementation of mannose in basal feed of animals may contribute to better health by interfering with colonisation and growth of pathogens in the gut (Raa, 2000).

Levan, a natural polymer of fructose with β (2,6) linkages produced extracellularly by many microorganisms like *Zymomonas mobilis*, *Bacillus subtilis*, *Bacillus polymyxa*, and *Acetobacter xylinum*, is shown to have immunostimulating properties in fish (Rairakhwada et al., 2007; Gupta et al., 2008). Dietary levan supplements at a concentration of 0.5% have been reported to activate non-specific phagocytes in *Cyprinus carpio* juveniles which resulted in higher survival rate when challenged with *Aeromonas hydrophila* (Rairakhwada et al., 2007). Likewise the histological study strongly suggests that levan supplementation at 1.25% ameliorates the effects of infection by *A. hydrophila* in *Labeo rohita* juveniles.

12. Non-starch polysaccharides degrading enzymes

Monogastric animals including fish lack the intestinal enzymes for the degradation of NSPs. The improvement in the digestibility of NSPs is achieved by supplementation of NSP-degrading enzymes in the diet. Such an approach has successfully been used in poultry diets but little work showing the potential of NSP-degrading enzymes in fish is available (Ai et al., 2007).

Various kinds of NSP-degrading enzymes have been reported in animal feed, which include cellulase, hemicellulase, xylanase, pectinase, β-glucanase and α-galactosidase. Their effects and benefits are illustrated in Table 5.

Table 5 Non-starch polysaccharides degrading enzymes in animal feed and their impact (adapted from Bhat, 2000)

Enzyme	Effect	Impact
Cellulases and hemicellulases	• Partial hydrolysis of lignocellulosic materials • Hydrolysis of β-glucans • Decrease in intestinal viscosity • Better emulsification and flexibility of feed materials	• Improvement in nutritional quality of animal feed • Improvement in performance of ruminants and monogastrics
β-Glucanase and xylanase	• Hydrolysis of cereal β-glucans and arabinoxylans • Decrease in intestinal viscosity • Release of nutrients from grains	• Improvement in feed digestion and absorption, and in weight gain of broiler chickens and hens
Hemicellulase with high xylanase actvity	• Increasein nutritive quality of pig feeds	• Reduction in the cost of pig feeds • Reduction in the cost of feeds for pigs
Cellulases, hemicellulases and pectinases	• Partial hydrolysis of plant cell wall during silage and fodder preservation	• Contribution to production and preservation of high quality fodder for ruminants • Improvement in quality of grass silage

β-Glucanases and xylanases have been successfully used in monogastric diets to hydrolyse NSPs such as barley β-glucans and arabinoxylans (Cowan, 1996). Moreover Yin et al. (2001) reported that β-glucanase (600 units/kg diet) and xylanase (745 units/kg diet) inclusion in pig feeds containing hulless barley decreased the viscosity in the distal part of the small intestine, lowered the plasma urea nitrogen concentration and increased the apparent ileal digestibility of energy and some amino acids. Similar beneficial effect of NSP-degrading enzymes in pigs has been reported by Yin et al. (2000). Besides, addition of NSP-degrading enzymes during feed production was found to degrade NSPs and markedly improved the digestion and absorption of feed components as well as growth performance in broiler chickens (Choct et al., 1995; Ghorbani et al., 2009). Almirall et al. (1995) showed that broiler chicks fed with high viscosity barley had lower amylase and lipase activities in the digesta, while addition of β-glucanase in the diet increased activities of the two enzymes and of trypsin as well. This observation was consistent with the result of Li et al. (2009) with tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) where 1

g/kg of NSP-degrading enzyme (50 FBG (Fungal beta glucanase units)/g; Roche Shanghai) increased activity of amylase in the hepatopancreas and intestine by 11.4% and 49.5%, respectively. The application of Natugrain-blend® (containing β-glucanase and β-xylanase) at concentrations of 75, 150 and 300 µl/kg of diet containing 30 % wheat or dehulled lupin had no remarkable effect on dry matter, energy or protein digestibilities when fed to silver perch (Stone et al., 2003a). According to Stone et al. (2003a) the missing effect of Natugrain-blend® inclusion on nutrient digestibility could be due to the intolerance of silver perch to high levels of galactose and xylose in blood (Stone et al., 2003b). Prolonged elevation of blood galactose has been reported to reduce growth performance in carps (Shikata et al., 1994).

The pretreatment of dietary plant materials with exogenous carbohydrases (α-amylase, β-glucanases and β-xylanases) enhances the energy digestibility by releasing more glucose, galactose and xylose (Kumar et al., 2006). The supplementation of two NSP-degrading enzymes, for example 400 mg 'VP' (contains mainly glucanase, pentosanase and cellulase, each at 50 IU per g) and 800 mg 'WX' (contains mainly xylanase, 1000 IU per g) in basal feed of Japanese seabass (*Lateolabrax japonicus*) significantly enhanced the specific growth rate, feed efficiency ratio, nitrogen retention and reduced ammonia excretion (Ai et al., 2007).

12.1. Mechanism responsible for non-starch polysaccharides -degrading enzymes

The mechanisms by which exogenous NSP-degrading enzymes enhance nutrient digestion and utilization from plant proteins in fish have not yet fully understood. However, based on various reports three types of mechanisms could be suggested for the action of NSP-degrading enzymes (Figure 6). These are discussed below.
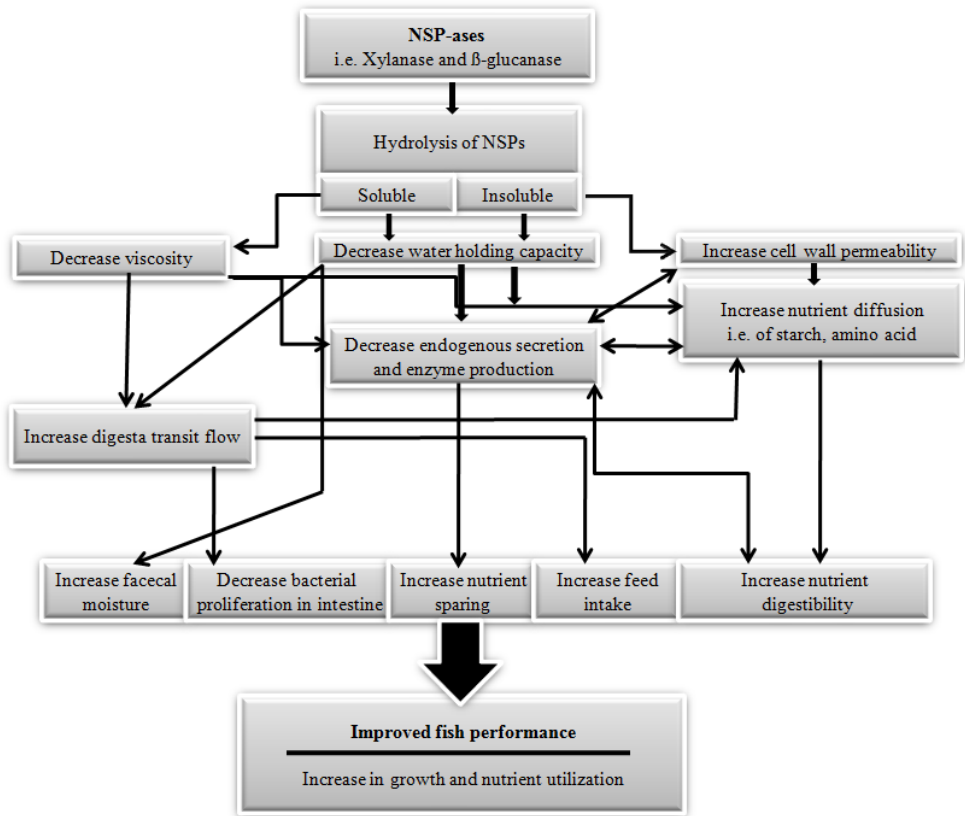
Fig. 6. Modes of action of non-starch polysachharides degrading enzymes (Wyatt et al., 2008)

12.1.1. Disruption of cell wall integrity

The cell wall in the cereals and legumes is constructed mainly of small amounts of cellulose, hemicellulose and arabinoxylan with minor β-glucan components. The activity of exogenous NSP-degrading enzymes creates 'holes' in the cell wall. This allows water hydration and permits pancreatic proteases and amylases to act, enabling better digestion of the starch and protein. Xylanases and to a lesser extent cellulases have proven most effective in broilers (Leslie et al., 2007). Mannanases and pectinases had shown good result in soy based diet (Jackson et al. 2004). However, in a corn-soy based diets xylanase and glucanase are more effective in breaking down the insoluble fibre fraction. Moreover, Li et al. (1996) suggested that the improvement in digestibility of nutrients in barley-based diets with

cellulase addition for young pigs was due to the increased degradation of β-linked components in barley that made them available to the host animal.

12.1.2. *Reduction of digesta viscosity*

Studies on monogastric animals have shown that reduced digesta viscosity due to NSP-degrading enzyme supplementation is the main factor responsible for the observed enhanced performance response on feeding plant materials rich in NSPs (Cowieson et al., 2006). However, Partridge (2001) demonstrated that pig small intestinal viscosity is relevant for the performance but it is of a lower order of importance than for broiler. To our knowledge no study is available which illustrates the effect of supplementing NSP-degrading enzymes on the intestinal viscosity in fish fed NSP-containing diet.

12.1.3. *Stimulation of bacterial population*

Addition of NSP-degrading enzymes in feed break down plant cell wall carbohydrates and reduce chain length producing smaller polymers and oligomers. These fragments further become small enough to act as a substrate for bacterial fermentation that can be beneficial with VFA production and altering the bacterial population. Several studies have shown that applications of exogenous enzymes significantly alter VFA production and the population profiles of gut associated microflora (Bedford and Apajalahti, 2001). Yin et al. (2000) studied the effects of xylanase supplementation in wheat bran based diet in young pigs, and found that the enzyme addition increased the ileal production of VFA.

However, care must be taken while selecting the feed enzyme because some products can be overdosed and reduce the size of the oligosaccharides down too far, to monosaccharides. If excess monosaccharides are produced, it may result in osmotic diarrhoea and /or poor performance (Schutte, 1990).

Currently, the use of various exogenous feed enzymes is considered to reduce FM inclusion by around 5% in most aquafeeds and there is a potential to reduce further the demand for FM by the aquaculture sector in the coming years (Felix and Selvaraj, 2004). However, lack of information on the nutritive value and the content and nature of anti-nutrients in feed ingredients make difficult the selection of suitable enzyme or enzyme mixture as feed additives. Further studies are required in this area. Another constraint is that feed enzymes are proteins, which are substrate specific,

work within a particular pH range and are themselves potentially open to digestion by endogenous enzymes. In the stomach pH is low and suited primarily to protein digestion (Yin et al., 2001). Consequently it is crucial that enzymes are protected to a sufficient extent against proteolysis and are available at the targeted site of activity (Yin et al., 2001).

13. Conclusions

In aquaculture the use of plant based protein is increasing at a fast pace due to their relatively low cost and ample availability. Consequently, the use of non-starch polysaccharides (NSPs), a class of anti-nutrients present in plant based diet will increase in the future. Currently there is relatively little information on the effects of dietary NSP on fish nutrition and physiology. Non-starch polysachharides, with their high water holding capacity can affect digesta viscosity in fish. High digesta viscosity, produced as a result of NSPs, delays gastric emptying and feed transit time, and decreases interaction of the intestinal enzymes with feed macromolecules, resulting in decreased nutrient availability. Eventually this leads to decreased growth performance. Non-starch polysaccharides also change the gut ecosystem, which could be beneficial or deleterious for the animals. The extent of fermentation of NSPs in fish intestine, the change in gut microbial ecology and the factors leading to adverse or beneficial effects in fish are not clear. The addition of NSP-degrading enzymes (NSPase) in diets containing plant sources could play a vital role in improving nutrient utilisation in fish, as observed for other monogastric animals. The use of such enzymes may offer feed producers a means to cut down the feed cost. Different plant sources contain different types of NSPs. The enzymes responsible for degradation of NSPs would differ, depending on the nature of NSPs. The efficacy of the enzymes would also differ with the physio-chemical conditions of the intestine and in particularly with the pH, which could differ from one fish species to another. In addition, the enzyme should also be resistant to intestinal proteases and should be active at the target site of action. Therefore, systematic studies are needed for better exploitation of NSP-degrading enzymes. Future studies should also be directed towards using the purified NSPs as immunostimulants in commercial fish rearing.

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Chapter 2

Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings

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Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings

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Abstract

Jatropha curcas kernel meal is rich in protein; however, it is toxic. Two durations of detoxification process were investigated: 30 min and 60 min; designated as J_a and J_b respectively. Common carp fingerlings (252 fish; 3.2±0.07 g) were fed diets: Control containing fishmeal (FM); S_{50}, J_{a50} and J_{b50}: 50% of FM protein replaced by soybean-meal (SBM), detoxified Jatropha kernel meal (DJ$_a$KM and DJ$_b$KM); S_{75}, J_{a75} and J_{b75}: 75% of FM protein replaced by SBM, DJ$_a$KM and DJ$_b$KM. Highest body mass gain, specific growth rate, metabolic growth rate and energy production value were observed for the J_{b50} group which were statistically similar to that for control group and significantly (P<0.05) higher than for all other groups. Lowest feed gain ratio was observed in control group, which was statistically similar to than in J_{b50} group. Lowest metabolic growth rate, protein efficiency ratio, protein productive value and lipid production value were observed in J_{a75} group. The dry matter and lipid digestibilities were statistically (P<0.05) highest in control group and lowest in J_{a50} group. Protein and energy digestibilties were statistically similar (P>0.05) for control and J_{b50} groups and these values were highest among the groups. Highest crude protein efficiency was observed in J_{b50} group, whereas, lowest in J_{b75} group. Relative intestinal length and hepatosomatic index were higher in plant protein fed groups. Intestinal amylase, protease and lipase activities for control group were statistically higher (P<0.05) than for plant protein fed groups. Conclusively, performance of J_{b50} group was similar to control group and better than other groups and, thus, is recommended as the most optimal diet for common carp fingerlings.

Introduction

Ongoing intensification of aquaculture in tropical countries has made it essential to develop suitable diets for carp using alternative protein sources. Traditionally, fish meal (FM) has been the main source of dietary protein for fish. In recent years, its increasing cost, decreasing availability in the market and poor quality have stimulated several studies on its partial or complete substitution with alternative protein sources (Kaushik et al., 1995; Fournier et al., 2004). Since, FM is a limited primary source and plants are widely available and reasonably priced, the use of plant protein sources in aqua feeds should be considered (SOFIA 2007). Our previous studies demonstrate that plant protein (*Moringa oleifera* leaf meal and *Sesbania aculeate* seed meal) could partially replace FM in the diet of tilapia, *Oreochromis niloticus* and common carp, *Cyprinus carpio* (Hossain et al., 2001; Richter et al., 2003a; Dongmeza et al., 2006).

Soybean meal (SBM) is one of the most nutritious of all plant protein sources (Lovell 1988). Soybeans are the leading oilseed crop produced globally, and its production for 2004–2005 was around 200 mmt (Gatlin et al., 2007). Because of its high protein content, high digestibility, relatively well-balanced amino acid profile, reasonable price, and steady supply, SBM is widely used as a cost-effective feed ingredient for many aquaculture animals (Storebakken et al., 2000). It is currently the most commonly used plant protein source in fish feeds (El-Sayed 1999). However, SBM competes with human food and hence there is a need to identify other protein rich plant resources that could be used in fish diets.

Jatropha curcas (physic nut) is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. Its seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into biodiesel upon transesterification and used as a substitute for diesel fuel (Makkar et al., 2007b). The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 58 to 62 % crude protein (Makkar et al., 2008; Makkar and Becker, 2009). The levels of essential amino acids (except lysine) are higher in *Jatropha* kernel meal than in the FAO reference protein for a growing child of 3–5 years (Makkar and Becker, 1999). However the presence of high levels of antinutrients like trypsin inhibitor, lectin and phytate (Makkar et al., 2008) and the major toxic components phorbol esters (PEs) (Makkar and Becker, 1997) restrict their use in fish feed.

Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for detoxification of Jatropha kernel

meal has been developed in our laboratory. It is based on removal of PEs and inactivation of trypsin inhibitors and lectin by heat treatment. The aim of this experiment was to evaluate the nutritional value of detoxified Jatropha kernel meal (DJKM) in common carp, and compare it with that of SBM and FM.

Material and methods

Preparation of the Jatropha kernel meal

Jatropha seeds were obtained from India and deshelled manually to obtain kernels. Defatting of Jatropha kernals was done using petroleum benzene (b.p. 40–60 ^{0}C) in a Soxhlet apparatus. Organic solvents were used to detoxify defatted Jatropha kernel meal. Two duration of PE removal were investigated: shorter (30 min) and longer (60 min) and the detoxified meals so obtained were designated as J_a and J_b respectively (patent application has been filed for the process of detoxification). After removal of PEs, the meal was autoclaved (121 ^{0}C) to remove heat labile antinutrients like trypsin inhibitor and lectin.

Diet formulation

FM (Seelöwe fishmeal) was procured from Vereinigte Fishmeh werke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; and wheat meal was purchased from a local market. SBM (dehulled, defatted and roasted) was obtained from Institute of Animal Nutrition (450), University of Hohenheim, Germany. Soya protein isolate (SUPRO® 500E IP) was purchased from Solae Europe S.A., 2, Chemin du Pavillon, CH-1218 Le Grand-Saconnex, Geneva, Switzerland. Source of sunflower oil was Thomy, Nestle Deutchland AG, 41415 Neuss; Reg. Trademark of Societe des, Produits Nestle S.A.. Vitamin premix and mineral premix were procured from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Source of lysine is Merck KGaA, 64271 Darmstadt, Germany.

Prior to feed formulation, the proximate composition of defatted Jatropha meal, wheat meal, SBM, soya protein isolates and FM was determined. A total of seven isonitrogenous and isoenergetic diets were formulated. Experimental diets containing crude protein 38%, crude lipid 8%, vitamin premix 2%, mineral premix 2% and titanium oxide (TiO$_2$) 1% were prepared. Lysine monohydrochloride (lysine 80% in this salt) was supplemented at the rate of 1% of DJKM inclusion in the diet. Each

experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg. The inclusion levels of the DJKM and SBM were as follows:

Control diet was prepared with FM and wheat meal, without any DJKM and SBM. The plant protein fed groups were: S_{50}: 50% of FM protein replaced by SBM; S_{75}: 75% of FM protein replaced by SBM; J_{a50}: 50% of FM protein replaced by DJ_aKM; J_{a75}: 75% of FM protein replaced by DJ_aKM; J_{b50}: 50% of FM protein replaced by DJ_bKM; J_{b75}: 75% of FM protein replaced by DJ_bKM. The final mixture of each diet was made into 2 mm diameter moist pellets (using a Bosch, Type UM60ST 2-M, Robort Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then freeze-dried (Table 1).

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets						
	Control	J_{a50}	J_{a75}	J_{b50}	J_{b75}	S_{50}	S_{75}
Fish meal	507.5	253.7	126.3	253.7	126.3	253.7	126.3
Soyabean meal	-	-	-	-	-	342.1	513
[1]Wheat meal	402.5	381.5	372	390	384.1	271	206
Jatropha meal	-	249.5	372	242.5	361.9	-	-
Soya concentrate	-	3.5	7	2	5	22	32
Sunflower oil	40	61.8	72.7	61.8	72.7	61.2	72.7
[2]Vitamin premix	20	20	20	20	20	20	20
[3]Mineral premix	20	20	20	20	20	20	20
TiO_2	10	10	10	10	10	10	10
Total	1000	1000	1000	1000	1000	1000	1000
Phytase (FTU/kg)	-	500	500	500	500	500	500
Lysine monohydrochloride (g)	-	2.5	3.7	2.4	3.6	-	-

Control: FM and wheat meal, without any DJKM and SBM
J_{a50}: 50% of FM protein replaced by DJ_aKM
J_{a75}: 75% of FM protein replaced by DJ_aKM
J_{b50}: 50% of FM protein replaced by DJ_bKM
J_{b75}: 75% of FM protein replaced by DJ_bKM
S_{50}: 50% of FM protein replaced by SBM
S_{75}: 75% of FM protein replaced by SBM
[1]Whole wheat meal.
[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.
[3]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 502; $MgSO_4$. $7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4$. H_2O, 3.12; $ZnSO_4$. $7H_2O$, 4.67; $CuSO_4$. $5H_2O$, 0.62; KI, 0.16; $CoCl_2$. $6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

Experimental system and animals

Common carp (*Cyprinus carpio* L.) fingerlings (about 2.0 – 3.0 g) from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. They were fed the Hohenheim standard fish diet containing approximately 38% protein, 8% lipid, 10% ash and with a gross energy content of 18.5 kJ g^{-1} dry matter. After an acclimatisation period of 20 days, 252 fish were randomly distributed into seven groups with four replicates; each replicate contained nine fish (av. wt. 3.2 ± 0.07 g) in an aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 – 27.1°C, pH 7.0 – 7.5, dissolved oxygen 6.9 – 7.4 mg l^{-1}, total NH_3 0.1– 0.2 mg l^{-1}, nitrite 0.07 – 0.1 mg l^{-1} and nitrate 1–3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass ($kg^{0.8}$) per day (equal to five times their maintenance energy requirement). In a preliminary study, feed at seven times maintenance energy requirement was offered to fish. This resulted in substantial presence of uneaten feed in the aquaria. However, no feed was left in the aquaria when feeds at five times maintenance energy requirement were offered. Since the aim of the study was to evaluate the performance of fish fed a diets containing detoxified Jatropha kernel meal, high level of feed consumption was preferred, in order to elicit adverse effects if any due to the presence of the detoxified kernel meal.

Total feed per day was split into five equal portions and each portion was given at 8:00, 10:30, 13:00, 15:30 and 18:00 h. The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment (av. wt. 3.2 ± 0.07 g) and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day. During last 2 weeks of the experiment, fish were fed with a diet containing a marker (TiO_2) for digestibility measurement (Mamun et al., 2007 and Dongmeza et al., 2009). The faeces collection was qualitative, as the experimental diets contained an inert marker (TiO2). During last two weeks of the experiment, faeces were

collected daily. After each feeding the aquaria were controlled for remaining feed; generally, there were no feed residues left. Every day prior to the faeces collection, aquaria were siphoned out to clean any residues. Faeces subsequently excreted by the fish were collected in separate beakers for each aquarium by siphoning with a short small pipe (Mamun et al., 2007). The collected mixture of water and faeces was centrifuged at 4000 × g for 10 min, the supernatant discarded and the faeces were then stored at -20°C until analysis. For the analysis, faeces from all the experimental periods from the same fish were pooled.

At start of the experiment, 18 fish of the same population were also killed and preserved at −20 °C for analysis of the initial body composition.

The experiment was terminated after 8 weeks and the fish were killed. Two fish per replicate were carefully dissected to obtain the intestine and stored in liquid nitrogen for determination of digestive enzymes activities. Two fish per replicate were stored at −20 °C for chemical composition analysis. Prior to the determination of the proximate composition, the fish were autoclaved at 121°C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and freeze-dried.

Extraction and estimation of phorbol esters (PEs) by high-performance liquid chromatography, and determination of antinutrients

PEs were determined according to Makkar et al., 2007a, which was based on the method of Makkar et al., 1997. Briefly, 0.5 g of the Jatropha meal and dried whole body fish sample were extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100, 5 µm (250 x 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007a). The four-PEs peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs is 3µg/g meal.

Trypsin inhibitor activity was determined essentially according to Smith et al., 1980 except that the enzyme was added last as suggested by Liu and Markakis, 1989. Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL^{-1} assay medium) that produced agglutination. The minimum amount was the

amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. The phytic acid content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis, MO, USA) as standard. Non-starch polysaccharides were estimated according to Englyst et al., 1994.

Amino acid analysis

The amino acid compositions of FM, DJKM, SBM, soya protein concentrate and wheat meal were determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 ^{0}C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. The tryptophan contents of the above-mentioned samples were quantified spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl, 1990.

Biochemical analysis

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Samples of animal origin (fish bodies and FM) were analysed for dry matter (DM), ash, crude protein (CP) and lipid (ether soluble lipid). Gross energy (GE) of diet ingredients, diets and fish bodies was determined with bomb calorimeter (IKA C7000, Janke & Kunkel GmbH, Satufen, Germany) using benzoic acid as a standard.

Growth parameters

Growth performance and diet nutrient utilisation were assessed in terms of body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed gain ratio (FGR), protein efficiency ratio (PER), protein productive value (PPV), lipid production value (LPV) and energy production value (EPV). These were calculated as follows:
BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100; SGR = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{\wedge 0.8}$ + (final body mass in g / 1000)$^{\wedge 0.8}$}/2] / number of trial days (Dabrowski et al., 1986); FGR = dry feed fed (g)/body mass gain (g); PER =

fresh body mass gain (g)/crude protein fed (g); PPV (%) = [(final fish body protein in g - initial fish body protein in g) / total protein   consumed in g] X 100; LPV (%) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid   consumed in g] X 100; EPV (%) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100.

Digestibility measurement and Fractional feed efficiency

Titanium dioxide in the feed and faeces was determined according to the method described by Richter et al. (2003b). The percentage of apparent dry matter digestibility of diets was calculated according to Maynard et al., 1981.

Apparent dry matter digestibility (%) = [1 – {(% TiO_2 in feed) / (% TiO_2 in faeces)}] X 100

Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets were calculated according to Maynard and Loosli, 1969.

The nutrient and energy digestibility were obtained using the following formula:

Apparent nutrient digestibility (%) = [1 – {(% TiO_2 in feed) / (% TiO_2 in faeces) X (% Nutrient or energy in faeces) / (% Nutrient or energy in feed)}] X 100

ADC of the test ingredients ADC were calculated based on the digestibility of the reference diet and the test diets using a equation used by Bureau et al., 1999:

$ADC_I = ADC_T + ((1-s) D_R/sD_I) (ADC_T - ADC_R)$

where: ADC_I = Apparent digestibility coefficient of test ingredient; ADC_T = Apparent digestibility coefficient of test diet; ADC_R = Apparent digestibility coefficient of the reference diet; D_R = % nutrient (or kJ/g gross energy) of the reference diet mash; D_I = % nutrient (or kJ/g gross energy) of the test ingredient; s = Proportion of test ingredient in test diet mash.

Fractional feed efficiency of nutrients and gross energy = (Nutrient and energy retained in the whole body/Digestible nutrient and gross energy) x 100

Digestible nutrients and energy = Total intake of nutrients and gross energy through feed X digestibility coefficient.

Relative intestinal length (RIL), hepatosomatic index (HSI), and intestinal somatic index (ISI)

RIL was measured and is expressed in relation to each animal weight expressed in mm g^{-1}.

RIL, HSI, and ISI are calculated as indicated below:

RIL = Intestine length (mm)/ body mass (g)

HSI = Liver mass (g) X 100 / body mass (g)

ISI = Intestine mass (g) X 100 / body mass (g)

Digestive enzymes assay

The reducing sugars produced due to the action of glucoamylase and α-amylase on carbohydrate was estimated using dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as mmole of maltose released from starch per min at 37 ^{0}C. Protease activity was determined by the casein digestion method of Drapeau, 1974. One unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta 0.001 A_{280}$ per minute at 37 ^{0}C and pH 7.8. Lipase activity was assayed by the method of Cherry and Crandall, 1932. One unit of enzyme was the hydrolysis of 1.0 microequivalent of fatty acid from a triglyceride in 24 h at pH 7.7 at 37^{0}C.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Duncan's multiple range test (P<0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results

Phorbol esters and antinutrients content in defatted Jatropha kernel meal and whole body fish

PE content in untreated defatted Jatropha kernel meal was 1.8 mg/g. However, PEs in detoxified Jatropha kernel meal (J_a and J_b) and dried whole body fish were undetectable. Trypsin inhibitor was not detected in autoclaved DJKM whereas phyate level in J_a and J_b were 8.9% and 9.1% respectively.

Fish behaviour and feed intake

Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was normal. There was no mortality during the entire experimental period.

It was particularly noted that the fish in the groups J_{a75} fed very slowly on their feed. Sometimes they ingested the feed pellets and spit them out after a few seconds and it was repeated 2 to 3 times before finally ingesting and swallowing the pellets, whereas fish in all other dietary groups fed actively on the experimental diets throughout the experiment.

Amino acid profile of experimental diets, proximate composition of experimental diets and whole body of fish

Proximate composition, antinutrients and amino acid compositions of feed ingredients are shown in Table 2. Amino acid compositions of experimental diets are shown in Table 3. All experimental diets have almost similar amino acid composition. The proximate composition of the different experimental diets (g kg^{-1} dry matter) and whole body tissue (wet basis g kg^{-1}) are presented in Tables 4 and 6 respectively. Diets contained about 38% crude protein and 18.5 kJ/g gross energy and were isonitrogenous and isoenergetic. Dry matter, crude lipid and ash were in the range of 94.4–96.1%, 8.3–8.8% and 10.3–11.1% respectively. There was no significant difference (P > 0.05) in moisture content, crude protein and ash of the whole body among the groups. Highest crude lipid deposition was observed in J_{b75} group, which is not statistically different (P > 0.05) to all plant protein fed groups, except J_{a75} group, wherein lowest lipid deposition was observed.

Table 2 Proximate composition, antinutrients content and amino acid composition of feed ingredients

	Fish meal	Jatropha meal (J$_a$)	Jatropha meal (J$_b$)	Soyabean meal	Soya protein concentrate	Wheat meal
Proximate composition (g kg $^{-1}$)						
Dry matter	940	941	945	955	940	941
Crude protein	635	646	665	471	900	143
Crude lipid	88	13.2	11.4	11.7	10	16.3
Crude ash	142	125	137	21.4	4.0	1.4
Crude fibre		89	91	38	10	25
Gross energy (KJ/g)	21.1	18.5	18.3	18.2	-	18.7
Antinutrients						
Trypsin inhibitor (mg trypsin inhibited per g sample)	ND	ND	ND	ND	ND	-
Lectin[a]	ND	ND	ND	ND	ND	
Phytate (% dry matter)	-	9.5	9.3	2.41	-	-
Amino acids composition (g kg $^{-1}$)						
Asparginine	60.5	66.1	68.7	66.6	122.8	7.2
Threonine	23	20.3	22	17.8	31.1	3.7
Serine	25.5	27.3	30.6	24.4	46	6.3
Glutamine	79.4	99.4	112.1	93.8	174.9	44.9
Glycine	59.8	27.6	31.5	21.3	37.2	5.6
Alanine	43.3	27	29.4	21.4	40.9	4.6
Cystine	4.3	2.2	2.3	6.5	9.8	2.9
Valine	29.3	28.5	31.6	21.2	37.4	5.1
Methionine	16	10.2	10.6	6.2	12.1	2
Iso leucine	22.8	24.3	26.7	19.6	36.5	4.2
Leucine	41.6	44.4	46.7	35.7	68.1	9.1
Tyrosine	14.8	15.5	18.8	15.8	31	3.3
Phenylalanine	21.8	27	30.4	24.3	43.2	6.5
Histidine	17.7	19.6	21.7	14.4	24.4	3.4
Lysine	40.9	19.7	23.3	29.1	52.1	3.3
Arginine	35.3	67.4	69.7	36	67.9	5.4
Proline	36.9	30.5	32.2	28.2	50.2	14.5
Tryptophan	4.9	7	7.1	6.4	10.4	1.4
Non-starch polysaccharides (NSP) (g kg $^{-1}$)						
Rhamnose	-	-	3	0	-	-
Fucose	-	-	1	0	-	-
Arabinose	-	-	31	24	-	-

Table 2 continued

Xylose	-	-	20	11	-	-
Mannose	-	-	5	6	-	-
Galactose	-	-	14	42	-	-
Glucose	-	-	57	32	-	-
Glucuronic acid	-	-	0	0	-	-
Galacturonic acid	-	-	30	24	-	-
Total-NSP	-	-	160	140	-	-

J_a and J_b: Detoxified Jatropha kernel meal obtained from shorter (30 min) and longer (60 min) duration of detoxification process respectively;

ND: Not detected

[a:]Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Table 3 Amino acid composition of the experimental diets (g kg^{-1} feed)

Amino acids	Control	J_{a50}	J_{a75}	J_{b50}	J_{b75}	S_{50}	S_{75}
Essential							
Arginine	20.09	28.07	32.02	28.10	32.10	24.23	26.21
Histidine	10.35	10.76	10.96	11.13	11.52	10.87	11.10
Iso leucine	13.26	13.58	13.74	13.97	14.34	14.43	14.97
Leucine	24.77	25.34	25.63	25.56	25.99	26.73	27.62
Lysine	22.09	19.23	17.79	19.82	18.73	22.37	22.44
Phenylalanine	13.68	14.90	15.52	15.52	16.47	16.56	17.94
Methionine	8.93	7.41	6.64	7.43	6.69	6.99	6.00
Threonine	13.16	12.42	12.05	12.68	12.44	13.61	13.79
Tryptophan	3.05	3.56	3.82	3.53	3.78	4.04	4.52
Valine	16.92	16.62	16.46	17.16	17.28	16.89	16.82
Non essential							
Alanine	23.83	19.62	17.51	19.99	18.08	20.45	18.70
Asparginine	33.60	35.02	35.77	35.06	35.88	42.79	47.22
Cystine	3.35	2.78	2.51	2.80	2.54	4.32	4.79
Glycine	32.60	24.32	20.16	25.07	21.29	24.79	20.82
Glutamine	58.37	62.69	64.93	65.19	68.72	68.25	72.99
Proline	24.56	22.68	21.75	22.93	22.13	24.04	23.72
Serine	15.48	15.85	16.04	16.44	16.94	17.54	18.51
Tyrosine	8.84	8.99	9.08	9.66	10.10	10.74	11.65

Amino acid compositions of the experimental diets were calculated from amino acid profile of individual feed ingredients

See footnotes to Table 1 .

Table 4 Proximate compositions of experimental diets (g kg^{-1} dry matter basis)

Treatment*	Dry matter	Crude protein	Crude lipid	Gross energy (kJ/g)	Ash
Control	948	385	87	184	105
J_{a50}	948	384	86	186	108
J_{a75}	954	385	86	185	111
J_{b50}	961	381	88	182	102
J_{b75}	949	382	87	184	100
S_{50}	944	383	83	187	110
S_{75}	949	382	85	194	103

* See footnotes to Table 1.

Growth performance and feed utilization

Weekly body mass developments of fish are given in Figure 1. The growth performance of the fish at the end of the experimental period and the nutrient utilization are presented in Tables 5 and 7. Weekly body mass gain indicates that second week onwards there was differential growth among the group, and lower body mass development was observed in J_{a50}, J_{a75} and S_{75} groups compared to other groups. This trend was maintained till the end of the experiment. Highest BMG, SGR, MGR and EPV were observed for the J_{b50} groups, which were statistically similar to that for control group and significantly (P < 0.05) higher than for all other groups. Lowest FGR was observed in control group, which is statistically similar to J_{b50} group. Highest LPV was observed in J_{b50} group, however; it was not significantly different from that of all other groups except J_{a75}. The LPV was lowest in J_{a75} group.

Lowest MGR, PER and PPV were observed in J_{a75} group whereas this group had lowest growth rate as well. In general, we found a decreasing trend of various growth and assimilation parameters (BWG, MGR, FGR, PER, PPV, and EPV) in the fish fed diets containing increasing levels of plant protein in the diet.

Table 5 Initial weight (IW), final weight (FW) and body mass gain (BMG) of common carp (*Cyprinus carpio* L.) fed with experimental diets for eight weeks

Treatment*	IW (g)	FW (g)	BMG (g)
Control	3.2 ± 0.1	32.0[a] ± 1.96	28.9[a] ± 1.95
J[a50]	3.2 ± 0.1	24.9[d] ± 3.31	21.7d ± 3.24
J[a75]	3.3 ± 0.0	20.9[e] ± 2.04	17.7[e] ± 2.03
J[b50]	3.2 ± 0.1	33.3[a] ± 0.64	30.1[a] ± 0.63
J[b75]	3.2 ± 0.1	28.3[bc] ± 1.21	25.1[bc] ± 1.25
S[50]	3.3 ± 0.1	30.6[ab] ± 0.72	27.3[ab] ± 0.68
S[75]	3.2 ± 0.1	27.7[c] ± 0.57	24.5[c] ± 0.64
SEM	0.01	0.88	0.88

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
* See footnotes to Table 1.

Table 6 Chemical composition of whole body of common carp (*Cyprinus carpio* L.) fingerlings of different experimental groups at the start and at the end of the experiment (gkg^{-1} wet basis ± SD)

Treatment*	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)
Initial fish	796 ± 0.6	133 ± 0.5	30 ± 0.1	44 ± 0.3	48 ± 0.3
Control	766 ± 6.4	146 ± 5.2	49[b] ± 2.3	22 ± 1.1	58[a] ± 1.6
J[a50]	778 ± 18.8	151 ± 5.1	57[ab] ± 17.8	23 ± 2.0	52[ab] ± 8.3
J[a75]	786 ± 7.0	150 ± 2.7	50[b] ± 6.1	22 ± 0.7	49[b] ± 2.6
J[b50]	769 ± 10.5	157 ± 2.4	66[ab] ± 10.7	22 ± 0.6	59[a] ± 02.2
J[b75]	768 ± 05.6	153 ± 3.1	69[a] ± 7.8	22 ± 1.9	55[ab] ± 2.3
S[50]	782 ± 11.5	14.5 ± 10.3	51[ab] ± 11.0	24 ± 1.3	49[b] ± 5.1
S[75]	786 ± 21.0	144 ± 14.9	57[ab] ± 4.8	22 ± 2.8	50[b] ± 4.3
SEM	2.8	1.4	2.1	0.3	1.1

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
* See footnotes to Table 1

Table 7 Growth performance and nutrient utilisation of common carp (*Cyprinus carpio* L.) fed with experimental diets for eight weeks

Treatment*	BMG	SGR	FGR	MGR	PER	PPV	LPV	EPV
Control	917.0[a] ± 59.63	4.1[ab] ± 0.11	1.00[b] ± 0.05	21.7[a] ± 0.5	2.6[a] ± 0.12	38.8[ab] ± 2.41	59.0[ab] ± 1.80	30.1[a] ± 1.91
J[a50]	684.7[c] ± 90.12	3.7[d] ± 0.21	1.06[ab] ± 0.03	19.3[b] ± 1.18	2.4[ab] ± 0.08	37.7[ab] ± 1.97	66.8[ab] ± 22.66	24.1[b] ± 4.76
J[a75]	542.8[d] ± 61.53	3.3[e] ± 0.18	1.15[ab] ± 0.03	17.6[c] ± 0.93	2.3[b] ± 0.05	34.7[b] ± 0.95	54.4[b] ± 7.54	20.8[b] ± 1.33
J[b50]	946.9[a] ± 26.34	4.2[a] ± 0.05	1.01[b] ± 0.02	22.0[a] ± 0.18	2.6[a] ± 0.04	41.3[a] ± 0.88	78.2[a] ± 13.04	29.5[a] ± 0.93
J[b75]	774.0[bc] ± 58.23	3.9[cd] ± 0.12	1.21[a] ± 0.24	20.1[b] ± 0.99	2.2[b] ± 0.37	34.4[b] ± 5.56	72.3[ab] ± 13.07	23.3[b] ± 4.01
S[50]	826.8[b] ± 25.95	4.0[bc] ± 0.05	1.06[ab] ± 0.02	21.1[ab] ± 0.20	2.5[ab] ± 0.06	38.0[ab] ± 1.87	68.4[ab] ± 10.39	24.2[b] ± 2.64
S[75]	758.1[bc] ± 39.13	3.8[cd] ± 0.08	1.02[b] ± 0.02	20.3[b] ± 0.32	2.6[a] ± 0.05	37.4[ab] ± 4.06	69.7[ab] ± 5.09	23.2[b] ± 2.00
SEM	27.24	0.06	0.02	0.31	0.04	0.71	2.71	0.82

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
BMG (%) - Body mass gain, SGR (%) – Specific growth rate and FGR – Feed gain ratio; MGR (gkg0.8 day-1) - Metabolic growth rate, PER - Protein efficiency ratio, PPV (%)- Protein productive value, LPV (%)-Lipid production value and EPV (%)-Energy production value.
* See footnotes to Table 1

Digestibility measurements of diets and feed ingredients

Digestibility of the dry matter, nutrients and energy of different experimental diets and feed ingredients (SBM and DJKM) are given in Tables 8 and 9 respectively. The dry matter and lipid digestibility were statistically (P < 0.05) highest in control group and lowest in J[a50] group. Protein and energy digestibilties were statistically similar (P> 0.05) for, control and J[b50] groups and these values were highest for these groups. This was also reflected in PER and EPV parameters for these two groups.

Highest dry matter digestibility was observed for J[b50] meal which was statistically similar to that S[50] and significantly higher than J[a50] meal. Protein and energy digestibilities for J[b50] meal was statistically higher than SBM and J[a] meal. It was generally observed that as plant protein inclusion increased in the diet, dry matter, protein and energy digestibility of feed ingredients (SBM, J[a] and J[b]) decreased gradually.
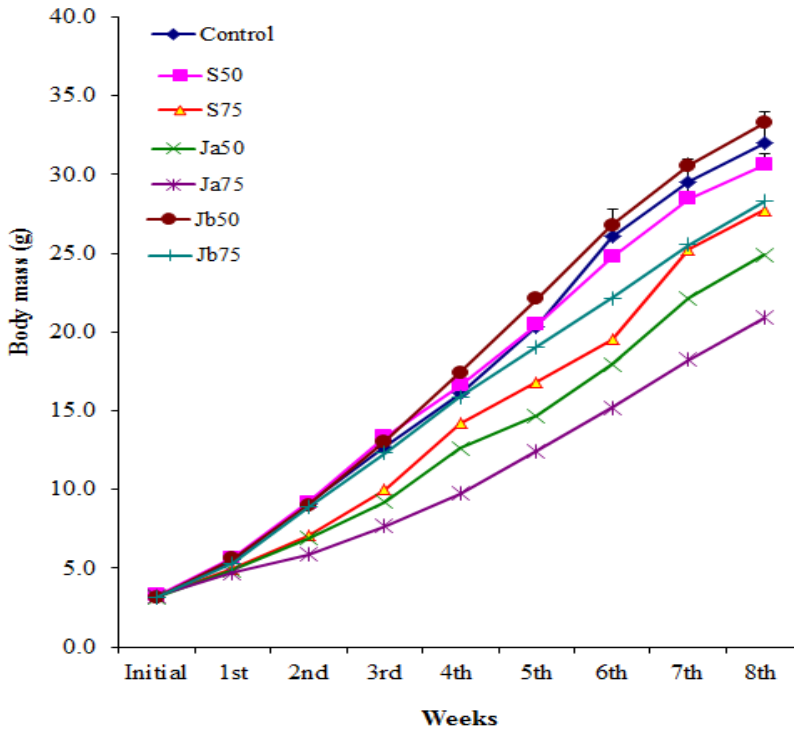
Figure 1 Body mass gain of common carp (*Cyprinus carpio*) fed with experimental diets for eight weeks

Table 8 Effects of experimental diets on apparent digestibility coefficient of the dry matter, nutrient and energy digestibility (%) in common carp (*Cyprinus carpio* L.) fingerlings

Treatment*	Dry matter digestibility	Protein digestibility	Lipid digestibilty	Energy digestibilty
Control	84.4[a] ± 0.40	92.3[a] ± 0.45	97.2[a] ± 0.68	87.7[a] ± 1.33
J$_{a50}$	73.1[f] ± 0.73	87.7[d] ± 0.75	89.7[d] ± 0.74	77.9[c] ± 3.14
J$_{a75}$	74.8[e] ± 0.93	87.3[d] ± 0.94	89.2[d] ± 1.31	78.0[c] ± 2.84
J$_{b50}$	82.5[b] ± 0.25	92.2[a] ± 0.39	95.0[b] ± 0.91	87.6[a] ± 1.11
J$_{b75}$	80.6[c] ± 0.12	90.6[b] ± 0.07	92.1[c] ± 0.90	83.1[b] ± 0.95
S$_{50}$	81.3[c] ± 0.45	91.3[b] ± 0.31	94.0[b] ± 0.82	83.6[b] ± 0.51
S$_{75}$	77.9[d] ± 0.29	88.6[c] ± 0.71	92.5[c] ± 1.11	82.8[b] ± 1.47
SEM	0.74	0.39	0.53	0.78

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
* See footnotes to Table 1.

Table 9 Dry matter, protein and energy digestibility of feed ingredients

Ingredients	Replacement of fish meal protein (%)	Dry matter digestibility	Protein digestibility	Energy digestibilty
Jatropha (J[a])	50%	60.6[c] ± 2.33	82.3[d] ± 1.89	61.6[c] ± 7.61
	75%	43.1[d] ± 2.68	79.4[e] ± 2.10	48.6[d] ± 12.51
Jatropha (J[b])	50%	77.1[a] ± 0.96	92.1[a] ± 1.12	87.4[a] ± 4.69
	75%	74.7[ab] ± 0.31	88.9[b] ± 0.15	74.8[b] ± 2.64
Soyabean	50%	75.9[a] ± 1.22	89.8[b] ± 0.81	75.0[b] ± 1.62
	75%	72.3[b] ± 0.54	85.8[c] ± 1.27	77.6[b] ± 3.04
SEM		2.85	0.96	2.55

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Fractional feed efficiency

Fractional feed efficiency of all experimental diets is shown in Table 10. Highest crude protein efficiency was observed in J[b50] group, which was statistically similar to that of control, S[50], S[75], J[a50] and J[a75] groups whereas lowest crude protein efficiency was found in J[b75] group. Crude lipid efficiency did not differ significantly among the groups. Crude energy efficiency was not statistically different (P>0.05) for control and J[b50] and significantly higher (P<0.05) compared to that for other groups.

Relative intestinal length (RIL), hepatosomatic index (HSI), and intestinalsomatic index (ISI)

RIL, HSI and ISI of fish fed different diets are presented in Table 11. There was no significant difference in ISI among different groups. HSI value of plant protein fed groups was statistically higher (P>0.05) than for control group. RIL value of plant protein fed groups except J[a50] and J[a75] were statistically higher (P>0.05) than for control group.

Digestive enzymes activity

Amylase, protease and lipase enzyme activities in intestine of fish are shown in Table 12. Amylase, protease and lipase activities for control group were higher (P<0.05) than for plant protein fed groups.

Table 10 Fractional feed efficiency of experimental diets, based on digestible nutrients

Treatments*	Crude protein efficiency	Crude lipid efficiency	Crude energy efficiency
Control	0.42[ab] ± 0.03	0.47 ± 0.15	0.34[a] ± 0.02
J_{a50}	0.43[ab] ± 0.02	0.57 ± 0.20	0.31[abc] ± 0.06
J_{a75}	0.39[ab] ± 0.01	0.47 ± 0.59	0.27[c] ± 0.02
J_{b50}	0.45[a] ± 0.01	0.65 ± 0.11	0.34[ab] ± 0.01
J_{b75}	0.38[b] ± 0.06	0.61 ± 0.11	0.28[bc] ± 0.05
S_{50}	0.42[ab] ± 0.02	0.55 ± 0.85	0.29[bc] ± 0.03
S_{75}	0.42[ab] ± 0.05	0.57 ± 0.46	0.28[bc] ± 0.03
SEM	0.08	0.23	0.09

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05). * See footnotes to Table 1.

Table 11 Effects of experimental diets on the relative intestinal length (RIL), hepatosomatic index (HSI) and intestinalsomatic index (ISI) of common carp (*Cyprinus carpio* L.) fingerlings

Treatment*	RIL	HSI	ISI
Control	2.55[bc] ± 0.13	2.12[b] ± 0.03	2.99 ± 0.26
J_{a50}	2.33[c] ± 0.10	2.20[b] ± 0.05	3.07 ± 0.17
J_{a75}	2.20[c] ± 0.08	2.23[ab] ± 0.10	3.12 ± 0.34
J_{b50}	3.30[a] ± 0.18	2.24[b] ± 0.04	3.08 ± 0.03
J_{b75}	2.93[ab] ± 0.10	2.35[a] ± 0.07	3.09 ± 0.36
S_{50}	3.10[a] ± 0.16	2.26[ab] ± 0.06	3.06 ± 0.15
S_{75}	2.75[b] ± 0.13	2.36[a] ± 0.12	3.23 ± 0.56
SEM	0.08	0.02	0.11

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05). * See footnotes to Table 1.

Table 12 Digestive enzymes activities (U/g protein) and of different experimental groups

Treatment*	Amylase	Protease	Lipase
Control	14.23[a] ± 1.71	31.12[a] ± 5.04	4.85[a] ± 0.74
J_{a50}	6.94[c] ± 0.70	13.65[d] ± 1.57	2.45[d] ± 0.54
J_{a75}	5.85[c] ± 0.97	10.51[e] ± 0.90	2.16[d] ± 0.37
J_{b50}	11.64[b] ± 1.80	24.79[b] ± 1.92	4.43[ab] ± 0.38
J_{b75}	11.02[b] ± 1.59	20.12[c] ± 2.37	4.21[bc] ± 0.62
S_{50}	10.51[b] ± 1.48	22.65[b] ± 1.89	4.16[ab] ± 0.61
S_{75}	10.47[b] ± 0.83	19.13[c] ± 1.32	3.95[c] ± 0.56
SEM	0.32	0.73	0.12

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
* see footnotes to Table 1.

Discussion

The results of the present investigation indicate that J_b meal at low inclusion levels (J_{b50}, 50% of FM protein replaced by DJKM) is a good dietary protein source for carp feed. The performance of SBM fed groups was not as good as of J_b fed groups, however it was better than that of J_a fed groups. SBM was found to be a moderately good protein source for carp at the 50% inclusion level, whereas, it was not promising at a high inclusion level (75% of FM protein replacement). J_a proved to be unsuitable even at low inclusion (50%) in carp diet.

Several factors such as acceptability of diets, presence of toxic and antinutritional factors and digestibility of protein and energy in the diets could contribute to the observed variation in the growth responses of carp. Although PEs was not detected by HPLC in J_a meal, the poor growth response, feed intake and feed utilization of carp fed diets containing J_a meal indicate that J_a meal was not detoxified properly. It is possible that PE might be present in strongly bounded form in the J_a meal, and was not extracted by our protocol for HPLC determination. PE could have been released from J_a meal in the fish intestine during digestion process hindering the growth performance and nutrient utilization. Experimental evidence for the toxic action of PE is well documented in common carp and rat. Feeding trials on common carp and rats with Jatropha meal containing PE has been reported to cause marked reduction in feed intake, diarrhoea and depression of growth (Becker and Makkar, 1998; Rakshit et al., 2008).

Growth performance and nutrient utilization

Growth performance and nutrient utilization of carp fed J_{b50} diet (50% FM protein replacement by DJKM) were better than of those fed SBM based diets and similar to those fed FM based diets. Slightly lower performance of fish fed the diet where 75% of FM protein was replaced by DJKM suggested that the capacity of DJKM to fully sustain growth was slightly lower compared to the diet based on FM only (control diet). The growth data were consistent with the feed conversion data, nitrogen retention and protein efficiency.

The lower growth response of 75% plant protein fed group could be due to lower protein availability from the SBM and DJKM (plant protein structure is much more compact than FM, so digestive enzymes act slowly on plant proteins), poor availability of crystalline lysine added to DJKM groups to equalize lysine content, and/or the presence of antinutrients such

as phytate and non-starch polysaccharides (NSP), which are present in high amounts in the kernel meal, which affects adversely the feed utilization.

Our result of growth performance and feed utilization in carp are supported by Hasan et al., 1997 in diet for carp wherein groundnut meal substituted 25% of FM protein without evidencing a negative effect on the feed intake and growth performance. Carp are not able to utilize high level (more than 50% of FM protein replacement) of plant derived protein in the diets because of low palatability, and high fibre and antinutrients content. Similar results were obtained by Viola et al., 1982 for carp and by Jackson et al., 1982 for tilapia.

Biochemical composition of whole body of fish

Moisture and lipid contents of whole body of fish tend to show greater variation than other carcass components (crude protein, ash and gross energy) and they appear to be inversely related (Atack et al., 1979; Focken and Becker, 1993; Hasan et al., 1997). Crude lipid content and gross energy content of carp fed different diets did not show large variations, although some of the values were statistically different (Table 4). Significantly higher crude lipid deposition was observed in all plant protein fed groups (except J_{a75} group), whereas lowest lipid deposition was observed in J_{a75} and control groups. Higher value of HSI in plant protein fed groups suggests higher lipid deposition in liver. HSI values of above 2, as observed here, are common in common carp (Yılmaz and Genc, 2006). The increase in whole body fat content with the use of dietary plant proteins based diets could be due to the higher content of total carbohydrate in plant protein based diets. The carbohydrates could get converted to lipid in the body by lipogenesis. Hasan et al., 1997 in their study on common carp fed with plant protein source such as mustard, sesame, linseed, copra and groundnut oil cakes, substituting for FM protein, also observed significantly higher deposition of crude lipid in whole body.

Whole body moisture and crude protein content was similar for all the groups. Whereas, Hasan et al., 1997 observed that carp fed with plant protein (mustard, sesame and linseed oil cake) exhibits lower moisture and higher crude protein content when compared to FM fed groups. However, in the same trial Hasan et al., 1997 found that whole body ash content of carp did not differ significantly among all the groups and same trend was followed in our study. In the present study the plant protein based diets were supplemented with phytase and that could have released minerals bound to phytic acid.

Digestibility measurement of experimental diets

Soybean meal and DJKM in combination with FM protein showed excellent dry matter, crude protein, lipid and energy digestibility in the present study. Generally, oil seed meal proteins have digestibilities of 80-95% for fish (Jauncey and Ross, 1982) and carp are also reported to be able to digest the plant proteins well, which is generally slightly better than monogastric mammals (National Research Council 1983). The protein digestibility coefficient is a key factor in the evaluation of the quality of the diet for fish and the potential of the diet for the synthesis of new tissue. Dry matter, protein, lipid and energy digestibility of experimental diets were 78-85%, 89-92%, 92-97% and 83-88% respectively, which indicate excellent utilization of feed ingredients. Higher apparent digestibility for protein from DJKM than from SBM implies that a greater amount of protein would have been available to the fish from the DJKM diets. Hasan et al., 1994 reported that apparent protein digestibility values ranged between 68.3-72.9% for carp fed plant protein (leucaena leaf meal) based diets. In the present study, crude protein digestibility of DJKM diets were high (above 90%) in common carp suggested DJKM to be an excellent protein source for carp diet. Energy digestibility of plant protein based diets (SBM and DJKM based diets) was considerably lower than protein digestibility due to their high carbohydrate content (Gomes et al., 1993; Gouveia et al., 1993). The lower lipid digestibility of the fish fed the SBM diet may be associated with the increase in the NSP content, which reduces fat absorption by disturbing micelle formation in the gastro intestinal tract (Krogdahl et al., 2003; Gatlin et al., 2007; Øverland et al., 2009).

Digestibility measurement of feed ingredients

The apparent protein digestibility (92.1%) of J_b meal at 50% replacement of FM was almost as good as FM. The reduction in the digestibility of protein in the fish fed the SBM compared to FM is in agreement with earlier findings (Storebakken et al., 2000; Aslaksen et al., 2007). However, the apparent digestibility of dry matter was generally poor, especially for plant feed ingredients where values ranged from 39.3% - 76.5%. The low-energy digestibility of these plant derived ingredients could be attributed to their high-carbohydrate content and poor digestibility by omnivorous fish (Lupatsch et al., 1997), although these values exhibit higher (48.3-87.4%) than reported by many researchers for fish. Interestingly it was found that J_b meal

at 50% replacement of FM protein in the carp diet has similar apparent energy digestibility as for FM. The results demonstrate that the carp were efficient in digesting protein and energy from the J_b meal, while fish fed the SBM in general exhibited lower digestibilities.

Fractional feed efficiency indicates retained nutrients and energy in whole body to the total digestible nutrients and gross energy. Highest crude protein and gross energy efficiency were observed in J_{b50} group, which indicate that carp have utilized J_b meal and retained nutrients in the body maximally.

Digestive enzyme activities and relative intestinal length (RIL)

Heat labile antinutrients (trypsin inhibitors and lectins) were not detected in the autoclaved DJKM and roasted SBM, whereas heat stable enzyme inhibitors (phytate) were present in those feed ingredients. Antinutritional factors such as phytic acid inhibit activities of some digestive enzymes such as pepsin, trypsin and alpha-amylase (Alarcon et al., 1999; Robaina et al., 1995), or form complexes with minerals (Teskeredzic et al., 1995; Sugiura et al., 1999) and proteins (Moyano et al., 1999), thereby modifying digestion processes and this could impair intestinal absorption. Carp showed a significant decrease in protease, amylase and lipase activities in intestine on inclusion of plant proteins in the diet. Lower protease activities, the level of which decreased with increase in SBM and DJKM in common carp diets, corresponded to decrease in protein availability from SBM and DJKM. Similar results were observed by Santigosa et al., 2008, Sandholm et al., 1976 and Krogdahl et al., 1994. They found that protein digesting enzyme (trypsin) activity decreases as plant protein inclusion increases in trout diet and they concluded that trypsin is highly sensitive to plant antinutrients. Escaffre et al., 1997 observed that increasing levels of dietary soya protein concentrate induced a significant decline in trypsin activity in common carp. The decrease in protease activity at higher inclusion level of DJKM might be caused by the presence of phytate. The lower activity of digestive enzymes in DJKM fed groups was correlated with lower nutrient digestibility of nutrients.

It is known that carnivorous and omnivorous fish require longer time to digest plant protein based diets (Buddington et al., 1997). Direct relationship between the amount of dietary plant protein and RIL has been reported earlier in fish (Kramer and Bryant 1995). In carp, plant protein based diets (except J_{a50} and J_{a75} groups) exhibited higher RIL than the control group. In addition, in the present study J_{b75} and S_{75} groups exhibited lower

RIL than J_{b50} and S_{50} and higher than control group. In another study, we observed that RIL value increases as the plant protein inclusion increases from 50% and 62.5% FM protein replacement in the common carp diets (Unpublished). This indicates that RIL value increased up to 62.5% replacement of FM protein by plant protein. Lower RIL at higher than 62.5% FM protein replacement by plant protein in common carp indicates that 75% level of replacement of FM is above the threshold level that could elicit higher RIL. Lower RIL of J_{a50} and J_{a75} groups than that of control could be attributed to the lower growth and lower nutrient utilization possible due to the presence of residual PEs.

From a physiological view point, a longer RIL would facilitate an increase in digestibility and retention time by enhancing contact time of the digestive enzymes and the feed components, resulting in increase in their digestion and absorption. Omnivorous fish like common carp species showed compensation mechanisms, such as an increase in RIL and as a result increase in digestive activity, to achieve a digestive balance and growth rates similar to those observed for FM fed group.

Conclusion

The DJKM can replace 50% FM protein in carp diets, without sacrificing the growth and nutrient utilization of fish. The DJKM can be used as one of the promising FM replacers in the diet of common carp. PE, the main toxic principle for Jatropha toxicity was not detected in fish muscle tissues, suggesting the fish is safe for human consumption.

Acknowledgement

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Chapter 3

Physiological, haematological and histopathological responses in common carp (*Cyprinus carpio* L) fingerlings fed with differently detoxified *Jatropha curcas* kernel meal

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Abstract

Protein rich *Jatropha curcas* kernel meal is toxic. It was detoxified using heat treatment and solvent extraction. Two duration of detoxification process were investigated: shorter (30 min) and longer (60 min) and the detoxified meals so obtained were designated as J_a and J_b respectively. Common carp fingerlings (252 fish; 3.2±0.07 g) were fed diets: Control containing fishmeal (FM); S_{50}, J_{a50} and J_{b50}: 50% of FM protein replaced by soybean-meal (SBM), detoxified Jatropha kernel meal (DJ_aKM and DJ_bKM); S_{75}, J_{a75} and J_{b75}: 75% of FM protein replaced by SBM, DJ_aKM and DJ_bKM. White blood cells count, mean cell volume and mean cell hemoglobin concentration, calcium and sodium ions and total bilirubin in blood did not differ significantly among the groups. Higher (P<0.05) RBC count was observed in 75% replacement of FM protein fed groups compared to other groups. Highest alkaline phosphatase and alanine transaminase activities in blood were observed in J_{a75}, which were not different (P>0.05) from those in J_{a50} group, but were higher than in other groups. No adverse histopathological changes in liver and muscle of any group were observed, but intestinal mucosa of J_{a75} groups showed severe pathological lesions. The results demonstrate that Jb was completely detoxified. Since the performance of J_{b50} group was similar to control group and better than other groups, optimum inclusion level of J_b is 50% replacement of FM protein.

Keywords: Common carp, *Jatropha curcas*, metabolic enzymes, haematology, histology

Introduction

Traditionally, fish meal (FM) has been the main source of dietary protein for fish. In recent years, its increasing cost, decreasing availability in the market and poor quality have stimulated several studies on its partial or complete substitution with alternative protein sources (Kaushik et al., 1995; Fournier et al., 2004). Soybean meal (SBM) is currently the most commonly used plant protein source in fish feeds (Yue and Zhou, 2009). The price of SBM, which is the main protein source for cultured animals, has increased sharply (Azaza et al., 2009). Nowadays, maize is using as an energy source in fish to reduce feed cost and also being used to satisfy the rising demand of the fast-growing bio-fuels industry (Azaza et al., 2009).

According to FAO (2008), the price of SBM increased to new record level, an increase of 60% from early May 2007. Based on the current supply and demand forecasts for the coming years, prices can be expected to remain high. This phenomenon has limited the expansion and profitability of aquaculture enterprises in most developing countries (Tacon, 2007). In many countries, SBM and soybean oil used in feed formulation are imported, which increase feed costs. This is the case in Europe and many tropical countries, especially in sub-Saharan Africa where soybean production is fairly limited due mainly to climatic and geographical constraints.

Most modern, nutrient-dense, aquaculture diets use some inclusion of plant protein ingredients. Many such ingredients have been assessed experimentally, but with the notable exception of SBM, few are used commercially (Carter and Hauler, 2000). The high cost of protein sources, their restricted availability and the unpredictability of their markets, increase the need for alternative sources of protein in fish feed.

Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. The International Jatropha Organization has projected that in 2017 there will be around 32.72 million hectares of land cultivated worldwide with *J. curcas,* producing 160 million tons of seeds and 95% of the total production will be in Asia (Siang, 2009). Jatropha seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar et al., 2007). The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 58 to 62 % crude protein (Makkar et al., 2008). The levels of essential amino acids, except lysine in *J. curcas* kernel meal are

higher than in SMB (except lysine). However, the presence of high levels of antinutrients such as trypsin inhibitor, lectin and phytate (Makkar et al., 2008) and the major toxic components phorbol esters (PE$_S$) (Makkar and Becker, 1997) restrict their use in fish feed. Heat labile antinutrients, protease inhibitors, and lectins are easy to inactivate by moist heating (Makkar and Becker, 2009). A method for detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

Jatropha plant can yield up to four tons seed per year from one hectare of plantation, which can produce approximately one ton of kernel meal rich in protein (Makkar and Becker, 1997). This means that there is a possibility of producing enough Jatropha kernel meal to meet growing aquaculture industry demand. Our previous studies have shown that detoxified Jatropha kernel meal (DJKM) is a good protein source for carp (*Cyprinus carpio*) (Kumar et al., 2008) and rainbow trout (*Oncorhynchus mykiss)* diets (Kumar et al., 2009). This study reports the physiological, haematological and histological responses of adding DJKM and SBM to common carp diets.

Material and methods

Preparation of the Jatropha meal

Jatropha seeds were obtained from India and deshelled manually to obtain kernels. Defatting of Jatropha kernels was done using petroleum benzene (b.p. 40–60 ^{0}C) in a Soxhlet apparatus. Organic solvents were used to detoxify defatted Jatropha kernel meal. Two durations of PE removal were investigated: shorter (30 min) and longer (60 min) and the detoxified meals so obtained were designated as J$_a$ and J$_b$ respectively (patent application has been filed for the detoxification process). After removal of PEs, the meal was autoclaved (121 ^{o}C) to remove heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation

Fish meal (Seelöwe fishmeal) was procured from Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; and wheat meal was purchased from a local market. Extracted soybean meal (dehulled, defatted and roasted) was obtained from the Institute of Animal Nutrition (450), University of Hohenheim, Stuttgart, Germany. Soya protein isolate

(SUPRO® 500E IP) was purchased from Solae Europe S.A., 2, Chemin du Pavillon, CH-1218 Le Grand-Saconnex, Geneva, Switzerland. Prior to feed formulation, the proximate composition of defatted Jatropha kernel meal, wheat meal, SBM, soya proteins isolate and FM was determined. A total of seven isonitrogenous and isoenergetic diets were formulated. Experimental diets containing crude protein 38%, crude lipid 8%, vitamin premix 2%, mineral premix 2% and TiO_2 1% were prepared. Lysine monohydrochloride (lysine 80% in this salt) was supplemented at the rate of 1% of DJKM inclusion in the diet. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg. The inclusion levels of the DJKM and SBM were as follows:

Control diet was prepared with FM and wheat meal, without any DJKM and SBM. S_{50}: 50% of FM protein replaced by SBM; S_{75}: 75% of FM protein replaced by SBM; J_{a50}: 50% of FM protein replaced by DJ_aKM; J_{a75}: 75% of FM protein replaced by DJ_aKM; J_{b50}: 50% of FM protein replaced by DJ_bKM; J_{b75}: 75% of FM protein replaced by DJ_bKM. The final mixture of each diet was made to 2 mm diameter moist pellets and then freeze-dried (Table 1).

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets						
	Control	J_{a50}	J_{a75}	J_{b50}	J_{b75}	S_{50}	S_{75}
Fish meal	507.5	253.7	126.3	253.7	126.3	253.7	126.3
Soyabean meal	-	-	-	-	-	342.1	513
[1]Wheat meal	402.5	381.5	372	390	384.1	271	206
Jatropha meal	-	249.5	372	242.5	361.9	-	-
Soya concentrate	-	3.5	7	2	5	22	32
Sunflower oil	40	61.8	72.7	61.8	72.7	61.2	72.7
[2]Vitamin premix	20	20	20	20	20	20	20
[3]Mineral premix	20	20	20	20	20	20	20
TiO_2	10	10	10	10	10	10	10
Total	1000	1000	1000	1000	1000	1000	1000
Phytase (FTU/kg)	-	500	500	500	500	500	500
Lysine monohydrochloride (g)	-	2.5	3.7	2.4	3.6	-	-

Control: FM and wheat meal, without any DJKM and SBM
J_{a50}: 50% of fish meal protein replaced by detoxified Jatropha kernel meal (30 min)
J_{a75}: 75% of fish meal protein replaced by detoxified Jatropha kernel meal (30 min)
J_{b50}: 50% of fish meal protein replaced by detoxified Jatropha kernel meal (60 min)
J_{b75}: 75% of fish meal protein replaced by detoxified Jatropha kernel meal (60 min)
S_{50}: 50% of fish meal protein replaced by soybean meal

S75: 75% of fish meal protein replaced by soybean meal

[1]Whole wheat meal.

[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[3]Mineral premix (g kg-1): CaCO3, 336; KH2PO4, 502; MgSO4. 7H2O, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; MnSO4. H2O, 3.12; ZnSO4.7H2O, 4.67; CuSO4.5H2O, 0.62; KI, 0.16; CoCl2. 6H2O, 0.08; ammonium molybdate, 0.06; 3 NaSeO3, 0.02.

Experimental system and animals

Common carp (*Cyprinus carpio* L.) fingerlings (about 2.0–3.0 g) from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahrensburg, Germany, were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. They were fed a standard fish diet containing approximately 38% protein, 8% lipid, 10% ash and with a gross energy content of 18.5 kJ g^{-1} dry matter. After an acclimatisation period of 20 days, 252 fish were randomly distributed into seven groups with four replicates; each replicate contained nine fish in an aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2–27.1°C, pH 7.0–7.5, dissolved oxygen 6.9–7.4 mg l^{-1}, total NH_3 0.1–0.2 mg l^{-1}, nitrite 0.07–0.1 mg l^{-1} and nitrate 1–3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass ($kg^{0.8}$) per day (equal to five times their maintenance energy requirement). No feed was left in the aquaria when the feeds at five times maintenance energy requirement were offered. Since the aim of the study was to evaluate the performance of fish fed diets containing DJKM, high level of feed consumption was preferred, in order to elicit adverse effects if any due to the presence of the detoxified kernel meal.

Total feed per day was split into five equal portions and each portion was given at 8:00, 10:30, 13:00, 15:30 and 18:00 h. The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment (av. wt. 3.2 ± 0.07 g) and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day.

The experiment was terminated after 8 weeks and the fish were killed. At the end of experiment, fish were anaesthetized by tricaine

methanesulfonate (MS222) at 250 ppm in water. Blood was drawn near caudal peduncle from two fish from each replicate and transferred into a heparinized tube. Blood from one fish was used for haematological study and the other was centrifuged at 1500×g for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at –20 °C for determination of cholesterol and triglycerides. Blood was drawn from one fish per replicate for serum collection. Serum was stored at –20 °C for lysozyme determination. Fish, one per replicate were carefully dissected to isolate muscle and stored at –20 °C for determination of cholesterol and muscle lipid peroxides.

The University animal welfare committee (University of Hohenheim Germany) approved all experimental procedures involving common carp.

Extraction and estimation of phorbol esters (PEs) by high-performance liquid chromatography, and determination of antinutrients

PEs was determined according to Makkar et al. (2007), which was based on the method of Makkar et al. (1997). Briefly, 0.5 g of the Jatropha kernel meal and dried whole body fish sample were extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100, 5 µm (250 x 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007). The four-PEs peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs is 3µg/g meal.

Trypsin inhibitor activity was determined essentially according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL^{-1} assay medium) that produced agglutination. The minimum amount was the amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis,

MO, USA) as standard. Non-starch polysaccharides (NSP) were estimated according to Englyst et al. (1994).

Amino acid analysis

The amino acid compositions of FM, DJKM, SBM, soya protein concentrate and wheat flour were determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 °C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. The tryptophan contents of the above-mentioned samples were determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

Proximate analysis

The proximate composition of diet ingredients and diets was determined using the standard methods of the Association of Official Analytical Chemists (AOAC, 1990). Samples of FM were analysed for dry matter, ash, crude protein and lipid. Gross energy of diet ingredients and diets was determined with bomb calorimeter (IKA C7000) using benzoic acid as a standard.

Spleen Index (SI)

Spleen Index is calculated as indicated below:
Spleen Index = spleen mass (g) / body mass (g)

Growth parameters

Growth performance in terms of body mass gain (BMG), Specific growth rate (SGR) and metabolic growth rate (MGR) were calculated as follows
BMG = final body mass (g) - initial body mass (g)
SGR (% day^{-1}) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR (g . $kg^{0.8}$. day^{-1}) = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$}/2] / number of trial days (Dabrowski et al., 1986).

Cholesterol and triglyceride estimation

The determinations of the plasma cholesterol and triglycerides were using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color intensity was determined photometrically and was directly proportional to the concentration of cholesterol and triglycerides in the plasma sample.

Assay of lipid peroxides

Lipid peroxides in fish muscle were determined using the procedure of Utley et al. (1967).

Blood chemistry (hemato-immunological parameters)

Total erythrocyte count (RBC) and total leucocyte count (WBC)

Red blood cells and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope.
The following formula was used to calculate the number of RBC per mm^3 of the blood sample:
Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid
N= Number of cells counted

Hemoglobin (Hb) and hematocrit (Hct) content

The Hb content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hematocrit was determined on the basis of sedimentation of blood. Heparinised blood (50 µl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Hematocrit [%] x 1000) ÷ (RBC count [millions/µL]); mean cell hemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/µL]) and mean cell hemoglobin concentration, MCHC [g/dL] = Hemoglobin [g/dL] ÷ Hematocrit [%].

Lysozyme activity in serum

Lysozyme activity of serum was measured by EnzChek Lysozyme Assay Kit (E-22013) Leiden, The Netherlands. The assay measures lysozyme activity on *Micrococcus lysodeikticus* cell wall, which is labeled to such a degree that the fluorescence is quenched. Lysozyme action relieves this quenching; yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase was measured by using spectrofluorometer that detects fluorescein. Lysozyme hydrolyzes β-(1-4)-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of the microorganism.

Blood parameters analysis by Vet Scan

VetScan Chemistry Analyzer (Scil Animal care company GmBh, Technischer service, Germany) was used for determinations of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium, creatinine, globulin, glucose, phosphorus, potassium, sodium, total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Histopathological studies

Immediately after killing the fish a total of 144 histological samples of different segments of the intestinal tract were fixed in Bouin´s fluid or in methanol/acetic acid for 48 h or 24 h respectively. The intestine was thereby subdivided in fore-, mid- and hindgut. After dehydration and embedding in paraffin serial sections of 5 μm thickness were prepared and processed for conventional histopathological studies. Haematoxylin and eosin (H&E) was used for staining the tissue sections.

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Duncan's multiple range test ($P < 0.05$). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results

Phorbol esters and antinutrient contents in defatted Jatropha kernel meal

Phorbol ester content in defatted Jatropha kernel meal was 1.6 mg/g. However PEs in the J_a and J_b detoxified Jatropha kernel meal was reduced to undetectable level. The sensitivity of the method is 5 µg/g kernel meal.

Trypsin inhibitor and lectins were not detected in SBM DJ_aKM and DJ_bKM. Phytate level in SBM, DJ_aKM and DJ_bKM was 9.5%, 9.3% and 2.5% respectively whereas NSP level in SBM, DJ_aKM and DJ_bKM was 14%, 16% and 16% on dry matter basis respectively.

Proximate composition, antinutrients and amino acid profile

Proximate composition and amino acid contents of feed ingredients are shown in Table 2. All experimental diets had almost similar amino acid composition. All the diets containing essential amino acid are essentially as per the requirement of the common carp (NRC, 1993). The proximate composition of the different experimental diets (% dry matter) is presented in Table 3. Diets contained about 38% crude protein and 18.5 MJ/kg gross energy. Dry matter, crude lipid and ash were in the range of 94.4–96.1%, 8.3–8.8% and 10.3–11.1% respectively.

Table 2 Composition of feed ingredients according to proximate and amino acid and non-starch polysaccharides analysis

	Fish meal	Jatropha meal (J_a)	Jatropha meal (J_b)	Soyabean meal	Soya protein concentrate	Wheat meal
Crude nutrients (g kg^{-1})						
Dry matter	940	941	945	955	940	941
Crude protein	635	646	665	471	900	143
Crude lipid	88	13.2	11.4	11.7	10	16.3
Crude ash	142	125	137	21.4	4.0	1.4
Crude fibre		89	91	38	10	25
Gross energy (KJ/g)	21.1	18.5	18.3	18.2	-	18.7
Essential amino acids (g kg^{-1})						
Arginine	35.3	67.4	69.7	36	67.9	5.4
Histidine	17.7	19.6	21.7	14.4	24.4	3.4
Iso leucine	22.8	24.3	26.7	19.6	36.5	4.2
Leucine	41.6	44.4	46.7	35.7	68.1	9.1

Table 2 continued

Lysine	40.9	19.7	23.3	29.1	52.1	3.3
Phenylalanine	21.8	27	30.4	24.3	43.2	6.5
Methionine	16	10.2	10.6	6.2	12.1	2
Threonine	23	20.3	22	17.8	31.1	3.7
Tryptophan	4.9	7	7.1	6.4	10.4	1.4
Valine	29.3	28.5	31.6	21.2	37.4	5.1
Non-essential amino acids (g kg $^{-1}$)						
Alanine	43.3	27	29.4	21.4	40.9	4.6
Asparginine	60.5	66.1	68.7	66.6	122.8	7.2
Cystine	4.3	2.2	2.3	6.5	9.8	2.9
Serine	25.5	27.3	30.6	24.4	46	6.3
Glutamine	79.4	99.4	112.1	93.8	174.9	44.9
Glycine	59.8	27.6	31.5	21.3	37.2	5.6
Tyrosine	14.8	15.5	18.8	15.8	31	3.3
Proline	36.9	30.5	32.2	28.2	50.2	14.5
Non-starch polysaccharides (NSP) (g kg $^{-1}$)						
Rhamnose	-	3	3	0	-	-
Fucose	-	1	1	0	-	-
arabinose	-	31	31	24	-	-
Xylose	-	20	20	11	-	-
Mannose	-	5	5	6	-	-
Galactose	-	14	14	42	-	-
Glucose	-	57	57	32	-	-
Glucuronic acid	-	0	0	0	-	-
Galacturonic acid	-	30	30	24	-	-
Total-NSP	-	160	160	140	-	-

J_a and J_b: detoxified Jatropha kernel meal obtained from shorter (30 min) and longer (60 min) duration of detoxification process respectively.

Table 3 Composition of experimental diets according to proximate and amino acid analysis (g kg^{-1} feed)

Treatment*	Control	J$_{a50}$	J$_{a75}$	J$_{b50}$	J$_{b75}$	S$_{50}$	S$_{75}$
Proximate (g kg^{-1})							
Dry matter	948	948	954	961	949	944	949
Crude protein	385	384	385	381	382	383	382
Crude lipid	87	86	86	88	87	83	85
Ash	105	108	111	102	100	110	103
Gross energy (kJ/g)	18.4	18.6	18.5	18.2	18.4	18.7	19.4
Essential amino acids (g kg^{-1})							
Arginine	20.09	28.07	32.02	28.10	32.10	24.23	26.21
Histidine	10.35	10.76	10.96	11.13	11.52	10.87	11.10
Iso leucine	13.26	13.58	13.74	13.97	14.34	14.43	14.97
Leucine	24.77	25.34	25.63	25.56	25.99	26.73	27.62
Lysine	22.09	19.23	17.79	19.82	18.73	22.37	22.44
Phenylalanine	13.68	14.90	15.52	15.52	16.47	16.56	17.94
Methionine	8.93	7.41	6.64	7.43	6.69	6.99	6.00
Threonine	13.16	12.42	12.05	12.68	12.44	13.61	13.79
Tryptophan	3.05	3.56	3.82	3.53	3.78	4.04	4.52
Valine	16.92	16.62	16.46	17.16	17.28	16.89	16.82
Non-essential amino acids (g kg^{-1})							
Alanine	23.83	19.62	17.51	19.99	18.08	20.45	18.70
Asparginine	33.60	35.02	35.77	35.06	35.88	42.79	47.22
Cystine	3.35	2.78	2.51	2.80	2.54	4.32	4.79
Glycine	32.60	24.32	20.16	25.07	21.29	24.79	20.82
Glutamine	58.37	62.69	64.93	65.19	68.72	68.25	72.99
Proline	24.56	22.68	21.75	22.93	22.13	24.04	23.72
Serine	15.48	15.85	16.04	16.44	16.94	17.54	18.51
Tyrosine	8.84	8.99	9.08	9.66	10.10	10.74	11.65

Amino acid compositions of the experimental diets were calculated from amino acid profile of individual feed ingredients.

*See footnotes to Table 1.

Fish behaviour and feed intake

Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behavior of fish was normal. There was no mortality during the entire experimental period. It was particularly noted that the fish in the groups J$_{a50}$ and J$_{a75}$ fed very slowly on their feed.

Sometimes they ingested the feed pellets and spit them out after a few seconds and it was repeated 2 to 3 times before finally ingesting and swallowing the pellets, whereas fish in all other dietary groups fed actively on the experimental diets throughout the experiment.

Growth performance

Weekly body mass gains of fish are given in Figure 1A. The growth performance of the fish at the end of the experimental period is presented in Table 4. Highest body mass gain was observed for the J_{b50} group, which was statistically not different (P > 0.05) from that for control group and was higher (P < 0.05) than for all other groups.

Table 4 Growth performance of common carp (*Cyprinus carpio* L.) fed experimental diets for eight weeks (Source: Kumar et al., 2010)

Treatment*	IBM (g)	FBM (g)	BMG (g)	SGR	MGR
Control	3.2 ± 0.1	32.0[a] ± 1.96	28.9[a] ± 1.95	4.1[ab] ± 0.11	21.7[a] ± 0.5
J_{a50}	3.2 ± 0.1	24.9[d] ± 3.31	21.7d ± 3.24	3.7[d] ± 0.21	19.3[b] ± 1.18
J_{a75}	3.3 ± 0.0	20.9[e] ± 2.04	17.7[e] ± 2.03	3.3[e] ± 0.18	17.6[c] ± 0.93
J_{b50}	3.2 ± 0.1	33.3[a] ± 0.64	30.1[a] ± 0.63	4.2[a] ± 0.05	22.0[a] ± 0.18
J_{b75}	3.2 ± 0.1	28.3[bc] ± 1.21	25.1[bc] ± 1.25	3.9[cd] ± 0.12	20.1[b] ± 0.99
S_{50}	3.3 ± 0.1	30.6[ab] ± 0.72	27.3[ab] ± 0.68	4.0[bc] ± 0.05	21.1[ab] ± 0.20
S_{75}	3.2 ± 0.1	27.7[c] ± 0.57	24.5[c] ± 0.64	3.8[cd] ± 0.08	20.3[b] ± 0.32
SEM	0.01	0.88	0.88	0.06	0.31

Initial body mass (IBM), final body mass (FBM), body mass gain (BMG), specific growth rate (SGR, % day^{-1}), and metabolic growth rate (MGR, g . kg$^{0.8}$ .day^{-1}).
Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05). *See footnotes to Table 1.
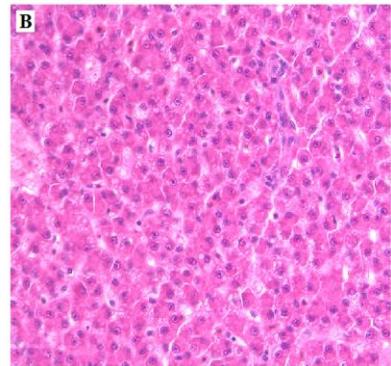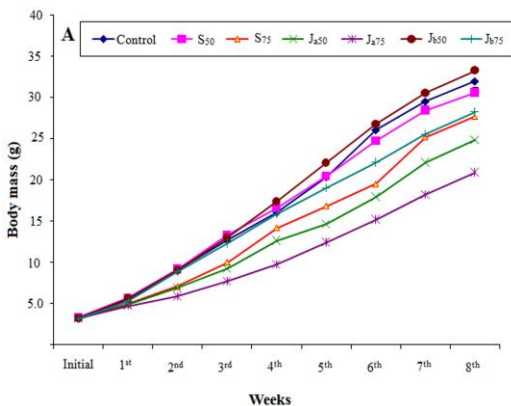
Fig 1. (A) Body mass gain of common carp (*Cyprinus carpio* L.) fed experimental diets for eight weeks, **(B)** liver of common carp (*Cyprinus carpio* L.) fed J_{b75} diet showing no pathological signs of steatosis/hepatic lipidosis.

Cholesterol and triglyceride levels in plasma, and cholesterol, phorbol esters and lipid peroxides in muscle

Cholesterol and triglycerides levels in plasma, muscle cholesterol and muscle lipid peroxide of different experimental groups are shown in Table 5. Plasma cholesterol level was highest in control group, which was statistically not different (P > 0.05) from those in S_{50} and J_{b50} groups and was higher than in other groups; whereas plasma triglyceride level was statistically lowest (P < 0.05) in control group. Muscle cholesterol level was highest in control group, which was statistically not different (P > 0.05) from that in S_{50} group and higher than those in other groups. Phorbol ester content in dried whole fish was undetectable. Muscle lipid peroxide value did not differ among the seven groups.

Table 5 Cholesterol and triglyceride in plasma (mg/dl), muscle cholesterol (mg/100 g), and muscle lipid peroxide (nmol malondialdehyde/100 g tissue) in common carp (*Cyprinus carpio*) fingerlings fed different experimental diets

Treatment*	Plasma cholesterol	Plasma triglycerides	Muscle cholesterol	Muscle lipid peroxide
Control	144.4[a] ± 16.3	77.2[b] ± 7.7	142.5[a] ± 94.7	1.60 ± 0.69
J_{a50}	108.7[bc] ± 25.4	142.3[a] ± 39.9	71.8[b] ± 7.1	2.20 ± 1.13
J_{a75}	107.6[bc] ± 19.5	133.5[a] ± 10.0	62.6[b] ± 3.8	2.70 ± 2.12
J_{b50}	136.4[ab] ± 9.0	140.4[a] ± 8.0	66.6[b] ± 2.1	3.83 ± 1.40
J_{b75}	103.9[bc] ± 26.9	120.4[a] ± 31.1	76.8[b] ± 5.6	2.95 ± 2.76
S_{50}	129.9[ab] ± 31.6	150.8[a] ± 31.8	85.3[ab] ± 5.2	1.57 ± 0.55
S_{75}	93.1[c] ± 15.2	145.8[a] ± 23.7	71.3[b] ± 5.7	1.52 ± 0.30
SEM	4.98	6.13	8.71	0.35

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
*See footnotes to Table 1.

Hemato-immunological blood parameters

Red blood cells, WBC, Hb level, Hct content, MCV, MCH, MCHC and spleen index are given in Table 6. White blood cells count and MCHC did not differ among the seven groups. Lowest MCV was observed in S_{75} group. Plant protein in the feed had significant effect on the total RBC count during the experimental periods. As the plant protein content increased, an increase in the RBC count was observed. Statistically similar (P > 0.05) RBC counts

were observed in S_{75}, J_{a75} and J_{b75} groups and these values were higher (P < 0.05) than those in other groups. Highest MCH was observed in J_{a50} group, which was statistically not different (P > 0.05) from those in control, S_{50}, J_{b50}, and J_{b75} groups but was higher (P < 0.05) than those in S_{50} and J_{a50} groups. Higher (P < 0.05) spleen index was observed in plant protein fed groups than control group.

Metabloic enzymes activities in blood

Highest ALP and ALT activities in blood were observed in J_{a75}, which was statistically not different (P > 0.05) from these in J_{a50} groups but were higher (P < 0.05) than those in other groups (Table 7).

Albumin, globulin and total protein concentration in the blood and lysozyme activity in serum

Albumin, globulin and total protein in the blood and lysozyme activity in the serum of different experimental groups are shown in Table 8. Highest albumin concentration in blood was observed in control group, which was statistically not different (P > 0.05) from that in J_{b50} group but was higher (P < 0.05) than those in other groups. Highest globulin concentration in blood was observed in J_{b50} group, which was statistically not different (P > 0.05) from that in S_{75} group but was higher (P < 0.05) than those in other groups. The concentration of total blood protein was highest in J_{a75} group, which was statistically not different (P > 0.05) to those in control, S_{50}, J_{a50}, and J_{b75} groups but was higher (P < 0.05) than those in other groups.

Glucose, total bilirubin, blood urea nitrogen, creatinine and ions in blood

Concentration of blood glucose, TBIL, BUN, creatinine and ions (calcium, phosphorus, sodium and potassium) are shown in Tables 9 and 10. Lower (P > 0.05) blood glucose concentration was observed in control group than plant protein fed groups. As plant protein content increased in the diet, blood glucose concentration also increased. Highest BUN was observed in J_{a75} group; whereas, TBIL did not differ among the seven groups.

Creatinine concentration in blood was significantly highest in control group. Calcium and sodium ions in blood did not differ significantly among the seven groups; whereas, there was significant difference in potassium and phosphorus ions in blood among the different groups.

Potassium and phosphorus ion concentrations in blood were higher (P < 0.05) in plant protein fed groups than control group.

Histopathology

Different experimental diets produced several changes in the intestinal mucosa but not in liver and muscle morphology in common carp. The liver did not show any pathological alteration or signs of steatosis/hepatic lipidosis in any of the experimental groups (Fig 1B). The intestinal loop sections appeared to be normal for fish fed control, S_{50}, $S_{75,}$ J_{b50} and J_{b75} diets. The section of J_{a75} group (Fig 2A) was characterized by reduced size of the intestinal loops compared with control section (Fig 2B). Additionally distinct parts of the anterior and posterior intestine showed severe pathological lesions in J_{a75} group. These lesions can mainly be described as "catarrhal enteritis" which is probably due to presence of PEs in DJ_aKM.
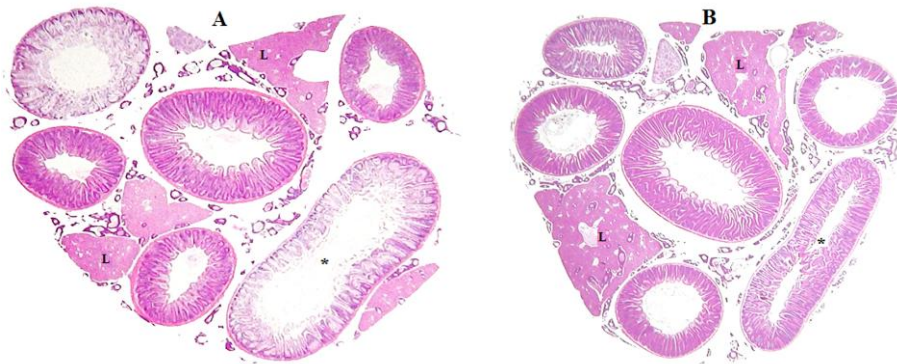
Fig 2. (A) Section of liver (L) and intestinal tract of J_{a75} group (reduced size of the digestive system organs and mucosal foldings; and disintegration of villi (*), **(B)** liver (L) and intestinal tract of control group (intestinal loops as well as the liver (L) are well-developed and show normal appearance).

Signs of necrosis characterize numerous enterocytes and denudations of the upper third of the lamina epithelial were accompanied by a massive influx of leucocytes into the lamina propria of the villi in J_{a75} group (Fig 3A). Hyperaemia and inflammation were additional symptoms in this group. There were mild lesions occasionally found in mucosal tissue samples of J_{b50} and J_{b75} groups, which were similar to those found in soybean meal containing diets. In these areas the pathological lesions were minor and all the other intestinal loops of both these groups seem to be unaffected in numerous different samples investigated. The intestinal mucosa was well

developed and no morphological alteration was found compared to control group (Fig 3B).
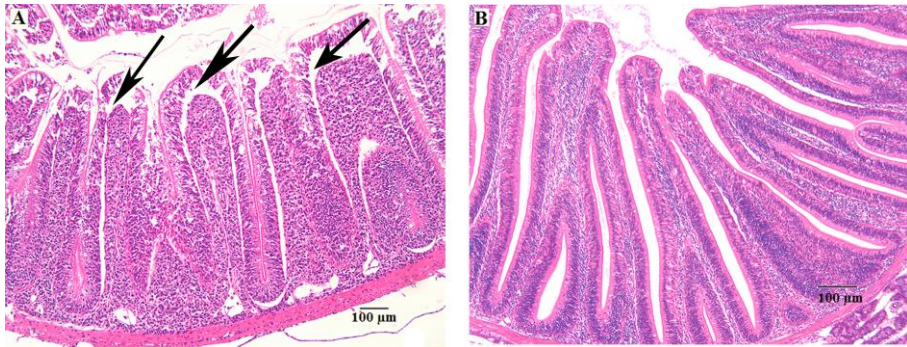
Fig 3. (A) Higher magnification of the mucosa of J_{a75} group demonstrates necrosis and denudation (arrows) of enterocytes and massive infiltration of immune-cells, **(B)** higher magnification of the mucosa of control group shows no signs of serious lesions.

Discussion

In the present study *Jatropha curcas* kernel meal was detoxified using heat treatment and solvent extraction. Two duration of detoxification process were investigated: shorter (30 min) and longer (60 min) and the detoxified meals so obtained were designated as J_a and J_b respectively. The results of the present investigation indicate that J_b meal at a lower inclusion level (J_{b50}, 50% of FM protein replaced by DJKM) is a good dietary protein source for carp feed. The performance of SBM fed groups was not as good as of J_b fed groups; however it was better than that of J_a fed groups. Soybean meal was found to be a moderately good protein source for carp at the 50% inclusion level, whereas, it was not promising at a higher inclusion level (75% of FM protein replacement). J_a proved to be unsuitable even at low inclusion (50%) in carp diet.

Several factors such as palatability, acceptability of diets, presence of toxic and antinutritional factors and digestibility of protein and energy in the diets could contribute to the observed variation in the growth responses of carp. Although PEs were not detected by HPLC in J_a meal, the poor growth response, feed intake, haematology and histopathological parameters of carp fed diets containing J_a meal indicate that J_a meal was not completely detoxified and PEs could be present at a concentration below the detection limit of the HPLC. It is possible that PEs might be present in strongly bounded form in the J_a meal, and were not extracted by our

protocol for the HPLC determination. Phorbol esters could have been released from J_a meal in the fish intestine during digestion process hindering the growth performance. Lately we have enhanced the sensitivity of the HPLC method for determination of phorbol esters from 10 ppm to 3 ppm and we found traces of phorbol esters (4 to 9 ppm) in Ja, and hence the effects are due to PEs. Experimental evidence for the toxic action of PEs is well documented in common carp and rat. Feeding trials on common carp and rats with Jatropha meal containing PEs have been reported to cause marked reduction in feed intake, diarrhoea and growth depression (Becker and Makkar, 1998; Rakshit et al., 2008).

Growth performance of carp fed J_{b50} diet (50% FM protein replacement by DJKM) was better than of those fed SBM based diets and similar to those fed FM based diets. Slightly lower performance of fish fed the diet where DJ_bKM replaced 75% of FM protein suggested that the capacity of DJKM to fully sustain growth was slightly lower compared to the control diet. This lower growth response of 75% plant protein fed group might be because of several factors such as lower digestibilities of protein and energy in the diets (Kumar et al., 2010), which could lead to lower protein and energy availability from the DJKM and SBM; and/or the presence of antinutrients such as phytate and NSP, which are present in high amounts in the SBM and DJKM and could adversely affect the feed utilization.

Cholesterol and triglycerides in plasma; blood glucose level

Dietary inclusion of DJKM in common carp reduced cholesterol level in plasma and muscle as compared to control group. The decrease in plasma cholesterol levels in fish fed diets with plant proteins has already been reported (Kaushik et al., 1995; Yamamoto et al., 2007). In terrestrial animals, plant products are generally considered to have a hypocholesteromic effect (de Schrijver, 1990), mainly due to the relatively high levels of estrogeno-mimetic isoflavones (Setchell and Cassidy, 1999). In humans, different plant constituents have been reported to lower plasma cholesterol levels (Wester, 2000). Although cholesterol metabolism in mammals and fish could differ, the fish hypocholesterolemia in response to dietary plant protein supply could be due either to an increased excretion of bile salts, to an inhibition of cholesterol intestinal absorption, or just to the withdrawal of FM rather than to the direct effects of plant protein (Kaushik et al., 2004). In any case, the significance of hypocholesterolemia in fish should be studied in depth.

Serum triglycerides act as a short-term indicator of the nutritional status (Bucolo and David, 1973). The increase in whole body fat content in plant protein fed groups (J_{b50} and S_{50}) (Kumar et al., 2010) along with the increase in plasma triglyceride concentrations also suggest significant lipid mobilisation in these groups.

Blood glucose concentration was affected by dietary treatments. Higher blood glucose concentration was observed in plant protein fed groups than in control group. Plant protein based diets contain higher amount of carbohydrates. Usually carbohydrates breakdown into smaller sugar compounds that concur with higher glucose level in blood. Similar trends were shown in fish fed diets containing SBM and corn gluten (Kikuchi et al., 1994; Kikuchi, 1999). On the other hand, Glencross et al. (2004) observed that dietary inclusion of yellow lupin in fish diet did not affect blood glucose level.

Hemato-immunological parameters

The WBC count, Hb content, Hct and MCHC did not differ significantly among the seven groups. As the plant protein content increased, an increase in the RBC count was observed. Higher RBC counts were observed in SBM and DJKM fed groups. Plant ingredients may cause early release of immature erythrocytes (Hemre et al., 2005), increasing the RBC count. Consequently, MCH value was changed at the same time.

Mean cell volume value differs significantly among the groups because RBC count was statistically different among the groups. Lower MCV was observed in plant protein fed groups (except J_{b50} group) than control group. Similarly, significant reduction of MCV on increase in the content of plant proteins in salmon diet was observed (Hemre et al., 2005). As this observation appeared to coincide with increased spleen size (Hemre et al., 2005), it was suggested that some of the plant ingredients might cause early release of immature erythrocytes. This appeared to be the case in the present study, since spleen size increased as the plant protein increased in diet (Table 6).

The Hb and Hct assays are normally used as general indicators of fish health (National Research Council, 1981). The Hb and Hct levels in all groups were within the normal range (Sun et al., 1995).

Blood protein and lysozyme activity in serum

The concentration of total protein in blood is used as a basic index for health and nutrititional status in fish (Martinez, 1976). Among the blood proteins, albumin and globulin are the major proteins, which play a significant role in the immune response. Albumin is used as an indicator of liver impairment (Silverman et al., 1986). Significantly lower blood protein content was observed in J_{a50} and J_{a75} groups that indicate nutritional deficiencies and impaired protein metabolism in the liver. However, in histopathological study we did not observe any pathological alteration or signs of steatosis/hepatic lipidosis in liver of these groups (Fig 2). Globulin is important for the immunological responses. Higher globulin concentration in blood was observed in DJKM and SBM fed groups compared to control groups. These results are consistent with the higher lysozyme activity observed in plant protein fed groups. This suggests an immunostimulating effect of DJKM and SBM inclusion in the diet of common carp fingerlings.

Lysozyme plays an important role in nonspecific immune response and it is found in mucus, serum and ova of fish. Innate immunity due to lysozyme is caused by lysis of bacterial cell wall and this stimulates the phagocytosis of bacteria. The suppression of the non-specific immune capacity by high concentrations of dietary soybean proteins has been reported in rainbow trout (Burrells et al., 1999). However, other reports wherein SBM was fed to rainbow trout (Rumsey et al., 1994) and Atlantic salmon (Krogdahl et al., 2000) or alginate to Atlantic salmon (Gabrielsen and Austreng, 1998), increased values of different non-specific immune parameters have been reported, which have been interpreted as immunostimulating effects of plant protein sources. Numerically higher lysozyme activity was observed in plant protein fed groups (except Ja50 and Ja75 groups); it might be due to immunostimulating effect of DJ_bKM and SBM in common carp.

Table 6 Effects of experimental diets on haematological parameters [spleen index, RBC (10^6 cells/mm³), WBC (10^5 cells/mm³), Hb (g/dl), Hct (%), MCV (fL), MCH (pg), MCHC (g/dl)] of common carp (*Cyprinus carpio*)

Treatment*	Spleen index	RBC	WBC	Hb	Hct	MCV	MCH	MCHC
Control	0.15[b] ± 0.01	1.58[b] ± 0.10	1.43 ± 0.07	5.00 ± 0.00	29.75 ± 2.73	189.3[ab] ± 4.16	31.8[ab] ± 1.99	17.14 ± 2.79
J$_{a50}$	0.16[ab] ± 0.02	1.67[ab] ± 0.16	1.65 ± 0.16	5.48 ± 0.96	30.00 ± 2.57	179.6[ab] ± 13.48	33.1[a] ± 7.07	19.30 ± 1.02
J$_{a75}$	0.17[a] ± 0.02	1.87[a] ± 0.03	1.60 ±0.03	5.00 ± 0.00	31.75 ± 1.71	170.0[ab] ± 8.03	26.8[b] ± 0.47	15.78 ± 0.83
J$_{b50}$	0.16[ab] ± 0.01	1.74[ab] ± 0.20	1.50 ± 0.08	5.00 ± 0.00	35.25 ± 5.74	205.3[a] ± 43.06	29.1[ab] ± 3.37	14.52 ± 2.73
J$_{b75}$	0.16[ab] ± 0.02	1.85[a] ± 0.13	1.85 ± 0.11	5.22 ± 0.43	31.75 ± 8.54	172.0[ab] ± 7.61	28.2[ab] ± 0.74	17.60 ± 1.93
S$_{50}$	0.16[ab] ± 0.01	1.71[ab] ± 0.08	1.73 ± 0.08	5.00 ± 0.00	32.25 ± 1.89	189.5[ab] ±18.53	29.3[ab] ± 1.35	15.54 ± 0.87
S$_{75}$	0.18[a] ± 0.01	1.87[a] ± 0.07	1.75 ± 0.07	5.00 ± 0.00	27.50 ± 5.00	147.6[b] ± 7.32	26.8[b] ± 0.97	18.63 ± 1.32
SEM	0.01	0.03	0.13	0.07	1.02	6.61	0.67	0.70

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
MCV: Mean cell volume (fL), MCH: Mean corpuscular hemoglobin (pg) and MCHC: Mean corpuscular hemoglobin concentration (g/dl)
*See footnotes to Table 1.

Metabolic enzymes

Alkaline phosphatase and ALT are released into blood during organ damage (Racicot et al., 1975). Alkaline phosphatase level rises during bile duct obstruction, and in intrahepatic infiltrative diseases of the liver (Goel et al., 1984). Alanine transaminase also called serum glutamic pyruvate transaminase is an enzyme present in hepatocytes. When liver cell is damaged, it leaks into the blood. Alanine transaminase rises dramatically in acute liver damage (Racicot et al., 1975). Thus, detection of blood level of ALP and ALT gives information on the damage of organs and in particular of liver cells. Significantly higher ALP and ALT activities were observed in J_{a50} and J_{a75} fed groups than other groups indicating toxicity, most probably due to residual PEs present in J_a meal. Phorbol esters could have damaged liver cells and ALP and ALT leaked into blood. Surprisingly we did not observe any liver cell damaged in histopathological study; whereas, we observed severe pathological lesions in mucosa, the first main organ interacting with the toxin. These lesions can mainly be described as "catarrhal enteritis". Hemre et al. (2005) and Sanden et al. (2006) also reported similar results on feeding SBM containing diets to Atlantic salmon.

Table 7 Effects of experimental diets on alkaline phosphatase (ALP, U/l) and alanine transaminase (ALT, U/l) in blood of *Cyprinus carpio* L. fingerlings

Treatment*	ALP	ALT
Control	85[b] ± 25.1	70[bc] ± 5.73
J_{a50}	159[a] ± 17.5	86[ab] ± 4.57
J_{a75}	162[a] ± 24.5	92[a] ± 6.63
J_{b50}	115[b] ± 52.1	80[b] ± 6.21
J_{b75}	75[c] ± 32.5	68[c] ± 3.62
S_{50}	107[ab] ± 21.8	77[b] ± 4.31
S_{75}	61[b] ± 24.2	71[b] ± 3.10
SEM	5.02	2.61

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute.
*See footnotes to Table 1.

Blood ions

Blood urea nitrogen concentrations were in the normal range (except for J_{a75} group) (Witters, 1986; Wedemeyer, 1996). The normal range for blood urea

nitrogen is 2 to 6 mg/dl (Witters, 1986; Wedemeyer, 1996). Blood urea nitrogen levels are thought to be associated with liver or gill dysfunction (Stoskopf, 1993), as these are the sites of urea production and excretion, respectively. Significantly higher (about 2 times) BUN was observed in J_{a75} group than other groups. This indicates liver and/or gill damage in this group. Blood urea nitrogen concentration were within normal range in all groups (except J_{a75} group) suggesting that SBM and DJ_bKM fed groups were normal and healthy.

The concentration of TBIL, an indicator of liver dysfunction (Tietz, 1986) was similar in all groups. Creatinine is used as an indicator of kidney damage or malfunction (Rock et al., 1986; Tietz, 1986), and is a by-product of creatine, which is involved in muscle energy metabolism. Blood creatinine is normally quite stable. The normal range for creatinine level in blood is 0.2 – 1.5 mg/dl (Tietz, 1986). Creatinine was highest in control group but was within the normal range. Creatinine being a degraded product of animal protein and its highest level in control is obviously due to highest content of FM in control diet. The plant protein based diets were supplemented with phytase and higher concentration of phosphorus in blood was observed in plant protein fed groups compared to control group, which could be due to release of phosphorus from bounded phytate and making it available for common carp.

Table 8 Lysozyme activity (IU/ml) in the serum, albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood of common carp (*Cyprinus carpio* L.) fingerlings fed different experimental diets

Treatment*	Lysozyme activity	Albumin	Globulin	Total protein
Control	336 ± 32.0	1.98[a] ± 0.12	0.88[c] ± 0.17	2.85[ab] ± 0.13
J_{a50}	328 ± 100.1	1.73[b] ± 0.11	1.03[bc] ± 0.19	2.75[b] ± 0.17
J_{a75}	287 ± 107.2	1.43[bc] ± 0.28	1.18[bc] ± 0.17	2.63[b] ± 0.13
J_{b50}	448 ± 172.9	1.35[c] ± 0.19	1.75[a] ± 0.30	3.10[a] ± 0.20
J_{b75}	402 ± 186.7	1.63[bc] ± 0.35	1.20[bc] ± 0.12	2.80[ab] ± 0.24
S_{50}	404 ± 101.9	1.63[bc] ± 0.21	1.15[bc] ± 0.13	2.80[ab] ± 0.16
S_{75}	457 ± 107.3	1.43[bc] ± 0.10	1.45[ab] ± 0.31	2.87[ab] ± 0.34
SEM	23.75	0.05	0.06	0.04

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

IU- The amount of enzyme required to produce a change in the absorbance at 450 nm of 0.001 units per minute at pH 6.24 and 25⁰C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

*See footnotes to Table 1.

Table 9 Effects of experimental diets on glucose (mg/dl), total bilirubin, TBIL (mg/dl), blood urea nitrogen, BUN (mg/dl) and creatinine (mg/dl) in blood of common carp (*Cyprinus carpio* L.) fingerlings

Treatment*	Glucose	TBIL	BUN	Creatinine
Control	72.75[b] ± 4.03	0.30 ± 0.00	3.00[c] ± 0.41	1.35[a] ± 0.70
J$_{a50}$	86.75[ab] ± 8.62	0.25 ± 0.06	5.50[b] ± 0.29	0.25[b] ± 0.06
J$_{a75}$	97.50[a] ± 2.82	0.25 ± 0.06	8.50[a] ± 0.29	0.23[b] ± 0.05
J$_{b50}$	87.75[ab] ± 4.03	0.22 ± 0.05	4.50[bc] ± 0.58	0.33[b] ± 0.15
J$_{b75}$	97.75[a] ± 5.63	0.22 ± 0.05	5.00[b] ± 0.00	0.20[b] ± 0.00
S$_{50}$	91.25[ab] ± 8.81	0.22 ± 0.05	3.00[c] ± 0.41	0.40[b] ± 0.14
S$_{75}$	99.00[a] ± 2.46	0.27 ± 0.05	3.00[c] ± 0.15	0.25[b] ± 0.06
SEM	2.95	0.01	0.40	0.09

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
*See footnotes to Table 1.

Table 10 Effects of experimental diets on blood ions [calcium (mg/dl), phosphorus mg/dl, sodium (mmol/l) and potassium (mmol/l)] of common carp (*Cyprinus carpio* L.) fingerlings

Treatment*	Calcium	Phosphorus	Sodium	Potassium
Control	10.43 ± 1.05	5.10[b] ± 1.69	131.25 ± 3.86	1.50[c] ± 0.00
J$_{a50}$	9.98 ± 0.59	7.63[a] ± 0.23	129.00 ± 1.63	2.18[b] ± 0.50
J$_{a75}$	10.60 ± 1.14	5.93[ab] ± 0.98	129.50 ± 1.91	2.80[b] ± 0.76
J$_{b50}$	10.15 ± 0.39	7.78[a] ± 1.13	131.00 ± 2.45	4.40[a] ± 0.57
J$_{b75}$	9.60 ± 0.42	6.30[ab] ± 1.10	130.75 ± 2.06	2.85[b] ± 0.17
S$_{50}$	10.53 ± 0.64	7.93[a] ± 0.38	130.25 ± 3.30	1.55[c] ± 0.10
S$_{75}$	10.35 ± 0.39	7.45[a] ± 0.87	133.50 ± 4.20	4.23[a] ± 0.51
SEM	0.14	0.43	0.55	0.31

Values are means (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
*See footnotes to Table 1.

Histology

The necrosis of enterocytes on the tips and sides of villi in J$_{a75}$ group (Fig 3A) appear to affect digestion and absorption of nutrients. The denudation of absorptive cells (enterocytes) and decreased contribution to digestion from the brush border enzymes resulted in overall reduction in absorptive surface. Mucosal lesions were accompanied by varying degrees of inflammatory cell infiltration in the lamina propria and cystic dilation of crypts in the J$_a$ group. These histological results concur with the decrease in nutrient and energy digestibilities and digestive enzyme (amylase, protease

and lipase) activities in common carp intestine fed J_a detoxified kernel meal (Kumar et al., 2010). The results of the present study demonstrate that the J_a meal was not detoxified completely.

Conclusions

The detoxified Jatropha kernel meal (DJ_bKM) can replace 50% FM protein in common carp diets, without sacrificing the growth and health of fish. The DJ_bKM can be used as one of the promising FM replacers in the diet of common carp. Phorbol esters, the main toxic principles for Jatropha toxicity were not detected in fish muscle tissues, suggesting the fish of DJ_bKM fed groups is safe for human consumption.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgement

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Chapter 4

Dietary inclusion of detoxified *Jatropha curcas* kernel meal: Effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L.

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Dietary inclusion of detoxified *Jatropha curcas* kernel meal: Effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L.

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Abstract

Jatropha curcas is a multipurpose and drought-resistant shrub or small tree widespread all over the tropics and subtropics. Its seeds are rich in oil and the Jatropha kernel meal obtained after oil extraction is rich in protein. However, presence of toxic and antinutritional constituents restricts its use in fish feed. Jatropha kernel meal was detoxified. Common carp, *Cyprinus carpio* fingerlings (15; av. body mass 10.9 ± 0.65 g) were randomly distributed in three groups with five replicates. A six-week feeding experiment was conducted in a respirometer system to evaluate the growth performance, nutrient utilization and energy budget. Fish were fed iso-nitrogenous diets (38% crude protein): Control diet (fish meal, $C_{control}$ group) containing fish meal (FM) based protein and two other diets replacing 75% FM protein with detoxified Jatropha kernel meal (DJKM, $J_{atropha}$ group) and soybean meal (SBM, S_{oybean} group). At the end of the experiment, body mass gain, metabolic growth rate, protein efficiency ratio, protein productive value, energy retention, efficiency of metabolised energy for growth and efficiency of energy retention were determined. These parameters were high and statistically similar for FM and $J_{atropha}$ groups and significantly lower ($P<0.05$) for S_{oybean} group. Whereas a reverse trend was observed for energy expenditure per g protein retained in fish body. No significant differences were found in heat released, gross energy uptake, metabolized energy intake, metabolisability, energy expenditure, energy expenditure per g protein fed and apparently unmetabolised energy. Conclusively, common carp fed plant protein (DJKM and SBM) and FM protein based diets exhibited equal average metabolic rate.

Key words Common carp, *Jatropha curcas,* Growth, Energy budget, Feed utilization

Introduction

Fish meal (FM) is often utilized in aqua feeds because it offers a balanced source of indispensable amino acids, essential fatty acids, vitamins, minerals, and generally enhances palatability. Current world production of FM is approximately 6–7 million tonnes annually and, while this level of production is expected to remain stable over the next 10 years (New and Wijkström 2002; Mazurkiewicz 2009), rapid expansion of aquaculture during this period will require lowering the inclusion rate of FM in aquafeeds and replacing it with plant-based protein sources (SOFIA 2007). Protein ingredients to substitute for FM, either partially or completely, included terrestrial plant meals and animal by-products readily available on the world markets (Samocha et al., 2004). Reducing the FM content of fish feeds is a strategy to increase the sustainability of carp aquaculture by reducing feed costs as well as reducing the environmental impact. However the studies on FM replacement in carp are sparse compared to other carnivore fishes. Hansan et al., 1997, Hossain and Jauncey, 1989, Hossain et al., 2001, Kumar et al., 2008, 2009a ,b, c and Mazurkiewicz, 2009 evaluated use of various oil seed as protein sources in common carp diet. Soybean meal (SBM) is currently the most commonly used plant protein source in fish feeds and amounts to 50% of the diet of freshwater omnivorous fish species (Yue and Zhou, 2009). Which indicates that commercial feed depends mostly on SBM as a FM replacer. However the over dependence will cause hike in price of SBM therefore utilization of other inexpensive plant protein source would be beneficial in reducing the feed cost (Yue and Zhou, 2009).

   Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. Its seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar et al. 2007a). The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 58 to 62 % crude protein (Makkar et al., 2008). The levels of essential amino acids (except lysine) are higher in *Jatropha* kernel meal than SBM (Unpublished). However the presences of high levels of antinutrients like trypsin inhibitor, lectin and phytate (Makkar et al., 2008) and the major toxic components phorbol esters (PEs) (Makkar and Becker, 1997) restrict their use in fish feed. Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction of PEs using organic solvents and inactivation of

trypsin inhibitors and lectin by heat treatment. Our previous study (Kumar et al., 2008) has shown that detoxified Jatropha kernel meal (DJKM) is a good protein source and better than SBM for common carp diet.

Growth and production can be described in terms of partition of dietary energy yielding components between catabolism as fuels and anabolism as storage in tissues. Metabolism, which includes all processes where transfer of energy is involved, can be quantified on the basis of the energy expenditure. The energy expenditure can be estimated from the gaseous exchange and the release of nitrogenous compounds (Brouwer, 1965). Many studies have been carried out on the relationship of dietary energy and protein content to growth of carp (Ogino et al., 1976; Schwarz et al., 1983; Gongnet et al., 1987). Some reports deal with the nutritional energetics related to the effect of diets on energy budgets (Cui et al., 1992; Helland and Helland, 1998; Francis et al., 2002), but little work has been done to examine the effects of dietary plant proteins on energy allocation in fish (Refstie and Tiestra, 2003). The present study was designed to investigate the effects of dietary DJKM on growth performance, nutrient utilization and interrelationships among the major components of the energy budget of common carp, *Cyprinus carpio*, and to compare these parameters with those obtained on dietary inclusion of SBM and FM.

Materials and Methods

Preparation of the Jatropha meal

Jatropha seeds were purchased from India and deshelled manually to obtain kernels. Defatting of Jatropha kernels was done using petroleum benzene (b.p. 40–60 °C) in a Soxhlet apparatus. Organic solvents were used to detoxify defatted Jatropha kernel meal (patent application has been filed for the process of detoxification, Makkar and Becker, 2008). After removal of PEs, the meal was autoclaved (121 °C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation

FM (Seelöwe fishmeal) was procured from Vereinigte Fishmeh werke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; and wheat meal was purchased from a local market. SBM (dehulled, defatted and roasted) was obtained from Institute of Animal Nutrition (450), University of Hohenheim, Germany. Soya protein isolate (SUPRO® 500E IP) was purchased from Solae

Europe S.A., 2, Chemin du Pavillon, CH-1218 Le Grand-Saconnex, Geneva, Switzerland. Source of sunflower oil was Thomy, Nestle Deutchland AG, 41415 Neuss; Reg. Trademark of Societe des, Produits Nestle S.A.. Vitamin premix and mineral premix were procured from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Source of lysine is Merck KGaA, 64271 Darmstadt, Germany. Phytase (Natuphos 5000 G (EU) 3-phytase (EC 3.1.8)) was purchased from BASF Ludwigshafen, Germany.

Prior to feed formulation, the proximate composition of DJKM, wheat meal, SBM, soya protein concentrate and FM were determined. A total of three isonitrogenous diets were formulated. Experimental diets containing crude protein 38%, crude lipid 9%, vitamin premix 2% and mineral premix 2% were prepared. The inclusion levels of the DJKM and SBM were as follows: Control diet (C_{ontrol}) was prepared with FM and wheat meal, without any DJKM and SBM; $J_{atropha}$: 75% FM protein replaced by DJKM; and S_{oybean}: 75% FM protein replaced by SBM (Table 1). The final mixture of each diet was made to 2 mm diameter moist pellets by pelletizer (using a Bosch, Type UM60ST 2-M, Robort Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then freeze-dried.

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets		
	C_{ontrol}	$J_{atropha}$	S_{oybean}
FM	505	126	126
Soybean meal	-	-	510
Wheat meal	415	368	219
Detoxified Jatropha kernel meal	-	381	-
Soya protein isolate	-	8	32
Lysine	-	4	-
Sunflower oil	40	73	73
[1]Vitamin premix	20	20	20
[2]Mineral premix	20	20	20
Total	1000	1000	1000
Phytase (FTU/kg)	-	500	500

[1]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[2]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 502; $MgSO_4.7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4.H_2O$, 3.12; $ZnSO_4. 7H_2O$, 4.67; $CuSO_4.5H_2O$, 0.62; KI, 0.16; $CoCl_2$. $6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

Experimental system

During the experiment, the fish were kept individually in respiration chambers 5 l capacity of the fully automated, computer controlled fish respirometer system Focken et al., 1994. Thirty-two measurements of oxygen consumption per individual fish were made every 24 h and recorded on the hard disk of the computer. The system was lit with fluorescent tubes to give a day length of 12 h. The water flow rate through the respirometer chambers (controlled by electronic flow meters connected to each chamber) was adjusted between 0.5 and 0.8 l min^{-1} to keep the oxygen saturation in water above 80% in the chambers. Everyday, about a tenth of the water in the system was replaced. Once a week, when the fish were weighed, the chambers were cleaned and the oxygen electrode calibrated. During the experimental period, all water quality parameters were maintained at optimum range (water temperature: 26.1 – 27.3°C, pH: 7.1 – 7.8, DO: 6.9 – 7.7 mg l^{-1}, NH_3-NH^{4+}: 0.1– 0.2 mg l^{-1}, nitrite: 0.07 – 0.1 mg l^{-1} and nitrate: 1–3 mg l^{-1}).

Experimental animals

Common carp (*Cyprinus carpio* L.) fingerlings obtained from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahrensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. They were fed the Hohenheim standard fish diet (containing approximately 38% protein, 8% lipid, 10% ash and with a gross energy content of 18 kJ g^{-1} dry matter) until their body weight reached approximately 10 g. Prior to the start of the experiment, 25 carp of comparable body mass (10.9 ± 0.65 g) were selected from a large population, weighed and 15 of them were placed individually in 15 respiration chambers of a device described by Focken et al., 1994. The remaining 10 fish were sacrificed by a sharp blow to the forehead and preserved for determination of the initial chemical composition. The fish were starved for 24 h prior to the start of feeding the experimental feeds. At the start of the experiment, the three experimental diets namely $C_{control}$, $S_{oyabean}$ and $J_{atropha}$, were assigned each to five fish in a random manner.

Feed regime

In a preliminary study, feeds at seven times maintenance requirement were offered to fish. This resulted in substantial presence of uneaten feed in the aquaria. However, no feed was left in the aquaria when feeds at five times maintenance requirement were offered. According to Becker et al., 1983, one time maintenance requirement equals 3.2 g feed per kg metabolic body mass ($kg^{0.8}$). So 16 g feed per kg metabolic body mass ($kg^{0.8}$) was fed for 5 times maintenance requirements, and the feed was split into 5 equal rations per day (at 8:00, 10:30, 13:00, 15:30 and 18:00 h). The feeds were dispensed using an automatic feeder (Graesslin, Rondomatic 400, Graesslin GmbH, St. Georgen/Schwarzw, Germany). Fish were weighed individually at the beginning of the experiment (av. wt. 10.9 ± 0.65 g) and at weekly intervals during the experimental period to adjust the feed amount for the subsequent week. The fish were not fed on the weighing day. At the end of six weeks, the experiment was terminated and the fish were weighed, sacrificed by a sharp blow to the forehead and immediately stored at -20 ^{0}C for chemical composition analysis. Prior to the determination of the proximate composition, the fish were autoclaved at 121 ^{0}C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and freeze-dried.

Extraction and estimation of phorbol esters by high-performance liquid chromatography, and determination of antinutrients

PEs were determined according to Makkar et al., 2007b, which was based on the method of Makkar et al., 1997. Briefly, 0.5 g of the Jatropha meal and dried whole body fish sample were extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100, 5 µm (250 x 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 ^{o}C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007b). The four-PEs peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs is 3µg/g meal.

Trypsin inhibitor activity was determined essentially according to Smith et al., 1980 except that the enzyme was added last as suggested by Liu and Markakis, 1989. Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL^{-1} assay medium) that produced agglutination. The minimum amount was the

amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. The phytic acid content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis, MO, USA) as standard. Non-starch polysaccharides were estimated according to Englyst et al., 1994.

Amino acid analysis

The amino acid compositions of FM, DJKM, SBM, soya protein concentrate and wheat meal were determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 ^{0}C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. The tryptophan contents of the above-mentioned samples were determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl, 1990.

Proximate analysis

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Samples of plant and animal origin (fish bodies and FM) were analysed for dry matter (DM), ash, crude protein (CP) and lipid (ether soluble lipid). Gross energy (GE) of diet ingredients, diets and fish bodies was determined with bomb calorimeter (IKA C 7000) using benzoic acid as a standard.

Calculation for growth performance, nutrient utilization and energy budget

All calculations were performed for each fish individually. Growth performance, diet nutrient utilisation and energy budget were assessed in terms of body mass gain (BMG,%), specific growth rate (SGR,%/day), metabolic growth rate (MGR, gkg$^{0.8}$ day^{-1}), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV,%), lipid productive value (LPV,%), energy expenditure (kJ g^{-1}), energy apparently not metabolised (AUE, kJ), energy retention (ER, kJ), average metabolic rate (AMR), metabolisable energy ingested, efficiency of metabolisable energy for growth, metabolisability and energy expenditure (EE, kJ)/g protein fed and retained. These were calculated as follows:

BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100; SGR (%/day) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$}/2] / number of trial days (Dabrowski et al., 1986); FCR = dry feed fed (g)/body mass gain (g); PPV (%) = [(final fish body protein in g - initial fish body protein in g) / total protein consumed in g] X 100; LPV(%) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid consumed in g] X 100; ER (%) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100; PER = fresh body mass gain (g)/crude protein fed (g).

EE (kJ) = Total oxygen uptake (g) X 14.85 (kJ/g) (Huisman, 1976); AUE (kJ) = Gross energy – (energy expenditure + energy retention); AMR = Oxygen consumed (mg/h)/[fish wt (g)]$^{0.8}$; heat released (kJ kg$^{0.8}$ d^{-1}) = (AMR X 24 X 14.85)/1000; metabolisable energy intake (kJ) = energy expenditure (kJ) + energy retention (kJ); efficiency of metabolisable energy for growth, metabolizability; EE/g protein fed (kJ g^{-1}) = Total EE (kJ)/ total protein consumed (g); EE/g retained protein (kJ g^{-1}) = Total EE (kJ)/ total protein retained in body (g).

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Duncan's multiple range test (P<0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results

Fish behavior and feed intake

Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behavior of fish was normal. There was no mortality during the entire experimental period.

It was particularly noted that the fish of the S$_{oybean}$ group fed slowly on their feed, whereas fish of other dietary groups fed actively throughout the experiment.

Phorbol esters content in defatted Jatropha kernel meal and antinutrients contents in detoxified Jatropha kernel meal and soybean meal

Phorbol esters content in untreated defatted Jatropha kernel meal was 1.8 mg/g. However, phorbol esters in the DJKM and dried whole body fish were undetectable. Lectin and trypsin inhibitor were not detected in DJKM and SBM; and phytate in DJKM and SBM was 9.3% and 2.4% respectively (Table 2). Non-starch polysaccharides level in DJKM and SBM were 16% and 14% respectively (Table 2).

Table 2 Proximate composition, amino acid composition and antinutrient content of feed ingredients

	Fish meal	Detoxified Jatropha kernel meal	Soybean meal	Soya protein isolate	Wheat flour
Proximate composition (g kg $^{-1}$)					
Dry matter	940	945	955	940	941
Crude protein	635	665	471	900	143
Crude lipid	88	11.4	11.7	10	16.3
Crude ash	142	137	21.4	40	14
Gross energy (kJ/g)	21.1	18.3	18.2	-	18.7
Essential amino acids composition (g kg $^{-1}$)					
Arginine	35.3	69.7	36	67.9	5.4
Histidine	17.7	21.7	14.4	24.4	3.4
Iso leucine	22.8	26.7	19.6	36.5	4.2
Leucine	41.6	46.7	35.7	68.1	9.1
Lysine	40.9	23.3	29.1	52.1	3.3
Phenylalanine	21.8	30.4	24.3	43.2	6.5
Methionine	16	10.6	6.2	12.1	2
Threonine	23	22	17.8	31.1	3.7
Tryptophan	4.9	7.1	6.4	10.4	1.4
Valine	29.3	31.6	21.2	37.4	5.1
Non-essential amino acids composition (g kg $^{-1}$)					
Alanine	43.3	29.4	21.4	40.9	4.6
Asparginine	60.5	68.7	66.6	122.8	7.2
Cystine	4.3	2.3	6.5	9.8	2.9
Glycine	59.8	31.5	21.3	37.2	5.6
Glutamine	79.4	112.1	93.8	174.9	44.9
Proline	36.9	32.2	28.2	50.2	14.5
Serine	25.5	30.6	24.4	46	6.3
Tyrosine	14.8	18.8	15.8	31	3.3

Antinutrients					
Trypsin inhibitor (mg trypsin inhibited per g sample)	ND	ND	ND	ND	-
Phytate (% dry matter)	-	9.3	2.41	-	-
Lectin[a]	-	ND	ND	-	-
Non-starch polysaccharides (NSP) (g kg^{-1})					
Rhamnose	-	3	0	-	-
Fucose	-	1	0	-	-
arabinose	-	31	24	-	-
Xylose	-	20	11	-	-
Mannose	-	5	6	-	-
Galactose	-	14	42	-	-
Glucose	-	57	32	-	-
Glucuronic acid	-	0	0	-	-
Galacturonic acid	-	30	24	-	-
Total-NSP	-	160	140	-	-

ND: Not detected

[a]: Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Proximate composition and amino acid profile of experimental diets and proximate composition of whole body of fish

Proximate composition, amino acid compositions and antinutrients of feed ingredients are shown in Table 2. Experimental diets contained about 39% crude protein and 19.2 kJ/g gross energy and were isonitrogenous and isocaloric. Dry matter, crude lipid and ash were in the range of 95.8–96.4%, 9.2–9.8% and 10.3–11.1% respectively (Table 3). The quantities of essential amino acids (except methionine) fulfilled the needs of the common carp (NRC 1993) in Table 3.

The chemical composition of the whole body of fish (wet basis %) is presented in Table 4. Highest whole body moisture content was observed in FM group, and lowest in J$_{atropha}$ group (Table 4). The moisture content of S$_{oybean}$ group was intermediate but was statistically not different (P > 0.05) to that of FM and J$_{atropha}$ groups. Whereas opposite trend was observed for whole body lipid content and gross energy content; highest lipid and gross energy content were observed in J$_{atropha}$ group. These parameters for S$_{oybean}$

group and FM group were statistically not different (P > 0.05). Highest crude protein and ash were observed in S_{oybean} group and lowest in FM group.

Table 3 Proximate composition and amino acid composition of the experimental diets (g kg^{-1} feed)

	C_{ontrol}	$J_{atropha}$	S_{oybean}
Proximate composition (g kg^{-1})			
Dry matter	958	964	959
Crude protein	392	391	390
Crude lipid	94	98	92
Crude ash	108	103	111
Gross energy (kJ/kg)	19.1	19.4	19.0
Essential amino acids composition (g kg^{-1})			
Arginine	20.1	32.1	26.2
Histidine	10.4	11.5	11.1
Iso leucine	13.3	14.3	15.0
Leucine	24.8	26.0	27.6
Lysine	22.1	18.7	22.4
Phenylalanine	13.7	16.5	17.9
Methionine	8.9	6.7	6.0
Threonine	13.2	12.4	13.8
Tryptophan	3.1	3.8	4.5
Valine	16.9	17.3	16.8
Non-essential amino acids composition (g kg^{-1})			
Alanine	23.8	18.1	18.7
Asparginine	33.6	35.9	47.2
Cystine	3.4	2.5	4.8
Glycine	32.6	21.3	20.8
Glutamine	58.4	68.7	73.0
Proline	24.6	22.1	23.7
Serine	15.5	16.9	18.5
Tyrosine	8.8	10.1	11.7

Amino acid composition of the experimental diets were calculated from amino acid profile of individual feed ingredients

Table 4 Chemical composition of whole body of common carp (*Cyprinus carpio* L.) fingerlings of different experimental groups at the start and at the end of the experiment (% wet basis)

Treatment	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)
Initial fish	77.9 ± 0.2	14.6 ± 0.01	3.7 ± 0.11	3.9 ± 0.10	4.0 ± 0.08
C[ontrol]	76.1[a] ± 0.8	16.3[b] ± 0.34	5.0[b] ± 0.44	2.6[b] ± 0.08	5.4[b] ± 0.21
J[atropha]	74.3[b] ± 0.3	16.7[ab] ± 0.26	6.3[a] ± 0.25	2.6[b] ± 0.03	6.0[a] ± 0.12
S[oybean]	75.5[ab] ± 1.3	17.3[a] ± 0.70	4.3[b] ± 1.29	3.0[a] ± 0.14	5.3[b] ± 0.52
SEM	0.28	0.15	0.29	0.04	0.11

Mean values in the same column with different superscript differ significantly ($P < 0.05$). Values are mean (n = 5) ± standard deviation.

Table 5 Growth performance and feed intake per day in common carp (*Cyprinus carpio* L.)

Treatment	IBM (g)	FBM (g)	Feed intake per day (g)	BMG	SGR	MGR
C[ontrol]	11.2 ± 1.14	49.0[a] ± 7.9	0.90 ± 0.13	338[ab] ± 51	3.50[ab] ± 0.3	15.3[a] ± 1.3
J[atropha]	10.6 ± 0.63	48.3[a] ± 3.0	0.92 ± 0.05	358[a] ± 45	3.62[a] ± 0.2	15.6[a] ± 0.9
S[oybean]	10.8 ± 0.97	39.1[b] ± 8.2	0.84 ± 0.10	262[b] ± 73	3.02[b] ± 0.5	13.1[b] ± 2.2
SEM	0.23	2.02	0.02	17.7	0.05	0.48

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly ($P < 0.05$).
IBM (g) - Initial body mass, FBM (g) – Final body mass, BMG (%) - Body mass gain, SGR (%/day) – Specific growth rate; and MGR (gkg$^{0.8}$ day^{-1}) - Metabolic growth rate

Growth performance and feed utilization

Growth performance parameters and daily feed intake are shown in Table 5. Weekly body mass gain indicates that second week onwards there was differential growth among the group, and lower body mass development was observed in S_{oybean} group compared to other groups. This trend was maintained till the end of the experiment. Highest body mass gain, specific growth rate, metabolic growth rate were observed for $J_{atropha}$ group which were statistically not different to that for FM group and significantly higher (P < 0.05) than for S_{oybean} group (Table 5). Whereas opposite trend was observed for FCR. The FCR for $J_{atropha}$ group was statistically not different to that for C_{ontrol} group (Table 6).

PER, PPV and LPV were statistically not different (P>0.05) for C_{ontrol} and $J_{atropha}$ groups and significantly higher (P<0.05) than for S_{oybean} group (Table 6).

Energy budget

Average metabolic rate and energy budget of fish in different experimental groups are shown in Tables 7 and 8 respectively. Highest ER was observed for $J_{atropha}$ group, which is not statistically different (P > 0.05) to that for C_{ontrol}. This value was lowest and significantly different for S_{oybean} group. Gross energy uptake, metabolisable energy intake, metabolisability, EE, AUE, AMR; heat released and EE per g protein fed did not differ significantly among the three groups. Lowest EE per g protein retained in the fish body was observed in $J_{atropha}$ group that was statistically not different from C_{ontrol} group and highest in S_{oybean} groups. Highest ER efficiency and efficiency of ME for growth were observed in $J_{atropha}$ group, which is statistically not different to C_{ontrol} group and significantly higher (P < 0.05) than S_{oybean} group.

Discussion

In the present study an excellent growth was observed. The fish body mass increased four to five times within six weeks; and growth performance and feed utilization of $J_{atropha}$ group was similar to FM fed group and better than S_{oybean} group. Highest ER was observed for $J_{atropha}$ group, which was statistically not different (P > 0.05) to that for C_{ontrol} group and higher than that for S_{oybean} group. Whereas other components of the energy budget and AMR did not differ significantly amongst the groups, suggesting that DJKM is a promising protein source for incorporation in carp feed.

Table 6 Nutrient utilization in common carp (*Cyprinus carpio*) fed with experimental diets for six weeks

Treatment	FCR	PER	PPV	ER	LPV
C_{ontrol}	$1.00^b \pm 0.05$	$2.58^a \pm 0.16$	$43.1^a \pm 2.4$	$33.5^a \pm 0.7$	$60.9^a \pm 4.0$
$J_{atropha}$	$1.03^b \pm 0.04$	$2.57^a \pm 0.15$	$44.2^a \pm 2.8$	$37.3^a \pm 1.2$	$75.0^a \pm 3.9$
S_{oybean}	$1.30^a \pm 0.27$	$2.07^b \pm 0.41$	$37.6^b \pm 5.3$	$27.3^b \pm 4.9$	$43.9^b \pm 17.9$
SEM	0.05	0.09	1.2	1.3	4.3

Mean values in the same column with different superscript differ significantly (P < 0.05).
Values are mean (n = 5) ± standard deviation.
PER - Protein efficiency ratio, PPV (%) - Protein productive value and ER (%) - Energy retention, LPV (%) - Lipid productive value

Table 7 Average metabolic rate, derived heat release and energy expenditure per g protein fed and retained in common carp (*Cyprinus carpio* L.)

	C_{ontrol}	$J_{atropha}$	S_{oybean}	SEM
Average metabolic rate ($mgO_2 kg^{0.8} h^{-1}$)	363 ± 83.3	442 ± 120.9	372 ± 60.6	23.9
Heat released ($kJ \, kg^{0.8} d^{-1}$)	129.3 ± 29.7	157.6 ± 43.1	132.7 ± 21.6	8.5
Energy expenditure (EE)/g protein fed (kJ)	19.6 ± 3.9	18.9 ± 1.1	20.0 ± 3.2	0.8
Energy expenditure (EE)/g protein retained (kJ)	$45.3^{ab} \pm 7.7$	$42.9^b \pm 2.4$	$53.4^a \pm 5.8$	1.88

Mean values in the same row with different superscript differ significantly (P < 0.05).
Values are mean (n = 5) ± standard deviation.
Metabolic rate = Oxygen consumed (mg/h)/[fish wt (g)]$^{0.8}$

Table 8 Energy budget of common carp (*Cyprinus carpio* L.) in different experimental groups

	C$_{ontrol}$	J$_{atropha}$	S$_{oybean}$	SEM
Initial GE content of carcass (kJ)	44.7 ± 4.6	41.9 ± 3.0	43.3 ± 3.9	1.06
Final GE content of carcass (kJ)	260.5 ± 32.7	285.1±25.0	208.1±44.1	12.95
Feed GE uptake (kJ)	645 ± 90.7	658 ± 45.2	600 ± 71.0	19.46
Energy expenditure (kJ)	289 ± 88.2	273 ± 17.2	270 ± 48.9	16.06
Energy expenditure (EE; % of GE fed)	44.3 ± 8.4	41.5 ± 0.9	45.1 ± 6.2	1.73
Energy retention (kJ)	216[ab] ± 29.5	243[a] ± 26.0	165[b] ± 43.8	12.78
Energy retention (ER; % of GE fed)	33.5[ab] ± 0.7	36.9[a] ± 1.5	27.3[b] ± 4.9	1.39
Metabolizable energy (ME) ingested (kJ)	505 ± 109	516 ± 42	435 ± 89	23.60
Metabolizability (ME % of diet)	77.8 ± 8.2	78.4 ± 1.1	72.3 ± 10.1	2.08
Efficiency of ME for growth	43.4[a] ± 4.4	47.1[a] ± 1.4	37.6[b] ± 3.1	1.31
Apparently unmetabolised energy (AUE) (kJ)	140 ± 50.8	142 ± 3.9	164 ± 58.4	12.84
Apparently unmetabolised energy (AUE; % of GE fed)	22.2 ± 8.2	21.6 ± 1.10	27.7 ± 10.1	2.23
Efficiency of energy retention (ER/EE)	0.77[a] ± 0.13	0.89[a]± 0.05	0.61[b]± 0.08	0.04

Mean values in the same row with different superscript differ significantly (P < 0.05).
Values are mean (n = 5) ± standard deviation.
EE = Oxygen uptake (g) X 14.85 kJ/g
AUE = Energy fed - energy expenditure - energy retention.

Feed intake, growth performance, nutrient utilization observed in this study was higher than those reported earlier in common carp (Viola et al., 1982; Hasan et al., 1997; Escaffre et al., 1997; Jahan et al., 2003; Kumar et al., 2008, 2009a; Mazurkiewicz, 2009). Although feed GE uptake did not differ significantly among the three groups, numerically, substantially higher (approximately 10%) feed GE uptake was observed in J$_{atropha}$ and FM groups compared to S$_{oybean}$ group. This concurs with the higher growth

performance of fish in J$_{atropha}$ and C$_{ontrol}$ groups, and indicates that higher energy (feed GE uptake) was available for growth in these groups compared to SBM fed groups. A similar observation was recorded for energy allocation in European minnow, *Phoxinus phoxinus* (L.) (Cui, 1987).

High and statistically not different feed efficiency, PER, LPV, ER and PPV values of fish in C$_{ontrol}$ and J$_{atropha}$ groups suggest that digestion and absorption of nutrients from DJKM and FM were similar. Nutrient utilization values found in this study signify very good utilization of the diets. These results were similar to those observed in our earlier study (Kumar et al., 2008) wherein DJKM fed groups were better than SBM in common carp.

A comparison was made of the growth rate and energy budget in the common carp fed different experimental diets. Throughout the experiment, oxygen consumption of fish in FM, DJKM and SBM fed groups did not differ significantly, indicating no difference in metabolic activity, from start to end of the experiment, among the groups. Similar results have been reported by Suárez et al., 2009, wherein for white shrimp (*Litopenaeus vannamei*) juveniles fed soybean-canola meal (at an 80% substitution level) energy budget did not differ significantly for plant protein and FM protein fed groups.

In our study, lowest ER in SBM fed group conforms to the lowest growth performance of this group. Whereas, ER in Jatropha group was statistically similar to that in C$_{ontrol}$ group and, reflecting higher growth performance in both groups. Lower growth performance and ER in SBM fed group compared to FM fed groups might be because of higher crude fibre content in this diet that could have led to losses of nitrogen and lipid through faeces (Hajra et al., 1987). Another reason could be due to lower protein and other nutrient availability from the SBM than from DJKM and FM. In another study (Kumar et al., 2009a) fractional digestibility of protein of SBM was lower than that of DJKM.

Cui and Liu, 1990 constructed average energy budgets for six teleost species fed *ad libitum* and found that heat loss was always the largest component, 50 – 69% of consumed energy, whereas the energy used for growth was much smaller; 21 – 35%. In the present study, this was the case for fish fed all experimental diets for which the energy retained for growth was between 27.5 and 37%, whereas energy expenditure was between 41.4 and 45.1%. Metabolizable energy of the diet ranges from 72 – 78% and utilization of metabolisable energy for growth was 38 – 47%. Highest metabolisable energy for growth was registered in J$_{atropha}$ group which is not statistically different from C$_{ontrol}$ group that indicate utilization of DJKM in

carp was better than SBM. Whereas highest EE per g protein retained in fish body was observed in S$_{oybean}$ group, which exhibit that utilization of SBM in carp require higher energy than DJKM and FM (Table 7).

Kumar et al., 1986 demonstrated that the toxic substance (di Nitro benzene, DNB) progressively decrease O$_2$ consumption as the concentration of DNB wastewater increase. It is reported that aromatic nitro compounds (for e.g. DNB) combine with haemoglobin and cause methaemoglobinemia in higher vertebrates (Hamblin, 1963). It may also be the case in fish. Fish have also been shown to exhibit stress reactions due to the presence of antinutrients like phorbol esters, trypsin inhibitors, and saponins through dietary (Makkar et al., 1998; Makkar and Becker, 1997; Becker and Makkar, 1998). Saponin can also increase O$_2$ consumption in common carp (Francis et al., 2002) and perch, *Anabas testudineus* (Roy and Munshi, 1989). The present study indicates that DJKM does not contain any toxic substance (for e.g. phorbol esters) since it did not effect on O$_2$ consumption. Similar conclusion was derived in our previous studies (Kumar et al., 2008, 2009a) based on determination of various blood enzymes and metabolites and organ enzymes and on histopathological investigations.

We observed a significant increased in fat content in J$_{atropha}$ group. This consequently resulted in increase in whole body energy content. Detoxified Jatropha kernel meal contains almost three times higher phytate content than soybean meal. Because of high phytate content, it might be that the availability of phosphorus and other minerals was low in the Jatropha group. Deficiency of phosphorus has been shown to increase body fat content (Sugiura et al., 2004). The whole body moisture content was lower and crude protein higher in plant protein fed groups, which is supported by Hasan et al., 1997; they observed that carp fed with plant protein (mustard, sesame and linseed oil cake) exhibited lower moisture and higher crude protein content compared to FM fed groups.

Conclusions

The results of this study showed that the detoxified Jatropha kernel meal fed group exhibited best growth performance (almost five times increased in fish body mass within six week) and good feed conversion ratio (about one). Overall, growth performance, feed utilization and energy budget of fish fed detoxified Jatropha kernel meal and fish meal were similar. This plant based protein-rich meal is a promising new ingredient for the aqua feed industry that could replace 75% of fish meal protein in the diets of common carp.

Acknowledgement

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Chapter 5

Influences of incorporating detoxified *Jatropha curcas* kernel meal (byproduct of biodiesel industry) in common carp (*Cyprinus carpio* L.) diet on the expression of growth hormone and insulin-like growth factor-1 encoding genes

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Influences of incorporating detoxified *Jatropha curcas* kernel meal (byproduct of biodiesel industry) in common carp (*Cyprinus carpio* L.) diet on the expression of growth hormone and insulin-like growth factor-1 encoding genes

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Abstract

Jatropha curcas is a drought-resistant shrub or small tree widespread all over the tropics and subtropics. The use of *J. curcas* (L) kernel meal in fish feed is limited owing to the presence of toxic and antinutritional constituents. In the current study, it was detoxified using heat treatment and organic solvent extraction method. The detoxification process was done for 60 min to obtain the detoxified meal. *Cyprinus carpio* L. fingerlings (n=252; av. wt. 3.2 ± 0.07 g) were randomly distributed in five treatment groups with four replicates and fed iso-nitrogenous diets (crude protein 38%) for eight-weeks. The inclusion levels of the detoxified Jatropha kernel meal (DJKM) and soybean meal (SBM) were as follows: Control diet was prepared with fish meal (FM) and wheat meal, without any DJKM and SBM; diets S_{50} and J_{50}: 50% of FM protein replaced by SBM and DJKM respectively; diets S_{75} and J_{75}: 75% of FM protein replaced by SBM and DJKM respectively. Highest body mass gain and insulin-like growth factor-1 (IGF-1) gene expression in brain liver and muscle were observed for the control group which were statistically similar to that for J_{50} group and significantly ($P < 0.05$) higher than for all other groups, whereas growth hormone gene expression in brain, liver and muscle exhibited opposite trend. IGF-1 concentration in plasma did not differ significantly among the five groups. Conclusively, growth performance was in parallel with IGF-1 gene expression and exhibited negative trend with GH gene expression.

Key words: Common carp, *Jatropha curcas*, Growth, Growth hormone, Insulin-like growth factor-1

Introduction

Fish meal (FM) is still the most important protein source for aquaculture industry, but this is a limited feed resource and there is serious concern on the long-term availability of this feedstuff for use in fish diets. Thus, for the continued development of aquaculture on the basis of sustainable and renewable feed resources, basic knowledge on the physiological consequences of a total or partial replacement of FM by alternative protein sources is needed. Given that plant feedstuffs are readily available, they have received considerable attention over the past years (Kumar et al., 2008, 2010a,b,c; Mazurkiewicz, 2009). Replacement of a substantial portion of FM by plant protein sources, particularly soybean meal in common carp diets has been achieved (Kumar et al., 2008, 2010a,b,c; Mazurkiewicz, 2009), but problems related to amino acid imbalances (NRC, 1993) and anti-nutritional factors (Francis et al., 2001) have not allowed so far a total substitution of FM. Presently most of the commercial feed depends mostly on soybean meal (SBM) as a FM replacer. However, the over dependence will cause hike in price of SBM. In addition, SBM is a human food. Therefore utilization of other inexpensive plant protein source would be beneficial in reducing the feed cost and contribute to food security.

Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. Its seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel. The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 58 - 62 % crude protein. The levels of essential amino acids (except lysine) are higher in *Jatropha* kernel meal than in SBM (Kumar et al., 2010a.b.c). However the presences of high levels of antinutrients like trypsin inhibitor, lectin and phytate and the major toxic components phorbol esters (PE$_S$) (Kumar et al. 2010a,b) restrict their use in fish feed. Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

The inclusion of detoxified Jatropha kernel meal (DJKM) or detoxified Jatropha protein isolate at 50% replacement of FM protein in fish diet show a beneficial effect on growth performance and feed utilization.

However, at levels greater than 50% replacement of FM protein a decreased performance has been recorded (Kumar et al., 2008, 2010a,b,c), which has been postulated to be due to lower digestion of Jatropha protein and/or absorption of amino acids and other nutrients from the plasma pool and their utilization (Kumar et al., 2008).

Nutritional factors may exert specific effects on the endocrine regulation of growth and metabolism where growth hormone (GH) and insulin-like growth factor-1 (IGF-1) play a central role. Growth hormone is produced and secreted from the pituitary, whereas circulating IGF-1is mainly released from the liver after GH stimulation In fish as in mammals IGF-I secretion also depends on the availability of energy and exerts a negative feedback effect on GH secretion (Duan, 1998). Both GH and IGF-1 treatment have been shown to stimulate growth in fish, but their relative contribution, and to what extent GH and IGF-1 interact and/or act independently to regulate growth and metabolic processes is not yet clear (Björnsson et al., 2002). The GH–IGF-1 axis is strongly influenced by the nutritional status of the fish, e.g. fasting or decreased dietary protein/energy ration is generally characterized by increased plasma GH concentrations and decreased plasma IGF-1 concentrations (Wagner and McKeown, 1986; Sumpter et al., 1991; Moriyama et al., 1994; Pérez-Sánchez et al., 1995; Johnsson et al., 1996; Martí-Palanca et al., 1996; Pierce et al., 2005). Recent studies also show that dietary protein sources may affect plasma GH concentrations, but not IGF-1 levels, in rainbow trout and gilthead sea bream (Gómez-Requeni et al., 2004, 2005). In addition, imbalanced dietary amino acid composition altered plasma GH and IGF-1 levels in gilthead seabream (Gómez-Requeni et al., 2003). However, the influence of specific dietary factors on the GH–IGF-1 axis in common carp has not been well studied. The aim of the present work was to evaluate the influence of incorporating DJKM (a byproduct of biodiesel industry) in diet on the expression of GH and IGF-1 encoding genes in common carp (*Cyprinus carpio* L.) and compare it with that of SBM and FM containing diets.

Materials and methods

Preparation of the Jatropha meal

Jatropha seeds were obtained from India and deshelled manually to obtain kernels. Defatting of Jatropha kernals was done using petroleum benzene (b.p. 40–60 °C) in a Soxhlet apparatus. Organic solvents were used to detoxify defatted Jatropha kernel meal. After removal of PEs, the meal was

autoclaved (121 ^{o}C) to remove heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation

FM (Seelöwe fishmeal) was procured from Vereinigte Fishmeh werke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; and wheat meal was purchased from a local market. SBM (dehulled, defatted and roasted) was obtained from Institute of Animal Nutrition (450), University of Hohenheim, Germany. Soya protein isolate (SUPRO® 500E IP) was purchased from Solae Europe S.A., 2, Chemin du Pavillon, CH-1218 Le Grand-Saconnex, Geneva, Switzerland. Source of sunflower oil was Thomy, Nestle Deutchland AG, 41415 Neuss; Reg. Trademark of Societe des, Produits Nestle S.A. Vitamin premix and mineral premix were procured from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Source of lysine is Merck KGaA, 64271 Darmstadt, Germany.

Prior to feed formulation, the proximate composition of defatted Jatropha kernel meal, wheat meal, SBM, soya proteins isolate and FM was determined. A total of seven isonitrogenous and isoenergetic diets were formulated. Experimental diets containing crude protein 38%, crude lipid 8%, vitamin premix 2%, mineral premix 2% and TiO$_2$ 1% were prepared. Lysine was supplemented at the rate of 1% of DJKM inclusion in the diet. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg. The inclusion levels of the DJKM and SBM were as follows:

Control diet was prepared with FM and wheat meal, without any DJKM and SBM. S$_{50}$: 50% of FM protein replaced by SBM; S$_{75}$: 75% of FM protein replaced by SBM; J$_{50}$: 50% of FM protein replaced by DJKM; J$_{75}$: 75% of FM protein replaced by DJKM. The final mixture of each diet was made to 2 mm diameter moist pellets pellets using a Bosch, Type UM60ST 2-M pelletizer (Robert Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then freeze-dried (Table 1).

Experimental system and animals

Common carp (*Cyprinus carpio* L.) fingerlings (about 2.0–3.0 g) from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. They were fed the Hohenheim standard fish diet

containing approximately 38% protein, 8% lipid, 10% ash and with a gross energy content of 18.5 kJ g^{-1} dry matter. After an acclimatisation period of 20 days, 180 fish were randomly distributed into five groups with four replicates; each replicate contained nine fish (av. wt. 3.2 ± 0.07 g) in an aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light: 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2–27.1 °C, pH 7.0–7.5, dissolved oxygen 6.9–7.4 mg l^{-1}, total NH$_3$ 0.1–0.2 mg l^{-1}, nitrite 0.07–0.1 mg l^{-1} and nitrate 1–3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass (kg$^{0.8}$) per day (equal to five times their maintenance energy requirement). Total feed per day was split into five equal portions and each portion was given at 8:00, 10:30, 13:00, 15:30 and 18:00 h. The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment (av. wt. 3.2 ± 0.07 g) and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day.

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets				
	Control	J$_{50}$	J$_{75}$	S$_{50}$	S$_{75}$
Fish meal	507.5	253.7	126.3	253.7	126.3
Soyabean meal	-	-	-	342.1	513
[1]Wheat meal	402.5	390	384.1	271	206
Jatropha meal	-	242.5	361.9	-	-
Soya concentrate	-	2	5	22	32
Sunflower oil	40	61.8	72.7	61.2	72.7
[2]Vitamin premix	20	20	20	20	20
[3]Mineral premix	20	20	20	20	20
TiO$_2$	10	10	10	10	10
Total	1000	1000	1000	1000	1000
Phytase (FTU/kg)	-	500	500	500	500
Lysine monohydrochloride (g)	-	2.4	3.6	-	-

Control: FM and wheat meal, without any DJKM and SBM
J$_{50}$: 50% of FM protein replaced by DJKM
J$_{75}$: 75% of FM protein replaced by DJKM

S_{50}: 50% of FM protein replaced by SBM

S_{75}: 75% of FM protein replaced by SBM

[1]Whole wheat meal.

[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[3]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 502; $MgSO_4$. $7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4$. H_2O, 3.12; $ZnSO_4$. $7H_2O$, 4.67; $CuSO_4$. $5H_2O$, 0.62; KI, 0.16; $CoCl_2$. $6H_2O$, 0.08; ammonium molybdate, 0.06; 3 $NaSeO_3$, 0.02.

The experiment was terminated after 8 weeks and the fish were killed. At the end of experiment, fish were anesthsized by tricaine methanesulfonate (MS222) at 250 ppm in water. Blood was drawn near caudal peduncle from one fish from each replicate and transferred into a heparinized tube. Blood was centrifuged at 1500×g for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at –20 °C for determination of insulin-like growth factor-1. One fish per replicate were carefully dissected to isolate muscle, liver and brain and stored in liquid nitrogen (at –180 °C) for analysis of the expression of GH and IGF-1 encoding genes.

Extraction and estimation of phorbol esters (PEs) by high-performance liquid chromatography, and determination of antinutrients

Phorbol esters were determined according to Makkar et al. (2007), which was based on the method of Makkar et al. (1997). Briefly, 0.5 g of the Jatropha kernel meal and dried whole body fish sample were extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100, 5 µm (250 x 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 °C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007). The four-PEs peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs is 3µg/g meal.

Trypsin inhibitor activity was determined essentially according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL-1 assay medium) that produced agglutination. The minimum amount was the

amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis, MO, USA) as standard. Non-starch polysaccharides (NSP) were estimated according to Englyst et al. (1994).

Amino acid analysis

The amino acid compositions of FM, DJKM, SBM, soya protein concentrate and wheat flour were determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 °C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. The tryptophan contents of the above-mentioned samples were determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

Proximate analysis

The proximate composition of diet ingredients and diets was determined using the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Samples of FM were analysed for dry matter, ash, crude protein and lipid (ether soluble lipid). Gross energy of diet ingredients and diets was determined with bomb calorimeter (IKA C7000) using benzoic acid as a standard.

Growth parameters

Growth performance in terms of body mass gain (BMG) calculated as follows
BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100

Radioimmunological determination of IGF-I

IGF-1 concentrations in plasma were determined radioimmunologically after acid ethanol extraction using a heterologous sensitive assay basing on hIGF-I as described and validated for measurements in porcine plasma (Claus and Weiler, 1996). Carp IGF-I is about 80% homologous to hIGF-I (Duan, 1998) and the reliability of the heterologous assay for determination of carp

samples was checked by the determination of parallelism as described below. In brief, samples of 50 µL of carp plasma were incubated with 400 µL HCl-ethanol and centrifuged. 100 µL of the supernatant were neutralized with 40 µL Tris-base (0.855 M) and further diluted with 200 µL assay buffer. 100 µL of this dilution was used in the assay in duplicate. 125I-hIGF-I was used as a tracer and had been prepared according to the Iododgen method (Salacinski et al., 1981). The specific activity was 95GBq/nmol. The first antibody has been raised in rabbit against high-I-thyroglobulin and was used at a final dilution of 1:90,000. The antiserum revealed a crossreactivity of 1.2% with pIGF-II, cross reactivity with carp IGF-II had not been determined. A second antibody (sheep anti rabbit) was used for precipitation of the first antibody. After centrifugation the supernatant was removed and the pellet counted in a γ-counter. Parallelism was determined by measuring aliquots of 100 to 10µL extracted and neutralized samples, the correlation between concentration and sample volume was 0.99. The lower limit of sensitivity was 15 pg/tube. The interassay variation ranged between 8.1% and 10.1%, the intra assay variation was 10.0%.

Expression of GH and IGF-1 genes

Isolation of total RNA

Total RNA was isolated from brain (including pituitary gland), liver and muscle tissues of common carp by the standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany). Tissue samples were homogenized in 1 ml of TRIzol® Reagent per 50 mg of the tissue. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse transcription (RT) reaction

The complete Poly(A)+ RNA isolated from common carp tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAid™ First

Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5μg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM of each dNTP, 50 μM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M- MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

Semi-Quantitative Real Time-Polymerase Chain Reaction (sqRT-PCR)

An iQ5-BIO-RAD Cycler (Cepheid. USA) was used to determine the common carp cDNA copy number. PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 μL 0.2 μM sense primer, 0.5 μL 0.2 μM antisense primer, 6.5 μL distilled water, and 5 μL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. Each experiment included a distilled water control.

The semi quantitative values of RT-PCR (sqRT-PCR) of GH (GH-F: 5′-CGG AAT TCG ACA ACC AGC GGC TGT-3′, GH-R: 5′-CGC GGA TCC TTA CTA CAG GGT GCA GTT G-3′, Li et al., 2003) and IGF-1 (IGF1-F: 5′-GTC TAG CGC TCT TTC C TT TC-3′, IGF1-R: 5′-TCC TAC GCT CTG TGC CTT TG-3′, Pedroso et al. 2006) genes were normalized on the bases of β-actin (β-actin-F: 5′-CAC ACT GTG CCC ATC TAC GA-3′, B-actin-R: 5′-TCC TTC TGC ATC CTG TCA GC-3′, Wong et al. 2001) expression.

At the end of each sqRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of Gene Expression

First the amplification efficiency (Ef) was calculated from the slope of the standard curve using the following formulae (Bio-Rad 2006):

$Ef = 10^{-1/slope}$

Efficiency (%) = (Ef – 1) x 100

The relative quantification of the target to the reference was determined by using the

ΔC_T method if E for the target (GH, IGF-1) and the reference primers (β-Actin) are the same (Bio-Rad 2006):

$\text{Ratio}_{(reference/ \, target \, gene)} = Ef^{C_T(reference) \, – \, C_T(target)}$

The amplification efficiency (E) for GH and IGF-1 were 1.993 (%E= 99.3) and 1.988 (%E=98.8), respectively. Whereas, the PCR conditions indicated that the slopes of GH and IGF-1 were -3.34 and -3.35, respectively.

Further, to ensure that the PCR efficiency ($E = 10^{-1/s}$ - 1) was similar between the sample and the standard which was close to 2, we analyzed whether the addition of RT products to the reaction mixture for the standard curve which was prepared for purified RNA affected the PCR efficiency.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test (P < 0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results

Phorbol esters and antinutrient contents in defatted Jatropha kernel meal

Phorbol ester content in defatted Jatropha kernel meal was 1.6 mg/g. However PEs in the detoxified Jatropha kernel meal was reduced to undetectable level. The sensitivity of the method is 5 μg/g kernel meal. Trypsin inhibitor and lectins were not detected in SBM and DJKM. Phytate level in, DJKM and SBM was 9.5% and 2.5% respectively whereas NSP level in SBM and DJKM was 14% and 16% respectively.

Proximate composition antinutrients and amino acid profile

Proximate composition and amino acid compositions of feed ingredients are shown in Table 2. The proximate and amino composition of the different experimental diets (% dry matter) is presented in Table 3. Diets contained about 38% crude protein and 18.5 MJ/kg gross energy and were

isonitrogenous and isoenergetic. Dry matter, crude lipid and ash were in the range of 94.4–96.1%, 8.3–8.8% and 10.3–11.1% respectively. All experimental diets had almost similar amino acid composition. All the diets containing essential amino acid are essentially as per the requirement of the common carp (NRC, 1993).

Feed intake and growth performance

Acceptability and palatability of the experimental diets were good based on visual observation during the feeding. There was no mortality during the entire experimental period. Weekly body mass gains (g) of fish are given in Figure 1. The body mass gain (%) of the fish at the end of the experimental period is presented in Figure 2. Weekly body mass gain indicates that second week onwards there was differential growth among the group, and lower body mass development was observed in J_{75} and S_{75} groups compared to other groups. This trend was maintained till the end of the experiment. Highest body mass gain was observed for the J_{50} group, which was statistically not different ($P > 0.05$) to that for control group and significantly ($P < 0.05$) higher than for all other groups.

Table 2 Proximate, antinutrients and amino acid composition of feed ingredients

	Fish meal	Detoxified Jatropha kernel meal	Soybean meal	Soya protein concentrate	Wheat meal
Proximate (g kg^{-1})					
Dry matter	940	945	955	940	941
Crude protein	635	665	471	900	143
Crude lipid	88	11.4	11.7	10	16.3
Crude ash	142	137	22.8	7.0	14
Gross energy (KJ/g)	21.1	18.3	18.2	22.6	18.7
Trypsin inhibitor (mg trypsin inhibited per g sample)	-	ND	ND	ND	-
Lectin[a]	-	ND	ND	ND	-
Phytate (% dry matter)	-	9.5	2.5	-	-

Table 2 continued

Non-starch polysaccharides (NSP) (g kg^{-1})				
Rhamnose	-	3	0	-	-
Fucose	-	1	0	-	-
Arabinose	-	31	24	-	-
Xylose	-	20	11	-	-
Mannose	-	5	6	-	-
Galactose	-	14	42	-	-
Glucose	-	57	32	-	-
Glucuronic acid	-	0	0	-	-
Galacturonic acid	-	30	24	-	-
Total-NSP	-	160	140	-	-
Essential amino acids (g kg^{-1})				
Arginine	35.3	69.7	36	67.9	5.4
Histidine	17.7	21.7	14.4	24.4	3.4
Iso leucine	22.8	26.7	19.6	36.5	4.2
Leucine	41.6	46.7	35.7	68.1	9.1
Lysine	40.9	23.3	29.1	52.1	3.3
Phenylalanine	21.8	30.4	24.3	43.2	6.5
Methionine	16	10.6	6.2	12.1	2
Threonine	23	22	17.8	31.1	3.7
Tryptophan	4.9	7.1	6.4	10.4	1.4
Valine	29.3	31.6	21.2	37.4	5.1
Non essential amino acids (g kg^{-1})				
Alanine	43.3	29.4	21.4	40.9	4.6
Asparginine	60.5	68.7	66.6	122.8	7.2
Cystine	4.3	2.3	6.5	9.8	2.9
Serine	25.5	30.6	24.4	46	6.3
Glutamine	79.4	112.1	93.8	174.9	44.9
Glycine	59.8	31.5	21.3	37.2	5.6
Tyrosine	14.8	18.8	15.8	31	3.3
Proline	36.9	32.2	28.2	50.2	14.5

ND: Not detected

[a:]Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.
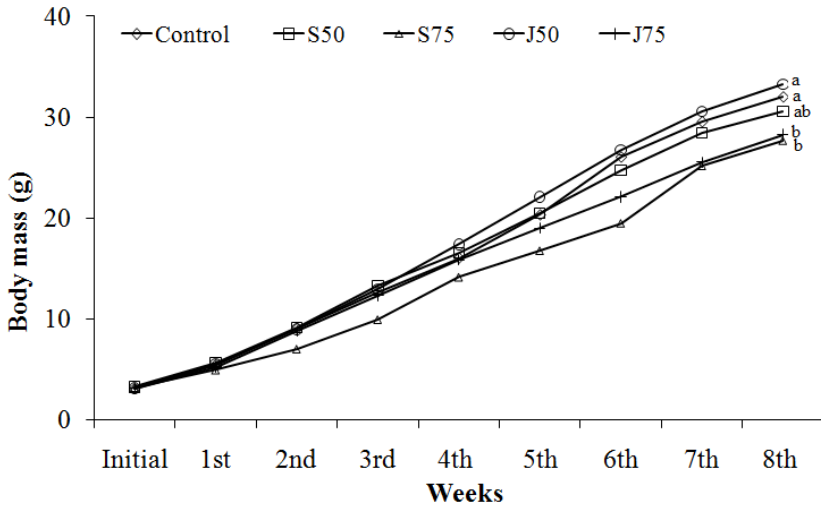
Figure 1 Weekly body mass gain of common carp (*Cyprinus carpio* L.) fed with experimental diets for eight weeks
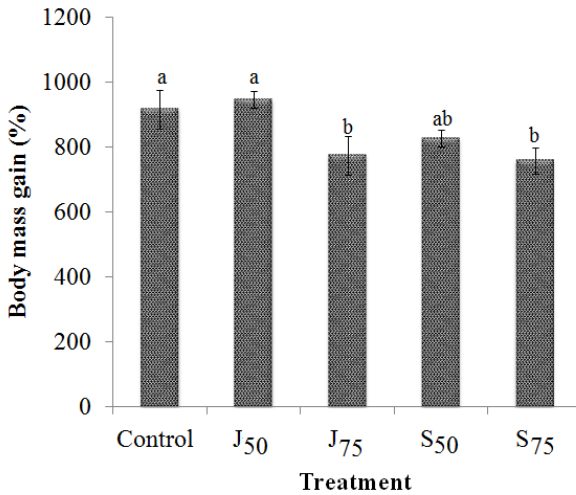
Figure 2 Body mass gain (%) of common carp (*Cyprinus carpio* L.) of different experimental groups. Each value is mean (n = 4) ± standard deviation. Mean values with different letters differ significantly (P < 0.05). (Kumar et al. 2010a).

IGF-1 concentration in plasma and expression of GH and IGF-1 genes

Data on plasma IGF-1 concentration; and expression of GH and IGF-1 genes in brain, liver and muscle are shown in Figures 3 - 5. IGF-1 gene expression was directly proportional to body mass gain of fish. Highest IGF-1 gene expression in brain, liver and muscle was observed in control group, which is statistically not different (P > 0.05) to J_{50} group and higher (P < 0.05) than other groups, whereas GH gene expression exhibited opposite trend. Dietary treatment did not affect significantly to the total amount of IGF-1 concentration in plasma. As a characteristic feature, plasma IGF-1 concentrations numerically decreased with the increase of FM replacement, but this effect appeared more evident in SBM fed groups fish.

Table 3 Proximate and amino acid composition of the experimental diets (g kg^{-1} feed)

Amino acids	Control	J_{50}	J_{75}	S_{50}	S_{75}
Proximate (g kg^{-1})					
Dry matter	948	961	949	944	949
Crude protein	385	381	382	383	382
Crude lipid	87	88	87	83	85
Ash	105	102	100	110	103
Gross energy (kJ/g)	18.4	18.2	18.4	18.7	19.4
Essential amino acids (g kg^{-1})					
Arginine	20.1	28.1	32.1	24.2	26.2
Histidine	10.5	11.1	11.5	10.9	11.1
Iso leucine	13.3	14.0	14.3	14.4	15.0
Leucine	24.8	25.6	26.0	26.7	27.6
Lysine	22.1	19.8	18.7	22.4	22.4
Phenylalanine	13.7	15.5	16.5	16.6	17.9
Methionine	8.9	7.4	6.7	7.0	6.0
Threonine	13.2	12.7	12.4	13.6	13.8
Tryptophan	3.0	3.5	3.8	4.0	4.5
Valine	16.9	17.2	17.3	16.9	16.8
Non essential amino acids (g kg^{-1})					
Alanine	23.8	20.0	18.1	20.4	18.7
Asparginine	33.6	35.1	35.9	42.8	47.2
Cystine	3.3	2.8	2.5	4.3	4.8
Glycine	32.6	25.1	21.3	24.8	20.8
Glutamine	58.4	65.2	68.7	68.2	73.0
Proline	24.6	22.9	22.1	24.0	23.7
Serine	15.5	16.4	16.9	17.5	18.5
Tyrosine	8.8	9.7	10.1	10.7	11.6

Amino acid compositions of the experimental diets were calculated from amino acid profile of individual feed ingredients.
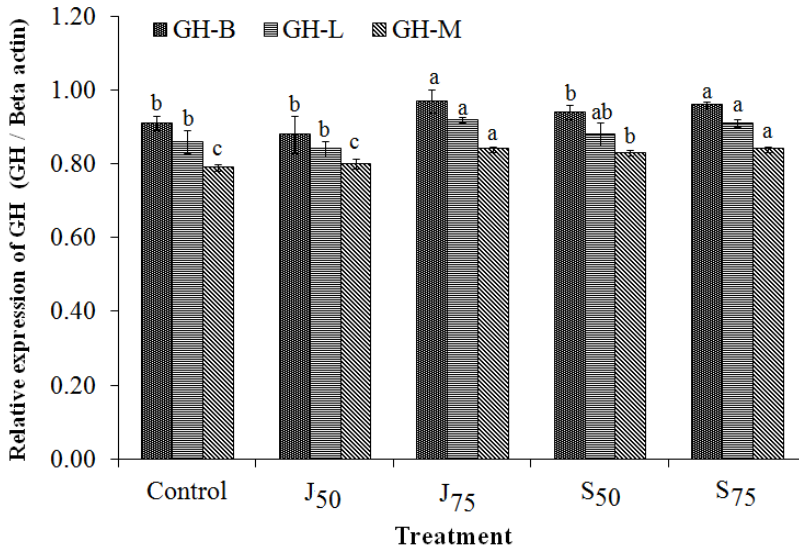
Figure 3 The semi quantitative values of growth hormone (GH) gene expression in brain (GH-B), liver (GH-L) and muscle (GH-M) of common carp (*Cyprinus carpio* L.) after eight weeks of different experimental groups. Each value is mean (n = 4) ± standard deviation. Mean values with different letters differ significantly (P < 0.05).
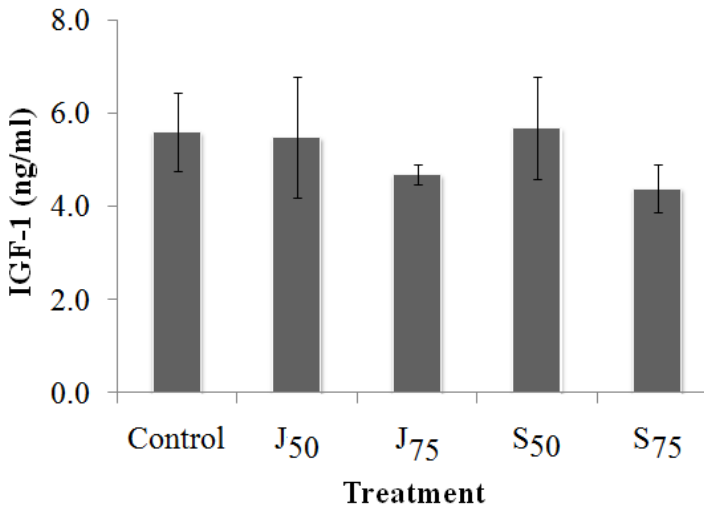
Figure 4 Plasma IGF-1 concentrations in common carp (*Cyprinus carpio* L.) after eight weeks of different experimental groups. Each value is mean (n = 4) ± standard deviation. Mean values with different letters differ significantly (P < 0.05).
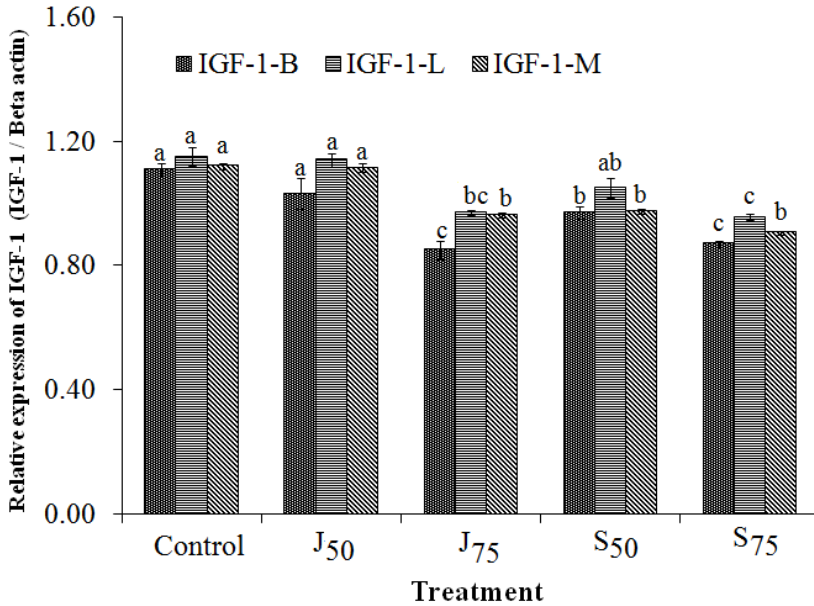
Figure 5 The semi quantitative values of Insulin like growth factor (IGF-1) gene expression in brain (IGF-1-B), liver (IGF-1-L) and muscle (IGF-1-M) of common carp (*Cyprinus carpio* L.) of different experimental groups. Each value is mean (n = 4) ± standard deviation. Mean values with different letters differ significantly (P < 0.05).

Discussion

In the present study, the results indicate that DJKM at lower inclusion levels (J$_{50}$, 50% of FM protein replaced by DJKM) is a good dietary protein source for carp feed. Growth performance of carp fed J$_{50}$ diet was better than SBM based diets and similar to that FM based diet. Slightly lower performance of fish fed the diet where DJKM replaced 75% of FM protein suggested that the capacity of DJKM to fully sustain growth was slightly lower compared to the control diet. Significantly lower growth response of 75% plant protein fed group might be because of several factors such as lower digestibilities of protein and energy in the diets (Kumar et al., 2010a,b), which could lead to lower protein and energy availability from the DJKM and SBM. Antinutrients such as phytate and NSP are available in high amount in the SBM and DJKM that could have adversely affect the feed utilization that leads to lower growth performance. It appears that fish fed diets with higher ratio plant protein showed a transitory energy deficit as the result mainly of a reduced in growth performance, which are in agreement with data in

common carp and rainbow trout (Gomes et al., 1993; Hasan et al., 1997; Mazurkiewicz, 2009; Kumar et al., 2010a,b,c).

The GH–IGF-1 network plays an integral role in mammalian growth (Jones and Clemmons, 1995). Cao et al. (2009) have suggested that IGF-1 is an important hormone involved in growth of carp. To the best of our knowledge, this is the first study to demonstrate a correlation with plasma IGF-1 concentration; GH and IGF-1 gene expression with growth performance when common carp fed with DJKM as a protein source.

The present study was aimed to measure the levels of GH and IGF-I mRNA in the brain (including pituitary), liver, and muscle to search for the underlying mechanism driving the differences in circulating hormone in elation to feeding and to suggest if GH is also expressed in extrapituitary tissues such as liver and muscle. Our findings were consistent with those of Yang et al (1999) and Tymchuk et al. (2009). They found that levels of GH mRNA were detected several tissues such as pituitary gland, brain, gill, heart, kidney, muscle and liver.

The GH-IGF-I axis provides an integrated signal for growth and nutrient partitioning (Beckman and Dickhoff 1998; Mingarro et al. 2002). In the present study for all diet groups, there is an over-all negative correlation between expression of GH gene and growth performance. Highest growth performance was observed for the J_{50} groups, which were statistically similar ($P > 0.05$) to that for control group and significantly ($P < 0.05$) higher than for all other groups whereas GH gene expression exhibited opposite trend. This is well in line with data from salmonids and other fish species showing elevated GH concentrations during extended periods of fasting or feed restrictions (Wagner and McKeown, 1986; Sumpter et al., 1991; Pérez-Sánchez et al., 1995; Pierce et al., 2005). The correlation results from the negative feedback effect of IGF-I on GH secretion, which ensures in fish as in mammals high GH levels during starvation to promote lipolysis (Duan, 1998). Although the fish in the present study were offered feed with five times their maintenance requirement, it thus appears that different physiological (decreased feed utilization) responses to the different diets, which leads to lower nutritional uptake in J_{75} and S_{75} groups than control and J_{50} groups, in turn leads to the increased in relative expression of GH gene in brain, liver and muscle. The reason for this may be that GH has important metabolic actions, and one of its functions during poor nutritional conditions may be to protect protein and mobilize energy from lipid (Björnsson, 1997). This is supported by the fact that in the present study, based on feed conversion ratio (date not shown) is opposite trend to growth performance and parallel to GH gene expression in brain, liver and muscle.

Our results are in concurs with Aksnes et al. (2006), wherein they observed that ≥75% FM protein replaced by a mixture of plant protein sources (SBM, soy protein concentrate, corn gluten meal, wheat gluten, extruded peas, rapeseed meal) balanced with indispensable amino acids in rainbow trout and gilthead sea bream leads to increased in GH concentration in plasma and liver GH gene expression. They concluded that growth performance was inversely related to GH gene expression, similar trend has been observed in our study.

Mounting evidence suggests that IGF-1 plays a similar role in the growth of fish (Duan, 1997,1998; Moriyama et al., 2000). Perez- Sanchez and Le Bail (1999) first proposed that the GH–IGF-1 network could be used as a marker of growth performance and nutritional status in cultured fish. The current study indicated that concentration of IGF-1 levels in plasma did not differ significantly among the treatment groups but numerically parallel with the IGF-1 gene expression in muscle liver and brain among the different dietary groups. Relative IGF-1 gene expression in muscle, liver and brain were highest for the J_{50} group, which were statistically not different to that for control group and significantly ($P < 0.05$) higher than for all other groups which was parallel to growth performance. Our results are in agreement with Gomez-Requeni et al. (2004), Dyer et al. (2004), Aksnes et al. (2006), Li et al. (2006), wherein they have observed that IGF-1 was in parallel to growth rates in trout, sea bream, channel cat fish, barramundi and Atlantic salmon. Results of the present study and the study of Dyer et al. (2004) suggest that mRNA expression and circulating plasma concentrations of IGF-1 is a useful tool to predict growth rates of fish.

Concentration of IGF-1 levels in plasma did not differ significantly among the treatment groups but numerically it was parallel growth performance data. Our results are concurs with Gómez-Requeni et al. (2004, 2005) where in they showed that dietary protein sources may affect plasma GH concentrations, but not IGF-1 levels, in rainbow trout and gilthead sea bream. IGF-1 is involved in the GH negative feedback loop (Perez-Sanchez et al., 1992; Weil et al., 1999), and consistent changes in plasma GH concentrations occur in response to a shift in ration size and dietary protein/energy ratio (Perez-Sa´nchez et al., 1995; Martı´-Palanca et al., 1996; Company et al., 1999). Gomez-Requeni et al. (2003) demonstrated that changes in the dietary essential and nonessential amino acids content in the diets are able to induce some state of liver GH resistance in conjunction with reduced growth rates. In comparison to fish fed the FM diet, no changes in the J_{50} and S_{50} groups (50% replacement of FM protein by SBM and DJKM meal respectively) were found, but in J_{75} and S_{75} groups (75% replacement of

FM protein) increased GH expression level paralleled with the decrease in IGF-I gene expression. All this revealed a state of GH-liver desensitization, which is due to a reduced expression of GH receptors in liver, a characteristic feature of catabolic states (Figure 3). In brief, our study demonstrates a concurrent down-regulation of GH expression when comparisons were made among the different groups while an opposite trend was noted for IGF-1 gene expression level (Figure 5).

Conclusions

Growth performance was in parallel with IGF-1 gene expression and exhibited negative trend to GH gene expression. PE, the main toxic principle for Jatropha toxicity was not detected in fish muscle tissues, suggesting the fish is safe for human consumption.

Acknowledgement

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Chapter 6

Section 1

Nutritional, physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal

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Abstract

Jatropha curcas is a multipurpose and drought resistant tree, widespread throughout tropics and subtropics. Its seeds are rich in oil and protein. It is being promoted as a biofuel plant. Jatropha kernel meal obtained after oil extraction is an excellent source of protein (58-66% crude protein). However, presence of toxic and antinutritional constituents restricts its use in fish feed. Jatropha kernel meal was detoxified. Using *Oncorhynchus mykiss* juveniles, a 12-week experiment was conducted to evaluate the nutritional quality of detoxified Jatropha kernel meal (DJKM). *Oncorhynchus mykiss* juveniles (36, av. wt. 4.2±0.4 g) were distributed in three groups with 12 replicates and fed iso-nitrogenous (crude protein 45%) diets: control (fish meal based protein), J_{50} and $J_{62.5}$ (50% and 62.5% of fishmeal protein replaced by DJKM). Growth performance, apparent lipid conversion, hepatosomatic index, nutrients and energy digestibilities; efficiency of digestible nutrients and energy; and glucose and creatinine levels in blood were statistically similar (P>0.05) for control and J_{50} but were higher (P<0.05) than for $J_{62.5}$. Opposite trend was observed for phosphorus, sodium, albumin, and total protein in blood. Feed conversion ratio, protein efficiency ratio, protein productive value, energy retention, gastro somatic index, muscle lipid peroxide; and red and white blood cells, hematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration; alkaline phosphatase and alanine transaminases activity, globulin, total bilirubin, blood urea nitrogen, calcium and potassium in blood and lysozyme activity in serum did not differ significantly among the groups. Intestinal enzyme activities; and plasma and muscle cholesterol levels for control were higher (P<0.05) than for DJKM groups; while, opposite trend was observed for relative intestinal length (mm g^{-1}). Conclusively, performance of J_{50} group was similar to control group and, thus, is recommended as the optimal diet for rainbow trout.

Introduction

Rainbow trout (*Oncorhynchus mykiss*) have been the most extensively studied aquaculture species for nutritional research. Intensive rainbow trout farming has undergone spectacular growth in the world especially in Europe in recent years due to its increase in demand for human consumption. This activity requires feeds with high levels of protein, which taxes finite global sources of fish meal (FM) supplies (SOFIA, 2007). Therefore, alternative feed ingredients are required to provide the essential nutrients for the growth and quality of aquaculture production. Several compounds have been tested as alternative protein sources, such as animal by-products, single cell proteins including micro algae (*Spirulina maxima*), bacterial single cell protein and yeast (El-Sayed, 1994; Perera et al., 1995) and plant proteins (Guillaume and Métailler, 2001; Hasan et al., 1997; Kumar et al., 2010a,b,c). Among the potential substitutes, plant ingredients appear to be the best candidates. Substantial progress has been made towards the replacement of FM with a number of different plant ingredients, including soyabean meal (SBM), lupin, peas, rapeseed meal and sunflower (Kaushik et al., 1995; Vielma et al., 2002), detoxified Jatropha kernel meal (DJKM) (Kumar et al., 2010a,b,c). Among plant ingredients SBM is currently the most commonly used plant protein source in fish feeds (Yue and Zhou, 2009). However, SBM competes with human food and hence there is a need to identify other protein rich plant resources that could be used in fish diets.

Many plant protein sources can be used to partially or almost totally replace dietary FM (Kaushik et al., 2004), provided that the essential amino acid requirements of the fish species is met, the palatability of the diets is improved and the levels of anti-nutritional factors are reduced (Guillaume and Métailler, 2001). Our previous studies demonstrate that plant protein sources (*Moringa oleifera* leaf meal, *Sesbania aculeate* seed meal and detoxified *Jatropha curcas* kernel meal) could partially replace FM in the diet of tilapia, *Oreochromis niloticus* and common carp, *Cyprinus carpio* (Hossain et al., 2001; Richter et al., 2003a; Dongmeza et al., 2006; Kumar et al., 2008, 2010).

Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. Its seeds have been extensively investigated as a source of oil. The seed kernel contains about 60% oil that can be converted into

biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar et al., 2007a). The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 58 to 66 % crude protein. The levels of essential amino acids (except lysine) are higher in *Jatropha* kernel meal than SBM (Kumar et al., 2010). However the presences of high levels of antinutrients like trypsin inhibitor, lectin and phytate (Makkar et al., 2008) and the major toxic components phorbol esters (PE$_S$) (Makkar and Becker, 1997) restrict their use in fish feed. Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

The International Jatropha Organization has claimed that in 2017 there will be around 32.72 million hectares of land cultivated worldwide producing 160 million tons of seeds and 95% of its total production will be concentrated in Asia (mostly in China and India). The total projected annual Jatropha oil production in Asian countries will be 46.88 million tons (Siang, 2009). Jatropha plant can yield up to 5 tons seed per year from one hectare of plantation, which can produce approximately one ton of kernel meal rich in protein (Makkar and Becker, 1997). This means that there is possibility of producing enough Jatropha seed meal to meet growing aquaculture industry demand. Our previous study (Kumar et al., 2008, 2010a,b,c) has shown that DJKM is a good protein source and better than SBM for carp diet. There is no study on the inclusions of DJKM into the diet of rainbow trout. This study reports the nutritional, physiological and haematological responses of adding DJKM in rainbow trout.

Material and methods

Preparation of the Jatropha meal

Jatropha seeds were purchased from India and deshelled manually to obtain kernels. Defatting of Jatropha kernals was done using petroleum benzene (b.p. 40–60 ^{0}C) in a Soxhlet apparatus. Organic solvents were used to detoxify defatted Jatropha kernel meal (patent application has been filed for the process of detoxification). After removal of PEs, the meal was autoclaved (121 ^{0}C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation

FM and wheat meal were purchased from Kurt Becker GmbH, Bremen, Germany, at a local market respectively. Source of fish oil (Menhaden, batch # 102k0126) and gluten (wheat, lot number # 100K0191) from Sigma-Aldrich, Chemie GmbH Steinheim, Germany. Source of sunflower oil was Thomy, Nestle Deutchland AG, 41415 Neuss; Reg. Trademark of Societe des, Produits Nestle S.A.. Vitamin premix and mineral premix were procured from Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Source of lysine is Merck KGaA, 64271 Darmstadt, Germany. Prior to feed formulation, the proximate composition of defatted Jatropha meal, wheat meal, wheat gluten and FM were determined. Three isonitrogenous and isoenergetic diets were formulated. Experimental diets containing crude protein 45%, crude lipid 24%, vitamin premix 2%, mineral premix 2% and Titanium oxide (TiO_2) 1% were prepared. TiO_2 was added for digestibility measurement. Lysine monohydrochloride was supplemented at the rate of 1.5% of DJKM inclusion in the diet. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg. The inclusion levels of the DJKM were as follows:

Control diet ($C_{control}$) was prepared with fishmeal and wheat meal, without DJKM. J_{50}: 50% FM protein replaced by DJKM; and $J_{62.5}$: 62.5% FM protein replaced by DJKM. The final mixture of each diet was made into 2 mm diameter moist pellets (using a Bosch, Type UM60ST 2-M, Robort Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then freeze-dried (Table 1).

Experimental system and animals

Rainbow trout (*Oncorhynchus mykiss*) juveniles (about 3 g) obtained from the Fischzucht, Peter Störk, Wagenhausen, Bad Saulgau, Germany were transferred to University of Hohenheim, Stuttgart and kept in 1000 l capacity tanks for acclimatisation. They were fed the Hohenheim standard fish diet containing approximately 45% protein, 24% lipid, 12% ash and with a gross energy content of 22 kJ g^{-1} dry matter.

In our initial trial, six fish per aquarium (45 l) were kept so as to have six fish per replicate. However this resulted in high mortality due to aggressive/hierarchical behaviour of rainbow trout. Therefore, after the acclimatisation, 36 fish were randomly distributed into three groups with 12 replicates (av. wt. 4.2 ± 0.4 g). Two fish kept in an aquarium (45 l capacity) were separated from each other by a perforated plastic sheet. All the aquaria

were supplied with water at 11 ± 2.0 °C from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 9.0 – 13.0 °C, pH 7.0 – 7.5, dissolved oxygen 6.2 – 7.6 mg l^{-1}, total NH_3 0.08– 0.18 mg l^{-1}, nitrite 0.06 – 0.09 mg l^{-1} and nitrate 1–2 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved. According to Becker et al., 1983, one time maintenance requirement equals 3.2 g feed per kg metabolic body mass ($kg^{0.8}$). So 16 g feed per kg metabolic body mass ($kg^{0.8}$) was fed for 5 times maintenance requirements, and the feed was split into 5 equal rations per day (at 8:00, 10:30, 13:00, 15:30 and 18:00 h). The feeds were dispensed using an automatic feeder (Graesslin, Rondomatic 400, Graesslin GmbH, St. Georgen/Schwarzw, Germany). Fish were weighed individually at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feed amount for the subsequent week. The fish were not fed on the weighing day. During last two weeks of the experiment, fish were fed with a diet containing a marker (TiO_2) for digestibility measurement (Mamun et al., 2007). The faeces collection was qualitative, as the experimental diets contained an inert marker (TiO2). During last two weeks of the experiment, faeces were collected daily. After each feeding the aquaria were controlled for remaining feed; generally, there were no feed residues left. Every day prior to the faeces collection, aquaria were siphoned out to clean any residues. Faeces subsequently excreted by the fish were collected in separate beakers for each aquarium by siphoning with a short small pipe (Mamun et al., 2007). The collected mixture of water and faeces was centrifuged at 4000 × g for 10 min, the supernatant discarded and the faeces were then stored at -20°C until analysis. For the analysis, faeces from all the experimental periods from the same fish were pooled. During the experiment there was no mortality. At the start of the experiment, 12 fish of the same population were killed and preserved at −20 °C for analysis of the initial body composition.

The experiment was terminated after 12-week and the fish were killed. At the end of experiment, fish were anesthsized by tricaine methanesulfonate (MS222) at 250 ppm in water. Blood was drawn near caudal peduncle from four fish from each group and transferred into a heparinized tube for hematological study. Blood was drawn from another four fish from each group and divided into two equal part, one part was centrifuged at 1500×g for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at −20 °C for determination of cholesterol

and triglycerides. Another part of blood was kept outside at room temperature for few minutes to collect serum. Serum was stored at −20 °C for lysozyme determination. Four fish per group were carefully dissected to isolate intestine and stored in liquid nitrogen for digestive enzymes assay. Muscle was isolated from same fish and stored at −20 °C for determination of cholesterol and muscle lipid peroxides value. Those four fish from which blood was drawn for haematological study were stored at −20 °C for chemical composition analysis. Prior to determination of the proximate composition, the fish were autoclaved at 121 °C for 20 min, thoroughly homogenised using an Ultra-Turrax T25 (Janke and Kunkel, GmbH & Co.KG, Grißheimer weg 5, Heitersheim, Germany), frozen overnight and freeze-dried.

The University animal welfare committee (University of Hohenheim Germany) approved all experimental procedures involving rainbow trout.

Table 1 Composition of the experimental diets (g kg^{-1} feed) for rainbow trout (*Oncorhynchus mykiss*) juveniles

Ingredients	Experimental diets		
	C_{ontrol}	J_{50}	$J_{62.5}$
Fish meal	687	339	255
[1]Wheat meal	93	53	57
Jatropha meal	-	343	433
Wheat gluten	-	10	5
Sunflower:Fish oil (1:1)	180	209	202
[2]Vitamin premix	20	20	20
[3]Mineral premix	20	20	20
Lysine monohydrochloride (g)	-	5	7
Total	1000	1000	1000
Phytase (FTU/kg)	-	500	500
TiO2	10	10	10

[1]Whole wheat meal.

[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[3]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 50$_2$; $MgSO_4$. $7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4$. H_2O, 3.12; $ZnSO_4$. $7H_2O$, 4.67; $CuSO_4.5H_2O$, 0.62; KI, 0.16; $CoCl_2.6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

Determination of phorbol esters, trypsin inhibitor, lectin, phytate and non starch polysaccharides

Phorbol esters (PEs) were determined according to Makkar et al., 2007b, which was based on the method of Makkar et al., 1997. Briefly, 0.5 g of the Jatropha meal and dried whole body fish sample were extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C_{18} LiChrospher 100, 5 µm (250 x 4 mm I.D. from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at room temperature (23 ^{O}C) and the flow rate was 1.3 ml/min using a gradient elution (Makkar et al., 2007b). The PEs peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs is 3 µg/g meal.

Trypsin inhibitor activity was determined essentially according to Smith et al., 1980 except that the enzyme was added last as suggested by Liu and Markakis, 1989. Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL^{-1} assay medium) that produced agglutination. The minimum amount was the amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis, MO, USA) as standard. Non-starch polysaccharides (NSP) were estimated according to Englyst et al., (1994).

Amino acid analysis

Amino acid composition of FM, DJKM and wheat meal was determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 ^{0}C for 24 h (Bassler and Buchholz 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. Tryptophan content of the above-mentioned samples was determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl, 1990.

Proximate analysis

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (1990). Samples were analysed for dry matter (DM), ash, crude protein (CP) and lipid (ether soluble lipid). Gross energy (GE) of diet ingredients, diets and fish bodies was determined with bomb calorimeter (IKA C7000, Janke and Kunkel, GmbH & Co.KG, Grißheimer weg 5, Heitersheim, Germany) using benzoic acid as a standard.

Growth parameters

Growth performance and diet nutrient utilization were assessed in terms of body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), apparent lipid conversion (ALC) and energy retention (ER). These were calculated as follows:

BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100; SGR = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR = (Body mass gain in g) / [{(initial body mass in g / $1000)^{0.8}$ + (final body mass in   g / $1000)^{0.8}$}/2] / number of trial days (Dabrowski et al., 1986); FCR = dry feed fed (g)/body mass gain (g); PER = body mass gain (g)/crude protein fed (g); PPV (%) = [(final fish body protein in g - initial fish body protein in g) / total protein   consumed in g] X 100; ALC (%) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid   consumed in g] X 100; ER (%) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100.

Digestibility measurement

Titanium dioxide in the feed and faeces was determined according to the method described by Richter et al., (2003b). The percentage of apparent dry matter digestibility of diets was calculated according to Maynard et al., (1981).

Apparent dry matter digestibility (%) = [1 – {(% TiO_2 in feed) / (% TiO_2 in faeces)}] X 100

Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets were calculated according to Maynard and Loosli, 1969. The nutrient and energy digestibility were obtained using the following formula:

Apparent nutrient digestibility (%) = [1 – {(% TiO$_2$ in feed) / (% TiO$_2$ in faeces) X (% Nutrient or energy in faeces) / (% Nutrient or energy in feed)}] X 100

Efficiency of digestible nutrients and gross energy = (Nutrient and energy retained in the whole body/Digestible nutrient and digestible energy) x 100
Digestible nutrients and energy = Total offered of nutrients and gross energy through feed X digestibility coefficient.

Relative intestinal length (RIL), spleen Index (SI), hepatosomatic index (HSI), and gastro somatic index (GSI)

RIL was measured excluding pyloric caeca and is expressed in relation to each animal weight and expressed in mm g^{-1}.
RIL, SI, HSI, and GSI are calculated as indicated below:
RIL = Intestine length (mm)/ body mass (g)
SI = Spleen mass (g) / body mass (g)
HSI = Liver mass (g) X 100 / body mass (g)
GSI = Intestine mass (g) X 100 / body mass (g)

Digestive enzymes assay

The reducing sugars produced due to the action of glucoamylase and α-amylase on carbohydrate was estimated using dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as mmol of maltose released from starch per min at 37 ^{O}C. Protease activity was determined by the casein digestion method of Drapeau, 1974, and one unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta 0.001 A_{280}$ per minute at 37 ^{O}C and pH 7.8. Lipase activity was assayed by the method of Cherry ad Crandell, 1932, and one unit of enzyme was the amount of enzyme that hydrolysis 1.0 microequivalent of fatty acid from a triglyceride in 24 h at pH 7.7 at 37 ^{O}C.

Cholesterol and triglyceride estimation and assay of lipid peroxides

The determinations of the plasma cholesterol and triglycerides were using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color

intensity was determined photometrically and was directly proportional to the concentration of cholesterol and triglycerides in the plasma sample. Lipid peroxides in fish muscle were determined using the procedure of Utley et al., 1967.

Haematological parameters

Total erythrocyte count (RBC) and total leucocyte count (WBC)

RBC and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope.

The following formula was used to calculate the number of RBC per mm^3 of the blood sample:

Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid

Haemoglobin (Hb) and hematocrit (Hct) content

The Hb content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hct was determined on the basis of sedimentation of blood. Heparinised blood (50 µl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Haematocrit [in %] x 1000) ÷ (RBC count [in millions/µL]); mean corpuscular haemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/µL]) and mean cell haemoglobin concentration, MCHC [in g/dL] = Haemoglobin [in g/dL] ÷ Haematocrit [in %].

Lysozyme activity in serum

Lysozyme activity of serum was measured by EnzChek Lysozyme Assay Kit (E-22013) Leiden, The Netherlands. The assay measures lysozyme activity on *Micrococcus lysodeikticus* cell wall, which is labeled to such a degree that the fluorescence is quenched. Lysozyme action relieves this quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase was measured by using spectrofluorometer that detects fluorescein. Lysozyme hydrolyzes β-(1-4)-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of the microorganism.

Blood parameters analysis by Vet Scan

VetScan Chemistry Analyzer (Scil Animal care company GmbH, Technischer Service, Germany) was used for determinations of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium (Ca^{++}), creatinine, globulin, glucose, phosphorus, potassium (K^+), sodium (Na^+), total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Duncan's multiple range test (P<0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results

Phorbol esters and antinutrients content in defatted Jatropha kernel meal

PE content in untreated defatted Jatropha kernel meal was 1.8 mg/g. However, PEs in DJKM was undetectable. Trypsin inhibitor and lectins were also not detected in DJKM; whereas phyate and NSP levels in DJKM were 9.1% and 16% respectively (Table 2).

Fish behaviour and feed intake

Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was normal. No left feed was observed in the aquaria. There was no mortality during the entire experimental period.

Proximate composition of feed ingredient, experimental diets and amino acid profile of feed ingredients and experimental diets

Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 2 and 3. Diets contained about 45% crude protein and 21.0 kJ/g gross energy and were isonitrogenous and isoenergetic. Crude lipid and ash were in the range of 23.9–24.7% and 13.6–14.1% respectively.

All experimental diets had almost similar amino acid composition (except sulfur containing amino acids such as methionine and cystine in J$_{62.5}$ group).

Table 2 Proximate composition, antinutrients content and amino acid composition of feed ingredients (dry matter basis)

	Fish meal	Jatropha meal	Wheat gluten	Whole wheat meal
Proximate composition (g kg^{-1})				
Dry matter	940	945	937	941
Crude protein	635	665	856	143
Crude lipid	88	11.4	13.4	16.3
Crude ash	142	137	8.7	14
Gross energy (KJ/g)	21.1	18.3	21.1	18.7
Antinutrients				
Trypsin inhibitor (mg trypsin inhibited per g sample)	ND	ND	ND	-
Lectin[a]	-	ND	ND	-
Phytate (% dry matter)	-	9.3	-	-
Non-starch polysaccharides (NSP) (g kg^{-1})				
Rhamnose	-	3	-	-
Fucose	-	1	-	-
arabinose	-	31	-	-
Xylose	-	20	-	-
Mannose	-	5	-	-
Galactose	-	14	-	-
Glucose	-	57	-	-
Glucuronic acid	-	0	-	-
Galacturonic acid	-	30	-	-
Total-NSP	-	160	-	-
Essential amino acids composition (g kg^{-1})				
Arginine	35.3	69.7	43.2	5.4
Histidine	17.7	21.7	21.4	3.4
Iso leucine	22.8	26.7	43.7	4.2
Leucine	41.6	46.7	69.4	9.1
Lysine	40.9	23.3	16.1	3.3
Phenylalanine	21.8	30.4	49.5	6.5
Methionine	16	10.6	17.0	2
Threonine	23	22	24.5	3.7
Tryptophan	4.9	7.1	10.2	1.4
Valine	29.3	31.6	43.3	5.1
Non-essential amino acids composition (g kg^{-1})				

Table 2 continued

Alanine	43.3	29.4	20.1	4.6
Asparginine	60.5	68.7	34.7	7.2
Cystine	4.3	2.3	17.6	2.9
Glycine	59.8	31.5	325.2	5.6
Glutamine	79.4	112.1	17.3	44.9
Proline	36.9	32.2	116.4	14.5
Serine	25.5	30.6	43.1	6.3
Tyrosine	14.8	18.8	28.2	3.3

ND: Not detected

[a]Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Table 3 Proximate and amino acid composition of experimental diets (dry matter basis) for rainbow trout (*Oncorhynchus mykiss*) juveniles

	C$_{ontrol}$	J$_{50}$	J$_{62.5}$
Proximate (g kg^{-1})			
Dry matter	947	948	944
Crude protein	467	464	461
Crude lipid	247	240	239
Crude ash	141	138	136
Gross energy (KJ/g)	21.0	21.2	20.8
Essential amino acids (g kg^{-1})			
Arginine	24.75	36.81	39.96
Histidine	12.48	13.92	14.31
Iso leucine	16.05	17.64	17.95
Leucine	29.43	31.49	31.93
Lysine	28.41	27.33	27.96
Phenylalanine	15.58	18.77	19.48
Methionine	11.18	9.39	8.94
Threonine	16.15	15.88	15.84
Tryptophan	3.50	4.30	4.49
Valine	20.60	21.60	21.82
Non-essential amino acids (g kg^{-1})			
Alanine	30.17	25.36	24.33
Asparginine	42.23	45.07	46.09
Cystine	3.22	2.59	2.37
Glycine	41.60	34.82	31.09
Glutamine	58.72	68.39	71.98
Proline	26.70	25.65	24.96
Serine	18.10	20.03	20.48
Tyrosine	10.47	11.99	12.33

Growth performance and feed utilization

Weekly body mass gains of fish are given in Figure 1. The growth performance of the fish at the end of the experimental period and the

nutrient utilization are presented in Table 4. Weekly body mass gain indicates that second week onwards there was differential growth among the group, and lower body mass development was observed in $J_{62.5}$ group compared to other groups. This trend was maintained till the end of the experiment. The BMG, SGR, MGR and ALC were statistically similar (P>0.05) for C_{ontrol} and J_{50} groups and significantly higher (P<0.05) compared to those for $J_{62.5}$. FCR, PER, PPV, and ER did not differ significantly among the three groups (Table 4).
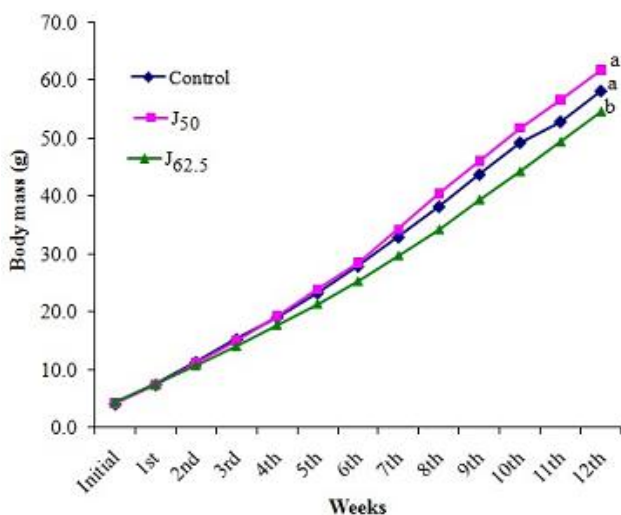
Figure 1: Body mass gain of rainbow trout (*Oncorhynchus mykiss*) juveniles fed with experimental diets for 12 weeks

Chemical composition of whole body of fish

Highest moisture content of the whole body was observed in C_{ontrol} group, which was statistically not different (P > 0.05) to that in $J_{62.5}$ group and was lowest in J_{50} groups; whereas, crude lipid in whole body had reverse trend (Table 5). Gross energy content of the whole body did not differ significantly among the groups. Highest crude protein and ash deposition were observed in J_{50} groups and these values were statistically not different (P > 0.05) for those in $J_{62.5}$ groups and were higher than those in control group (Table 5).

Table 4 Initial body mass (IBM), growth performance and nutrient utilization of rainbow trout (*Oncorhynchus mykiss*) juveniles fed with experimental diets for 12 weeks

Treatment	IBM (g)	BMG (%)	SGR (%)	MGR (g kg$^{0.8}$ day^{-1})	FCR	PER	PPV (%)	ALC (%)	ER (%)
C$_{control}$	4.12 ± 0.26	1385[a] ± 157	3.2[a] ± 0.12	11.4[a] ± 0.37	1.3 ± 0.10	1.6 ± 0.10	22.8 ±1.10	31.0[a] ± 2.30	24.8 ± 0.45
J$_{50}$	4.17 ± 0.54	1394[a] ± 185	3.2[a] ± 0.15	11.4[a] ± 0.42	1.2 ± 0.10	1.7 ± 0.11	26.3 ±2.37	31.9[a] ±1.80	25.5 ± 1.49
J$_{62.5}$	4.31 ± 0.49	1178[b] ± 257	3.0[b] ± 0.24	10.8[b] ± 0.75	1.3 ± 0.20	1.6 ± 0.40	25.2 ± 4.59	27.5[b] ±1.89	23.2 ± 2.18
SEM	0.07	38.66	0.03	0.10	0.02	0.04	0.91	0.80	0.49

BMG - Body mass gain, SGR – Specific growth rate and MGR - Metabolic growth rate; FCR – Feed conversion ratio, PER - Protein efficiency ratio, PPV - Protein productive value, ALC - Apparent lipid conversion and ER - Energy retention
Values are mean (n = 12) ± standard deviation for BMG, SGR, MGR, FCR and PER; Values are mean (n = 4) ± standard deviation for PPV, ALC and ER
Mean values in the same column with different superscript differ significantly ($P < 0.05$).

Table 5 Chemical composition of whole body of rainbow trout (*Oncorhynchus mykiss*) juveniles of different experimental groups at the start and at the end of the experiment (g kg^{-1}, wet basis ± SD)

Treatment	Moisture	Crude protein	Crude lipid	Ash	Gross energy (KJ/g)
Initial fish	736 ± 2.0	173 ± 0.50	56 ± 1.20	30 ± 1.10	59 ± 1.50
C$_{control}$	745[a] ± 2.70	139[b] ± 4.90	92[ab] ± 4.40	23[b] ± 1.50	67 ± 1.60
J$_{50}$	720[b] ± 4.80	159[a] ± 4.20	96[a] ± 1.60	25[a] ± 0.80	70 ± 3.10
J$_{62.5}$	736[a] ± 8.10	153[a] ± 6.30	86[b] ± 5.80	25[a] ± 1.00	66 ± 2.90
SEM	3.50	2.90	1.70	0.50	0.90

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly ($P < 0.05$).

Dry matter and nutrient digestibility and efficiency of digestible nutrients and energy

The digestibility of dry matter and nutrients of different experimental diets are given in Table 6. The dry matter protein, lipid and energy digestibility were highest for control group and statistically not different (P>0.05) from J_{50} group but significantly higher (P<0.05) than $J_{62.5}$ group.

Efficiency of digestible nutrients and energy of experimental diets are shown in Table 6. Efficiency of digestible nutrients and energy of experimental diets did not differ significantly among the three groups.

Digestive enzymes activities

Amylase, protease and lipase activities were highest in control group, followed by J_{50} and $J_{62.5}$ groups; all being significantly different (Table 6).

Relative intestinal length (RIL), spleen Index (SI), hepatosomatic and gastro somatic index (GSI)

RIL, SI, hepatosomatic and GSI of different experimental groups are given in Table 7. Highest HSI was observed in control group and lowest in $J_{62.5}$ group; both these value were statistically different (P < 0.05). Whereas SI and RIL exhibited opposite trend. An increase in the RIL was noticed in J_{50} and $J_{62.5}$ with the longest length in the latter. There was no significant difference in GSI among the different groups.

Cholesterol and triglyceride levels in plasma, and cholesterol, phorbol esters (PEs) and lipid peroxides in muscle

Cholesterol and triglycerides levels in plasma, muscle cholesterol and muscle lipid peroxide of different experimental groups are shown in Table 7. Plasma cholesterol and muscle cholesterol levels were highest in control group, and lowest in $J_{62.5}$ groups. Plasma triglycerides increase with the addition of DJKM. PEs content in dried whole fish was undetectable. Muscle lipid peroxide value did not differ significantly among the three groups.

Table 6 Effects of experimental diets on the apparent digestibility coefficient of dry matter, nutrient and energy digestibility (%); utilization of digestible protein, lipid and energy; and digestive enzymes activities (U/mg protein) in rainbow trout (*Oncorhynchus mykiss*) juveniles

Treatment	Dry matter digestibility	Protein digestibility	Lipid digestibility	Energy digestibility	DPE (%)	DLE (%)	DEE (%)	Amylase	Protease	Lipase
C[ontrol]	78.6[a] ± 0.81	89.8[a] ± 0.55	95.2[a] ± 0.43	86.8[a] ± 0.83	25.4 ± 1.08	32.6 ± 2.55	28.7 ± 2.15	4.6[a] ± 0.40	50.3[a] ± 3.59	13.9[a] ± 1.02
J[50]	79.5[a] ± 0.42	89.7[a] ± 0.83	95.2[a] ±0.80	86.1[a] ± 0.71	29.3 ± 2.58	33.5 ± 2.10	29.4 ± 4.12	3.2[b] ± 0.20	41.0[b] ± 2.16	10.8[b] ± 0.38
J[62.5]	73.3[b] ± 2.11	84[b] ± 1.41	89.8[b] ± 0.86	81.8[b] ± 1.13	29.9 ± 5.05	30.6 ± 1.87	26.2 ± 3.43	2.5[c] ± 0.15	32.5[c] ± 1.29	8.6[c] ± 0.48
SEM	0.90	0.85	0.79	0.71	1.06	0.68	0.98	0.27	2.28	0.68

DPE: digestible protein efficiency; DLE: digestible lipid efficiency; DEE: digestible energy efficiency
Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 7 Cholesterol and triglyceride (mg/dl) level in plasma; and muscle cholesterol (mg/100 g) level and muscle lipid peroxide (nMol MDA (malondialdehyde) /100g tissue) level, relative intestinal length (RIL) (mm g^{-1}), spleen index (SI), hepatosomatic index (HSI) and gastro somatic index (GSI) of rainbow trout (*Oncorhynchus mykiss*) juveniles

Treatment	Plasma cholesterol	Plasma triglycerides	Muscle cholesterol	Muscle lipid peroxide	RIL	SI	HSI	GSI
Control	246[a] ± 23.9	162[b] ± 3.5	93[a] ± 2.0	3.70 ± 2.12	0.47[a] ± 0.02	0.19[a] ± 0.01	1.3[a] ± 0.20	2.25 ± 0.65
J[50]	182[b] ± 11.2	235[a] ± 3.9	85[b] ± 5.5	4.23 ± 1.40	0.56[b] ± 0.02	0.22[b] ± 0.01	1.2[ab] ± 0.10	1.95 ± 0.34
J[62.5]	171[b] ± 3.4	255[a] ± 6.4	75[b] ± 4.8	3.95 ± 2.76	0.63[c] ± 0.01	0.24[c] ± 0.00	1.1[b] ± 0.10	1.74 ± 0.14
SEM	12.7	14.3	2.8	0.55	0.02	0.01	0.04	0.13

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 8 Effects of experimental diets on the haematological parameters (RBC (10^6 cells/mm^3), WBC (10^3 cells/mm^3), Hb (g/dl), Hct (%), MCV (fL), MCH (pg), MCHC (g/dl), albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood and lysozyme activity (IU/ml) in the serum of rainbow trout (*Oncorhynchus mykiss*) juveniles

Treat	RBC	WBC	Hb	Hct	MCV	MCH	MCHC	Albumin	Globulin	Total protein	Lysozyme activity
C$_{control}$	0.96 ± 0.05	19.7 ± 3.69	4.5 ± 0.5	45.6 ± 5.32	466 ± 30.8	46.6 ± 3.08	10.18 ± 1.41	2.18[b] ± 0.28	1.64 ± 0.30	3.8[b] ± 0.20	494 ± 52
J$_{50}$	0.97 ± 0.07	19.8 ± 3.18	4.3 ± 0.4	45.2 ± 4.10	442 ± 24.6	44.2 ± 2.46	9.54 ± 0.95	2.66[a] ± 0.15	1.40 ± 0.32	4.10[a] ± 0.30	505 ± 45
J$_{62.5}$	1.05 ± 0.08	19.4 ± 2.94	4.5 ± 0.5	47.2 ± 8.04	432 ± 67.1	43.2 ± 6.71	10.57 ± 3.07	2.36[b] ± 0.53	1.46 ± 0.34	3.8[b] ± 0.50	564 ± 94
SEM	0.02	0.79	0.10	2.49	11.41	1.14	0.69	0.15	0.08	0.10	23.02

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05)
MCV: Mean cell volume (fL); MCH: Mean corpuscular hemoglobin (pg); MCHC**:** Mean corpuscular hemoglobin concentration (g/dl)
IU- The amount of enzyme required producing a change in the absorbance at 450 nm of 0.001 units per minute at pH 6.24 and 25^{0}C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

Table 9 Effects of experimental diets on alkaline phosphatase (ALP, U/l), alanine transaminase (ALT, U/l), glucose (mg/dl), total   bilirubin (TBIL, mg/dl), blood urea nitrogen (BUN, mg/dl) and creatinine (mg/dl) in blood, blood ions (calcium (mg/dl), phosphorus mg/dl, sodium (mMol/l) and potassium (mMol/l) of rainbow trout (*Oncorhynchus mykiss*) juveniles

Treatment	ALP	ALT	Glucose	TBIL	BUN	Creatinine	Calcium	Phosphorus	Sodium	Potassium
C$_{control}$	101 ± 8.3	58.4 ± 13.1	117[a] ± 10.4	0.22 ± 0.04	2.0 ± 0.01	1.70[a] ± 0.86	12.4[b] ± 0.49	14.9[b] ± 0.98	141[b] ± 4.04	1.48 ± 0.36
J$_{50}$	96 ± 25.0	75.2 ± 41.8	90[b] ± 6.8	0.20 ± 0.01	2.2 ± 0.45	0.98[ab] ± 0.22	13.3[a] ± 0.35	17.3[a] ± 1.57	143[ab] ± 3.16	1.5 ± 0.31
J$_{62.5}$	84 ± 30.7	71.0 ± 35.5	86[b] ± 2.75	0.24 ± 0.05	2.0 ± 0.01	0.34[b] ± 0.21	12.2[b] ± 0.82	16.7[a] ± 0.62	146[a] ± 2.68	1.6 ± 0.32
SEM	5.91	8.40	5.57	0.01	0.01	0.19	3.26	0.38	1.01	0.08

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute).

Blood chemistry (Hemato-immunological parameters)

Haematological, biochemical and metabolic response parameters are presented in Tables 8 - 9. RBC and WBC counts; hemoglobin, hematocrit level, MCV, MCH, MCHC, globulin, total bilirubin, BUN, calcium and potassium contents; and ALP and ALT activities in blood and lysozyme activities in serum did not differ significantly among the three groups.

Blood glucose level was lower ($P < 0.05$) in DJKM fed groups than control group. Creatinine level in blood was statistically similar for C_{ontrol} and J_{50} and significantly higher than that of $J_{62.5}$ group. On the other hand, opposite trend was observed for the phosphorus and sodium ion, albumin, and total protein in blood.

Discussion

A large number of investigations have been conducted on the replacement of FM with SBM as a protein source in feeds for rainbow trout (Sanz et al., 1994; Kaushik et al., 1995; Barrows et al., 2008). Similarly, other plant sources such as peas (Gouveia et al., 1993; Pfeffer et al., 1995), lupin (Gouveia et al., 1993; Bangoula et al., 1993), faba beans (Gouveia et al., 1993; Bangoula et al., 1993), rapeseed, canola (Bangoula *et al.*, 1993; Stickney et al., 1996), and sunflower meal (Sanz et al., 1994) have been used in trout feeds. To our knowledge this is the first study on the replacement of FM by DJKM in rainbow trout. Results of this study showed that DJKM could replace 50% of dietary FM without affecting growth performance, nutrient utilization, and metabolic and hematological parameters.

Growth performance and nutrient utilization

Growth performance and nutrient utilization of rainbow trout fed J_{50} diet (50% FM protein replacement by DJKM) were better than that of $J_{62.5}$ group and similar to the control group (FM based diet). These results are in concurrence with other studies on SBM wherein SBM protein could substitute 50% of FM protein without evidencing a negative effect on the feed intake and growth performance (Refstie et al., 2000; de Francesco et al., 2004; Aksnes et al., 2006; Refstie et al., 2006). Since there were no refusals for any of the diets, the observed differences in growth in this experiment were not due to different palatability of the diets. Significantly lower growth response of $J_{62.5}$ group might be because of several factors such as:

> Lower digestibilities of protein and energy in the diets (Table 6), which could lead to lower protein and energy availability from the DJKM (plant protein structures in general are much more compact than FM protein, so digestive enzymes act slowly on DJKM proteins), and/or the presence of antinutrients such as phytate and NSP, which are present in high amounts in the DJKM and could affect adversely the feed utilization at higher level of its incorporation;

> The digestibility of synthetic amino acid (lysine) is lesser than natural amino acids which are present in feed ingredients; and

> The total sulfur amino acid composition in $J_{62.5}$ diet was slightly lower than control diet. According to NRC (1983) the sulfur containing amino acids (methionine and cystine) requirement of rainbow trout is 13 g/kg diet. In our present study the total sulfur amino acid was 11.3 g/kg $J_{62.5}$ diet which is slightly lower than optimum requirement that could lead to lower growth performance in this group.

Another Constraint related to the digestion of plant sources is their relatively high carbohydrate content that is generally not well digested by salmonids (Singh and Nose, 1967). The negative effects at high inclusion levels of plant protein sources such as sunflower meal, SBM and maize gluten (> 75 % replacement of FM protein) on growth performance are well documented in earlier work on trout (Kaushik et al., 1995; Sanz et al., 1994; Refstie et al., 2000; Morris et al., 2005).

We did not observe any significant difference in FCR, PER, PPV and ER among the groups. Our results are in contrast with those obtained in earlier studies wherein SBM has been evaluated in trout diets and diets of several other species by many researchers (Refstie et al., 2000; Refstie et al., 2006; Opstvedt et al., 2003). They observed slightly reduced nutrient utilization (PER and PPV) when 50 - 75% of FM protein was replaced by SBM. These differences could due to the inherent differences between the nature of the two plant protein sources, soyabean meal and detoxified Jatropha kernel meal.

Biochemical composition of whole body of fish

In the present study the decrease in protein content in the whole body of fish fed control diet was associated with increase in whole body lipid content. Moisture content exhibited inverse relationship with crude lipid in the whole body of rainbow trout. Highest lipid concentration was observed in J_{50}

group, which was similar to that in control group, and lowest deposition was observed in $J_{62.5}$ group. Similarly, de Francesco et al., 2004 observed higher lipid content in rainbow trout fed plant protein. There is evidence that replacement of FM by plant protein sources such as corn gluten meal and soy protein concentrates affects increaes hepatic lipogenic enzyme activities in seabass (Dias, 1999; Kaushik et al., 2004) that leads to higher whole body lipid. In salmonids, increases found in whole body fat content with the use of dietary plant proteins, were explained by imbalances in amino acid concentrations (Kaushik et al., 2004; Bjerkeng et al., 1997). Furthermore, it is suggested that unbalanced amino acid composition influences energy metabolism. Vilhelmsson et al., 2004 found an up-regulation of several proteins involved in energy metabolism in rainbow trout liver when fed plant (maize gluten meal, wheat gluten, extruded whole heat, extruded peas and rapeseed meal) protein and concluded that the plant protein increase the energy demands of fish. Possible reason could be higher supply of some of the dispensable amino acids such as glutamic acid in excess by the plant protein fed diets that could have lead to higher lipid retention (Barrows et al., 2008). These authors also indicate the involvement of possible metabolic or endocrine mechanisms in eliciting such differences in whole body lipid deposition. It may be noted that in our study as well, glutamic acid concentration in DJKM based diets was higher.

Efficient protein synthesis requires sufficient availability of all essential amino acids (Dabrowski and Guderly 2002). Unbalanced amino acid concentrations in a diet resulted in increased protein degradation (Langar et al., 1993; von der Decken and Lied, 1993), and thereby increased protein turnover (Martin et al., 2003). Many researchers (Refstie et al., 2000; Pack et al., 1995; Cheng et al., 2003) reported that the plant protein (SBM) based diets lower nitrogen retention in salmon and trout because these diets have less digestible energy and an amino acid profile that is suboptimal for muscle growth. Interestingly in our study crude protein content in whole body was higher in DJKM fed groups. Similarly Barrows et al., 2008 and Cheng et al., 2003 also found that the body protein content increased significantly when SBM replaced FM in trout diet. This indicates that DJKM contain optimum digestible energy and balanced amino acid profile optimal for trout growth.

DJKM based diets (J_{50} and $J_{62.5}$) were supplemented with phytase and that could have released minerals bound to phytate. Consequently, leading to increase in body minerals contents, as observed in J_{50} and $J_{62.5}$ groups. On the other hand, Elangovan and Shim, 2000 and Barrows et al., 2008 observed that SBM containing diets up to (35%) inclusion (without the addition of

phytase) had no effect on body ash content. Phytate content in SBM is approximately three-fold lower than in DJKM and therefore it is not expected to have any significant effect on the ash content.

Similar values of HSI in control and J_{50} groups and these values being higher than that in $J_{62.5}$ group suggest higher lipid deposition in liver of control and J_{50} group. It may be noted that the whole body lipid content followed a similar pattern. HSI values of above 1, as observed here, are common in rainbow trout and European seabass (de Francesco et al., 2004; Dias, 1999; Ballestrazzi et al., 1998) where hepatic fat deposition is very high (Dias, 1999).

Digestibility measurement and efficiency of digestible nutrients and energy

Detoxified Jatropha kernel meal in combination with FM protein showed excellent dry matter, crude protein, lipid and energy digestibilities in the present study. Generally, oil seed meal proteins have digestibilities of 80-95% for fish (Jauncey and Ross, 1982). Dry matter, protein, lipid and energy digestibilities of experimental diets were 73-80%, 84-90%, 90-95% and 82-87% respectively, which indicate excellent utilization of feed ingredients. The protein digestibility is a key factor in the evaluation of the quality of a diet for fish and its potential for the synthesis of new tissues.

The ADCs observed are comparable to values obtained for good quality proteins in fish diets (Cho and Kaushik, 1990). Dry matter digestibilities were above 73% for all diets and these values were higher than those (56-60%) observed by Hilton and Slinger, 1986 and Abdou et al., 1990. Higher ADCs for protein of J_{50} diet than $J_{62.5}$ diet implies that a greater amount of protein would have been available to the fish from the J_{50} diets. While ADCs of proteins was little affected by dietary treatments, inclusion of DJKM at 62.5% replacement of FM protein, decreased dry matter, lipid and energy digestibilities. The low-energy digestibility of the plant (DJKM) based diet ($J_{62.5}$) can be attributed to their NSP content and its poor digestibility by rainbow trout, although the energy digestibility values were higher (82-87%) than those reported by many researchers for fish (Gomes et al., 1993; Gouveia et al., 1993).

In our study higher inclusion of DJKM in rainbow trout diet decreased lipid digestibility. Several studies have concluded that dietary plant protein lower the lipid digestibility in salmonids (Storebakken et al., 1998; Romarheim et al., 2006; Yamamoto et al., 2007). The lower lipid digestibility of the fish fed the $J_{62.5}$ diet may be associated with the increase in the NSP content, which reduces fat absorption by disturbing micelle

formation in the gastro intestinal tract; and another reason could be that NSP entrap bile salts, thereby reduce their effectiveness in solubilizing fats (Krogdahl et al., 2003; Gatlin et al., 2007; Øverland et al., 2009).

Interestingly it was found that J_{50} diet had similar ADC of dry matter, protein and lipid and apparent energy digestibility as for control diet. These results demonstrate that the rainbow trout were efficient in digesting protein, lipid and energy from the DJKM meal at 50% replacement level, while fish fed at higher level (62.5% replacement of FM) in general exhibited lower digestibilities. Crude protein digestibilities of DJKM diets were high (above 84%) in rainbow trout, suggesting DJKM to be an excellent protein source for this species.

Efficiency values of digestible nutrients and energy indicate retained nutrients and energy in whole body relative to the total digestible nutrients and digestible energy. Efficiency of digestible nutrients and energy of diets did not differ significantly among the three groups, indicating that rainbow trout has utilized DJKM well and retained nutrients in the body maximally and similar to control group. The value for the efficiency of digestible protein, lipid and energy were in the range of 25-30%, 31-34% and 26-29% respectively.

Digestive enzyme activities and relative intestinal length (RIL)

Heat labile antinutrients such as trypsin inhibitors and lectins were not detected in the autoclaved DJKM, whereas heat stable antinutrient (phytate) was present in the DJKM. Phytate is known to inhibit activities of digestive enzymes such as pepsin, trypsin and alpha-amylase (Robaina et al., 1995; Alarcon et al., 1999), or to form complexes with minerals (Teskeredzic et al., Sugiura et al., 1999) and proteins (Moyano et al., 1999), thereby modifying digestion processes and impairing intestinal absorption. In our study digestive enzymes activities decreased on inclusion of DJKM in the rainbow trout diet. Decrease in digestive enzyme (amylase, protease and lipase) activities in trout intestine might be because of phytate present in the DJKM based diets. For plant based feeds, phytase has been added in the feed at a level of 500 FTU per kg (Forster et al., 1999; Cheng et al., 2004). We used 500 FTU phytase per kg feed, which might not be sufficient because of the high phytate content in DJKM. For any new feed resource such as Jatropha kernel meal separate systematic studies on optimization of phytase level in the diet need to be undertaken. In our laboratory, work is in progress to optimize phytase level in the DJKM based diets for trout, common carp (*Cyprinus carpio* L.) and tilapia (*Oreochromis niloticus*).

Lower protease activities corresponded to decrease in protein availability from DJKM and the presence of unutilised phytate, as DJKM level increased in rainbow trout diets. Similar results were observed by Santigosa et al., 2008, Sandholm et al., 1976 and Krogdahl et al., 1994. They found that protein digesting enzyme (for example trypsin) activity decreases as plant protein inclusion increases in trout diet, and they concluded that trypsin is highly sensitive to plant antinutrients. Reduced nutrient digestibility and morphological changes in the distal intestinal mucosa leading to digestive and absorptive dysfunctions, with digestive enzyme activities decreasing in a dose-dependent manner with increasing SBM inclusion levels in Atlantic salmon (Krogdahl et al., 2003) have been reported. The lower activity of digestive enzymes in DJKM fed groups was correlated with lower nutrient digestibility.

It is known that carnivorous and omnivorous fish require longer time to digest plant protein based diets compared to animal protein based diets (Buddington et al., 1997). Direct relationship between the amount of dietary plant protein and RIL has been reported earlier in fish (Kramer and Bryant, 1995). In rainbow trout, DJKM based diets exhibited higher RIL than the control group. RIL value increases as the plant protein inclusion increases in the trout diets (Øverland et al., 2009). From a physiological view point, a longer RIL would facilitate an increase in digestibility and retention time by enhancing contact time of the digestive enzymes and the feed components, resulting in increase in their digestion and absorption. Carnivorous fish like rainbow trout species showed compensation mechanisms, such as an increase in RIL and as a result increase in digestive activity, to achieve a digestive balance and growth rates similar to those observed for FM fed group.

Cholesterol and triglycerides; and blood glucose level

Dietary inclusion of DJKM in the trout reduced cholesterol level in plasma and muscle as compared to control group. The decrease in plasma cholesterol levels in fish fed diets with plant proteins has already been reported in rainbow trout (Kaushik et al., 1995; Romarheim et al., 2006;Yamamoto et al., 2007). In terrestrial animals, plant products are generally considered to have a hypocholesteromic effect (De Schrijver, 1990), mainly due to the relatively high levels of estrogeno-mimetic isoflavones (Setchell and Cassidy, 1999). In humans, different plant constituents have been reported to lower plasma cholesterol levels (Wester, 2000). Although cholesterol metabolism in mammals and fish could differ, the fish

hypocholesterolemia in response to dietary plant protein supply could be due to an increased excretion of bile salts, an inhibition of cholesterol intestinal absorption, or just the withdrawal of FM rather than to the direct effects of plant protein (Kaushik et al., 2004). In any case, the significance of hypocholesterolemia in fish should be studied in depth. Plasma triglycerides increased in concentrations with increased dietary DJKM level. The increased in whole body lipid content in J_{50} group along with the increased in plasma triglyceride concentrations. Whereas Shimeno et al., 1993 observed opposite trend in the yellowtail (*Seriola dumerilii*).

Blood glucose level was affected by dietary treatments. Lower blood glucose level was observed in DJKM fed groups than control group. The presence of higher starch content in control group and higher intestinal amylase activity in this group explain higher blood glucose level recorded. Detoxified Jatropha kernel meal based diets contain higher amount of NSP. Usually NSP in monogastric animals can delay intestinal absorption of glucose, possibly through a reduced rate of gastric emptying, leading to delayed absorption (Knudsen, 2001). Opposite trends were shown in fish fed diets containing SBM and corn gluten as a substitute (Kikuchi et al., 1994; Kikuchi, 1999), but Glencross et al., 2004 observed that dietary inclusion of yellow lupin in trout diet did not affect blood glucose level.

Blood chemistry

Red blood cells and WBC counts; and Hct and Hb level did not differ significantly among the groups and their ranges were in the normal range reported by Blaxhall and Daisley, 1973 for healthy trout. A corresponding similar Hb and Hct content accompanied the similar number of red blood cells. Consequently, MCV, MCH and MCHC value was not changed. MCV did not differ significantly among the groups because RBC counts and Hct values were not different among the groups. Whereas in another study (Hemre et al., 2005) significant reduction of MCV on increase in the content of SBM in salmon diet was observed. As reduction of MCV appeared to coincide with increased spleen size (Hemre et al., 2005), it was suggested that some of the plant ingredients might cause early release of immature erythrocytes. In our study spleen size increased as the plant protein increased in the diet.

The hematocrit assay is normally used as a general indicator of fish health (NRC, 1981). Hematocrit level in all groups was within the normal range and did not differ significantly among the groups (Blaxhall and Daisley, 1973; Sun et al., 1995). In another study Soltan et al., 2008 observed

that FM protein replaced by mixture of plant proteins in Nile tilapia diets that lead to the lower hematocrit levels could be attributed to the binding of phytate to minerals (iron) and/or amine group of amino acids causing their low availabilities in the body and increase in erythrocyte fragility.

Blood protein and lysozyme activity in serum

Total blood protein content is taken as an index of nutritional status (Martinez 1976). Among the blood protein, albumin and globulin are the major proteins, which play a significant role in the immune response. Total blood protein concentration in fish fed J_{50} diet, was significantly elevated over that of the control group, suggesting an immunostimulating effect of DJKM at low inclusion level in rainbow trout juveniles. The blood protein content values are similar to those found for rainbow trout (Martinez, 1976).

Lysozyme plays an important role in nonspecific immune response and it is found in mucus, serum and ova of fish. Innate immunity due to lysozyme is caused by lysis of bacterial cell wall and this stimulates the phagocytosis of bacteria. The suppression of the non-specific immune capacity by high concentrations of dietary soybean proteins has been reported in rainbow trout (Burrells et al., 1999). However, other reports wherein SBM was fed to rainbow trout (Rumsey et al., 1994) and Atlantic salmon (Krogdahl et al., 2000) or alginate was fed to Atlantic salmon (Gabrielsen and Austreng, 1998), increased values of different non-specific immune mechanisms, which have been interpreted as immunostimulating effects. In the present study lysozyme activity was statistically not different amongst the groups but numerically higher lysozyme activity was observed in plant protein fed groups (J_{50} and $J_{62.5}$), which might be due to immunostimulating effect of detoxified Jatropha kernel meal in rainbow trout.

Metabolic enzymes

Alkaline phosphatase and ALT are released into blood during organ damage (Racicot et al., 1975). Thus, detection of high levels of ALP and ALT in blood gives information on the damage of organs and in particular of liver cells. Levels of ALP and ALT were similar in all the diets, indicating normal organ function on feeding of DJKM. Hemre et al., 2005 and Sanden et al., 2006 also reported similar results on feeding SBM containing diets to Atlantic salmon.

Blood ions

Blood urea nitrogen levels are associated with liver or gill dysfunction (Stoskopf, 1993) and in our study these levels were in the normal ranges (Witters, 1986; Wedemeyer, 1996). Furthermore, these values did not differ significantly among the groups. These results show that DJKM fed groups were normal and healthy.

TBIL, an indicator of liver dysfunction (Tietz, 1986) was similar for all groups. Creatinine was highest in control group. Creatinine is a metabolite of animal protein and its highest level in control is due to highest content of FM in the diet. Creatinine level reflects kidney dysfunction, and its level in all the experimental group were in the normal range. Detoxified Jatropha kernel meal based diets were supplemented with phytase and higher phosphorus and other ions in blood observed in DJKM fed fish could be due to increased release of phosphorus, sodium and calcium from feed and making them available for trout.

Conclusions

Rainbow trout can efficiently use DJKM as a source of protein. DJKM could replace 50% FM protein in rainbow trout diets, without sacrificing growth and nutrient utilization; and without affecting physiological and haematological parameters. The results of this study enlarge the portfolio of plant protein sources that can be used in fish diets, and open a new market opportunity for use of a new feed resource in the feed industry.

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Chapter 6:

Section 2

Effects of detoxified *Jatropha curcas* kernel meal on the histology of rainbow trout (*Oncorhynchus mykiss*) juveniles

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This study is in continuation of the previous study (Chapter 6, *section 1*).

Materials methods:

Experimental set up and feeding formulation: Described in chapter 6 (*section1*).

Histopathological studies:

Immediately after killing the fish a total of 15 histological samples of different segments of the intestinal tract, stomach and liver were fixed in Bouin´s fluid or in methanol/acetic acid for 48 h or 24 h respectively. After dehydration and embedding in paraffin, serial sections of 5 µm thickness

were prepared and processed for conventional histopathological studies. Haematoxylin and eosin (H&E) was used for staining the tissue sections.

Results and discussion:

Serial sections of the gut (Figure 1), at level 1 include: the liver (L), the cranial part of the stomach (S), and pyloric appendices of the mid gut (P) and hindgut (E). Level 2 includes the stomach, the loop formation of the pyloric appendices and the hindgut. At level 3, can be seen the caudal flexure of the stomach, a few distal parts of the pyloric appendices and the most distal part of the hindgut. The stomach of all investigated samples in rainbow trout appears as a tube of slightly varying diameter. The luminal folds have different shapes and the thickness of the muscle layers varies depending on the levels at which the sections were cut. The gastric glands are well developed and the epithelium lining the luminal surface that consists of highly columnar cells that produce protective mucous is not altered. The same is true for the branched tubular glands at the bottom which are also well developed (Fig. 2A and 2B). The shape and cellular morphology of pepsin and hydrochloric acid producing cell-types (oxyntopeptidic cells) remain unchanged and no signs of inflammation or increased leucocyte immigration are found in the DJKM fed groups. The development of intestinal loops, pyloric appendices and the terminal intestine (hind gut) remains largely unchanged (Fig. 3A) and the villi of the appendices or terminal intestine are well constructed. The surface epithelium layer is composed of unchanged, slender, highly columnar enterocytes (Fig. 3B). No pathological alterations are seen in the hindgut or terminal gut segment. Regarding liver and pancreatic morphology, the typical arrangement of parenchymal cells in both glands (dissiminated pancreas) remains unchanged. No signs of hepatic steatosis or lipidosis were found in rainbow trout in detoxified Jatropha kernel meal fed group (Fig 4).
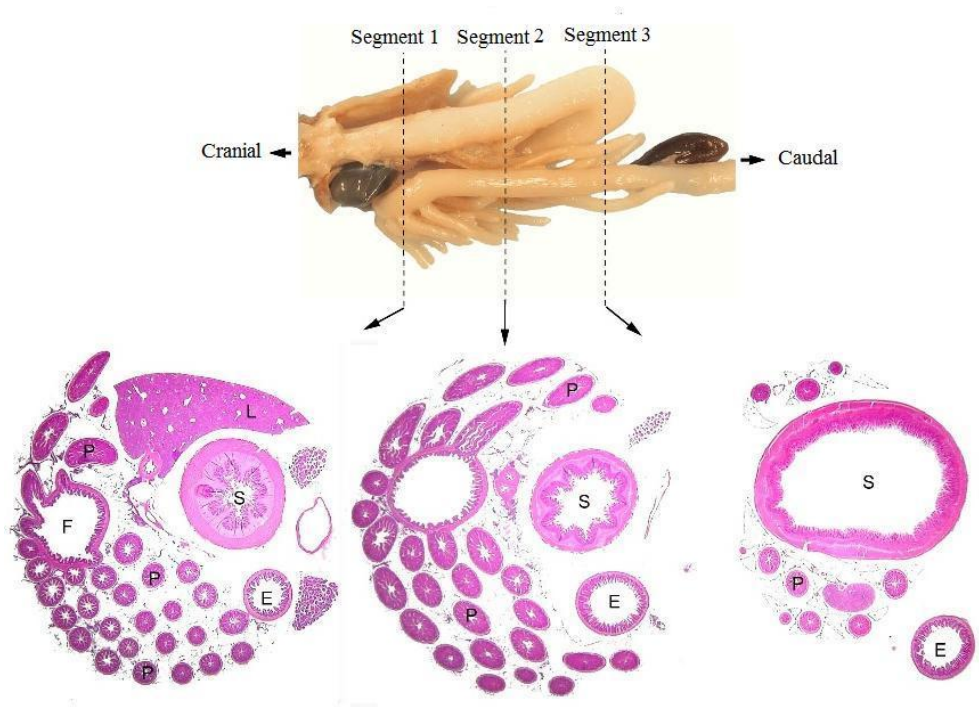
Fig. 1: On top: different section-planes through the digestive tract of the rainbow trout. Below: sections through segment 1, 2 and 3 consisting of: liver (L), stomach (S), foregut (F) with pyloric appendices (P), hind gut/rectum (E).
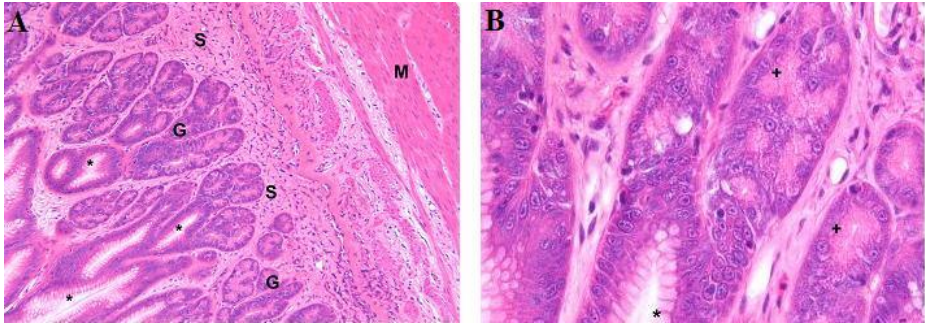
Fig. 2: (A) Trout stomach showing well developed gastric glands (G), submucosa (S) and the tunica muscularis (M) of $J_{62.5}$ group. **(B)** Higher magnification of branched tubular gastric glands. Shape and cellular morphology of mucous (*) and pepsin and hydrochloric acid producing cell-type (+) (oxyntopeptidic cells) of $J_{62.5}$ group did not show any signs of pathological alterations compared with control group.

Fig. 3: (A) Cross section through pyloric appendices (P), disseminated pancreatic cells (arrows) and fat tissue (F) of $J_{62.5}$ group. **(B)** Detail of the figure 3A showing several villus tips and enterocytes. Typical and unchanged appearance of absorbing cells provided with a well developed brush border lining the cell surface and also visible single penetrating lymphocytes (*) and apoptotic enterocytes (arrows) in $J_{62.5}$ group.
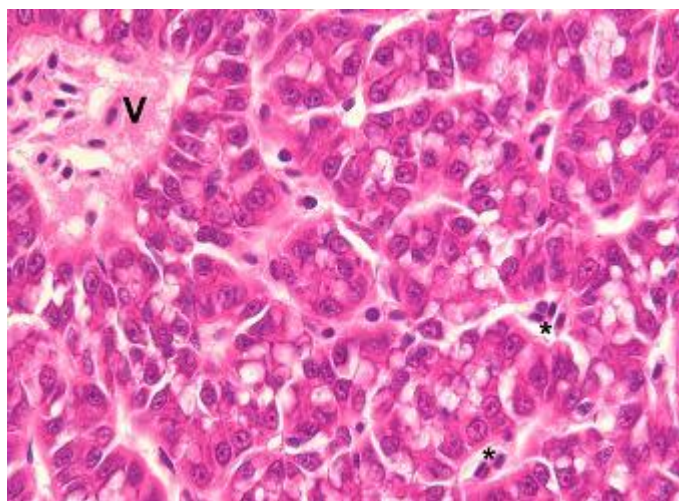
Fig. 4: The liver of $J_{62.5}$ group did not show any pathological alterations or signs of steatosis (hepatic lipidosis). Cords of hepatocytes separated by sinusoids containing erythrocytes.

Chapter 7

Substitution of fish meal by *Jatropha curcas* kernel meal: Effects on growth performance and body composition of white leg shrimp (*Litopenaeus vannamei*)

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Substitution of fish meal by *Jatropha curcas* kernel meal: Effects on growth performance and body composition of white leg shrimp (*Litopenaeus vannamei*)

Till Harter, Frederik Buhrke, Vikas Kumar, Ulfert Focken, Harinder P. S. Makkar, Klaus Becker

Abstract

Jatropha curcas (L.) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. Its seeds are rich in oil and protein (contains: 60 – 66% crude protein similar to fish meal). It is being promoted as a biofuel plant. Jatropha kernel meal obtained after oil extraction is an excellent source of protein. However, presence of toxic and antinutritional constituents restricts its use in fish and shrimp feed. An eight week experiment was conducted to evaluate the nutritional quality of the detoxified Jatropha kernel meal (DJKM) in white leg shrimp (*Litopenaeus vannamei*). Shrimp (60) with an initial average body weight of 4.46 ± 0.64 g were randomly distributed into three treatments with four replicates and fed iso-nitrogenous and iso-energetic diets (crude protein 35%, crude lipid 9%): Control (fish meal based protein), JC_{25} and JC_{50} (25% and 50% of fish meal protein replaced by DJKM). Higher ($P < 0.05$) body mass gain, specific growth rate and metabolic growth rate were observed in DJKM fed groups than in Control group. However, lower ($P > 0.05$) feed conversion ratio was observed in DJKM fed groups, while protein efficiency ratio exhibited an opposite trend. Protein, ash and gross energy content of the whole shrimp body were higher ($P < 0.05$) in DJKM fed groups compared to Control group. Moisture and lipid content of the whole shrimp body did not differ significantly among the three groups. Cholesterol level in plasma was highest ($P < 0.05$) in the Control group, followed by JC_{25} and JC_{50} groups; all being significantly different. In conclusion, DJKM is a promising fish meal replacer in shrimp diets.

KEY WORDS: White leg shrimp, *Jatropha curcas*, growth, nutrient utilization, fish meal replacer

Introduction

The current trend to intensify shrimp farming is making imperative the use of alternative protein sources in their diets. As captured fish production has remained stagnant for the last few decades and aquaculture sector has had phenomenal growth rate which is projected to continue, the fish meal (FM) demand is expected to outstrip the world's supply by 2050 (Halweil, 2008). These developments have led to rising and fluctuating FM prices. There is a need to investigate the possibility of using other more economical alternative protein sources with a nutritive value comparable to that of FM.

In quest for indigenous sources of renewable liquid fuels, *Jatropha curcas* (Physic nut) has received increasing interest since the beginning of the 21st century (Ye et al., 2009). Its seeds are rich in oil (25-35%) that can be used as biofuel (Makkar et al., 2008). The use of Jatropha seed meal was impeded so far, because of its toxicity, which is mainly ascribed to the presence of phorbol esters (PEs). The meal also contains antinutritive factors such as trypsin inhibitors, lectin and phytate (Makkar and Becker, 2009). The crude protein content in the detoxified kernel meal is approximately 60 - 66 % and the protein has a good amino acid balance. The levels of essential amino acids (except lysine) are higher in Jatropha kernel meal than soybean meal (Kumar et al., 2010a,b). Recently a process to detoxify *J. curcas* kernel meal has been developed which makes it suitable for use as animal feeds (Makkar and Becker, 2010). This new development offers an array of opportunities to use the detoxified kernel meal in animal nutrition, including the substitution of FM in shrimp feeds.

Our previous study (Kumar et al., 2010a,b,c,d) has shown that detoxified Jatropha kernel meal (DJKM) is a good protein source for common carp and trout diets. The objective of the present study was to investigate the potential of the detoxified meal to substitute FM in shrimp diets. Growth rate and nutrient utilization in shrimp fed a diet replacing 25 and 50 % FM protein by the detoxified kernel meal have been reported.

Material and methods

Jatropha seeds were purchased from India and deshelled and defatted in Germany. Organic solvents were used to detoxify defatted Jatropha kernel meal (patent application has been filed for the process of detoxification, Makkar and Becker, 2010). After removal of PEs, the meal was autoclaved (121 °C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitors

and lectin.

Three diets were formulated to contain 35% crude protein and 9% lipid. The inclusion levels of the DJKM were as follows: Control diet was prepared with FM as protein source, without any DJKM; and for JC_{25} and JC_{50} diets 25% and 50% of FM protein was replaced by DJKM respectively. The final mixture of each diet was made into 3 mm diameter moist pellets (using a Bosch, Type UM60ST 2-M, Robert Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then dried in an oven at 40 °C overnight. Compositions of the experimental diets are listed in Table 1.

Table 1 Composition of the experimental diets (g kg^{-1} feed) for white leg shrimp (*Litopenaeus vannamei*)

Ingredients	Experimental diets		
	Control	JC_{25}	JC_{50}
[1]Fish meal	492.3	369.2	246.2
[2]Wheat starch	312.5	292.0	271.5
Jatropha meal	—	124.6	249.2
[3]Dextrose	50	50	50
[4]Cellulose	69.3	74.2	79.1
[5]Sunflower oil	20.9	20.2	19.4
[6]Fish oil	—	14.8	29.5
[7]*Vitamin premix	20	20	20
[8]**Mineral premix	20	20	20
Cholesterol	5.0	5.0	5.0
Soy lecithin	10	10	10
Total	1000	1000	1000

[1]Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany.

[2]Cerestar, Deutchland GmbH, Zülpich, Germany.

[3]Sigma-Aldrich Chemie, Batch (104K0016), GmbH, Steinheim, Germany.

[4]Lot 109H0196, Batch (104K0016), Sigma-Aldrich Chemie, GmbH, Steinheim, Germany.

[5]Thomy, Nestle Deutschland AG, 41415 Neuss; Reg. Trademark of Societe des Produits Nestle S.A. Germany.

[6]Menhaden, batch # 102k0126, Sigma-Aldrich, Chemie GmbH Steinheim, Germany.

[7 & 8] Altromin Spezialfutter GmbH & Co. KG, Lage, Germany.

*Vitamin premix (g or IU kg^{-1} premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10; cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

**Mineral premix (g kg^{-1}): $CaCO_3$, 336; KH_2PO_4, 50₂; $MgSO_4. 7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4. H_2O$, 3.12; $ZnSO_4. 7H_2O$, 4.67; $CuSO_4. 5H_2O$, 0.62; KI, 0.16; $CoCl_2. 6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

L. vannamei juveniles (2 – 4 g) were obtained from the northern German aqua farm EAP (Ecological Aquaculture Production AG) in

Strande/Kiel Germany. A total of 60 shrimp juveniles were randomly distributed into three groups with four replicates; each replicate contained five shrimp (av. wt. 4.46 ± 0.65 g) per aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 – 27.5°C, salinity 16 – 19‰, pH 7.0 – 7.5, dissolved oxygen 6.9 – 7.4 mg l^{-1}, total NH$_3$ 0.1– 0.2 mg l^{-1}, nitrite 0.07 – 0.1 mg l^{-1} and nitrate 1–3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before the start of the experiment, the shrimp were fasted. During the experimental period shrimp were fed diets at 5% of animal biomass per day and feeding frequency was set to five times per day. Two of the feedings were set at night time, to better suit the animal's natural feeding behaviour.

Survival was calculated by using the following formula:

Survival (%) = (Number of shrimp at the end of experiment/Number of shrimps stocked initial)*100

The experiment was terminated after eight weeks and the shrimps were killed. At the end of the experiment, shrimps were anaesthetized by tricaine methanesulfonate (MS222) at 250 ppm in water. Haemolymph was drawn from two shrimps from each group and transferred into a tube, which was centrifuged at 1500×g for 5 min at room temperature (24 ^{o}C) to obtain plasma, which was then stored at −20 ^{o}C for determination of cholesterol. Shrimp were stored at −20 ^{o}C for chemical composition analysis. Prior to determination of the proximate composition, the shrimp were autoclaved at 121 ^{o}C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and then freeze-dried.

Phorbol esters were determined according to Makkar et al. (2007), which was based on the method of Makkar et al. (1997). The results were expressed as equivalents to the standard, phorbol-12-myristate 13-acetate. Detection limit of PEs was 3 µg/g meal. Trypsin inhibitor activity was determined essentially according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). The haemagglutination activity was expressed as the minimum amount of material (mg mL^{-1} assay medium) that produced agglutination. The minimum amount was the amount of material mL^{-1} assay medium in the highest dilution that was positive for agglutination; the lower this value, the higher the lectin activity. Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Results were

expressed as g phytic acid per 100 g material using phytic acid sodium salt (Sigma, St Louis, MO, USA) as standard. Non-starch polysaccharides (NSP) were estimated according to Englyst et al. (1994). Amino acid composition of FM and DJKM was determined using an automated amino acid analyser after hydrolysing the samples with 6 M HCl at 110 °C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. Tryptophan content of the above-mentioned samples was determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

The proximate composition of diet ingredients, diets and whole body of shrimp was determined using the standard methods of the AOAC (1990). Samples were analyzed for dry matter, ash, crude protein and lipid (ether soluble lipid). Gross energy of diet ingredients, diets and shrimp bodies was determined with a bomb calorimeter (IKA C7000) using benzoic acid as a standard.

The determination of the plasma cholesterol was using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889). The color intensity was determined photometrically and was directly proportional to the concentration of cholesterol in the plasma sample.

Feed intake: Feed offered was used as feed intake since no leftovers were observed. The leaching losses were considered as negligible.

Growth performance and nutrient utilization were assessed in terms of body mass gain (BMG), specific growth rate (SGR, % day^{-1}), metabolic growth rate (MGR, g kg$^{0.8}$ day^{-1}), feed conversion ratio (FCR) and protein efficiency ratio (PER).

BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100; SGR (% day^{-1}) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR (g kg$^{0.8}$ day^{-1}) = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{\wedge 0.8}$ + (final body mass in g / 1000)$^{\wedge 0.8}$}/2] / number of trial days; FCR = dry feed fed (g)/body mass gain (g) and PER = body mass gain (g)/crude protein fed (g).

Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Tukey HSD test (P < 0.05). The software used was Statistica 7.0 (StatSoft, Inc., 2300 E. 14th St., Tulsa, Oklahoma, 74104, USA). Values are expressed as means ± standard deviation.

Results and discussion

Toxic compound (phorbol esters), antinutrients, proximate and amino acid composition

Phorbol esters content in untreated defatted Jatropha kernel meal was 1.8 mg g^{-1} whereas, PEs in DJKM were undetectable. Trypsin inhibitors and lectins were also not detected in DJKM; whereas phytate and NSP levels in DJKM were 9.5% and 16% respectively (Table 2).

Table 2 Proximate composition and amino acid composition of feed ingredients

	Fish meal	Jatropha meal
Proximate composition (g kg^{-1})		
Dry matter	940	945
Crude protein	650	665
Crude lipid	88	11.4
Crude ash	142	82
Gross energy (KJ/g)	21.1	18.3
Antinutrients		
Trypsin inhibitor (mg trypsin inhibited per g sample)	–	ND
Lectin[a]	–	ND
Phytate (% dry matter)	–	9.5
Essential amino acids composition (g kg^{-1})		
Arginine	35.3	69.7
Histidine	17.7	21.7
Iso leucine	22.8	26.7
Leucine	41.6	46.7
Lysine	40.9	23.3
Phenylalanine	21.8	30.4
Methionine	16	10.6
Threonine	23	22
Tryptophan	4.9	7.1
Valine	29.3	31.6
Non-essential amino acids composition (g kg^{-1})		
Alanine	43.3	29.4
Asparginine	60.5	68.7

Table 2 continued

Cystine	4.3	2.3
Glycine	59.8	31.5
Glutamine	79.4	112.1
Proline	36.9	32.2
Serine	25.5	30.6
Tyrosine	14.8	18.8
Non-starch polysaccharides (NSP) (g kg $^{-1}$)		
Rhamnose	-	3
Fucose	-	1
arabinose	-	31
Xylose	-	20
Mannose	-	5
Galactose	-	14
Glucose	-	57
Glucuronic acid	-	0
Galacturonic acid	-	30
Total-NSP	-	160

ND: Not detected

[a]Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 2 and 3. Diets contained about 35% crude protein, 9.5 – 9.9% crude lipids and 19.0 kJ/g gross energy and were isonitrogenous, isolipidic and isoenergetic. Ash content was in the range of 11.3 –14.2%. All experimental diets had an almost similar essential amino acid composition except lysine which was slightly lower in DJKM based diets compared to control diet.

Shrimp behaviour, feed intake and survival, growth performance and nutrient utilization

Based on visual observation during feeding time, acceptability of feed was good and the behaviour of shrimp normal throughout the experiment. No left over feed was observed in the aquaria. In the present study survival rate were 100%. There was no mortality during the entire experimental period. This implies that environmental conditions were well suited for *L. vannamei*. Furthermore, it can be assumed, that there were no serious nutritional deficiencies or incompatibilities generated by any of the offered experimental diets.

Weekly body mass gains of white leg shrimp are given in Figure 1. These indicate that from the second week on there was differential

growth among the groups and lower body mass development was observed in the control group than in other groups. This trend was maintained till the end of the experiment.

**Table 3** Proximate composition and amino acid composition of the experimental diets (g kg^{-1} feed)

	Control	JC$_{25}$	JC$_{50}$
Proximate composition (g kg $^{-1}$)			
Dry matter	955	961	962
Crude protein	356	353	346
Crude lipid	95	96	99
Crude ash	142	127	113
Gross energy (KJ/g)	19.3	19.3	19.5
Crude fibre	35	59	78
Acid detergent fibre	41	64	83
Essential amino acids composition (g kg $^{-1}$)			
Arginine	17.4	21.7	26.1
Histidine	8.7	9.2	9.8
Iso leucine	11.2	11.7	12.3
Leucine	20.5	21.2	21.9
Lysine	20.1	18.0	15.9
Phenylalanine	10.7	11.8	12.9
Methionine	7.9	7.2	6.6
Threonine	11.3	11.2	11.1
Tryptophan	2.4	2.7	3.0
Valine	14.4	14.8	15.1
Non-essential amino acids composition (g kg $^{-1}$)			
Alanine	21.3	19.6	18.0
Asparginine	29.8	30.9	32.0
Cystine	2.1	1.9	1.6
Glycine	29.4	26.0	22.6
Glutamine	39.1	43.3	47.5
Proline	18.2	17.6	17.1
Serine	12.6	13.2	13.9
Tyrosine	7.3	7.8	8.3

Amino acid compositions of the experimental diets were calculated from the amino acid profile of individual feed ingredients

Growth performances and nutrient utilization parameters were

affected by dietary treatments (Table 4). Our previous studies indicate that 50% FM protein replaced by DJKM exhibited similar growth performance and nutrient utilization to FM fed group in common carp and rainbow trout (Kumar et al., 2010a, b, c, d). Interestingly in the present study growth performance and nutrient utilization of shrimp groups fed DJKM were better than that of FM fed group. A number of researchers (Colvin and Brand, 1977; Davis and Arnold, 2000; Fox et al., 2004; Gonzalez-Rodriguez and Abdo de la Parra, 2004; Amaya et al., 2007) reported that FM protein could be replaced to the extent of 40–80% by plant protein (soybean meal, corn gluten meal, corn fermented solubles and cottonseed meal) in shrimp diets; and higher inclusion (more than 80% replacement of fish meal) of plant protein in shrimp diets leads to lower growth because of lower feed intake. Paripatananont et al. (2001) reported that soy protein concentrate can replace 50% FM protein without hindering the growth performance and nutrient utilization. Suárez et al. (2009) reported that FM substitutions as high as 80% for a mixture of soybean meal and canola meal and proves, that diets based mainly on plant protein are feasible for *L. vannamei*. In the present study the higher growth response of DJKM fed groups could be due to higher protein availability from the DJKM than FM, which enhances the feed utilization. There is a possibility of synergistic effects between the used feed ingredients (FM and DJKM); both were complementary to each other in their amino acid composition. Therefore, DJKM protein in combination with FM protein probably induces excellent nutrient and energy digestibility and lead to higher growth performance and nutrient utilizations in shrimp. Amino acid composition of the diets tested indicates that the requirements of shrimp (Akiyama and Tan, 1991; Van Wyk, 1999) for these nutrients were met in all the dietary treatments. Over all, shrimp can efficiently use DJKM as a source of protein. Detoxified Jatropha kernel meal could replace 50% FM protein in shrimp diets, without sacrificing growth and nutrient utilization parameters. Results from this study provide important information regarding the potential of white leg shrimp (*L. vannamei*) to utilize alternative feed formulations with low levels of animal protein under experimental conditions.
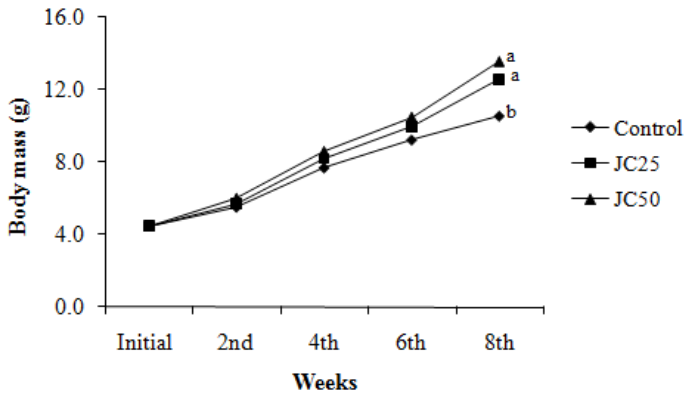
Figure 1 Body mass gain of white leg shrimp (*Litopenaeus vannamei*) fed experimental diets for eight weeks

Chemical composition of whole shrimp body and cholesterol level in plasma

Whole body chemical composition is shown in Table 5. Moisture contents of whole shrimp body did not differ significantly among the three groups. A similar trend has been observed by Paripatananont et al. (2001). In their study, tiger shrimp (*Penaeus monodon*) fed with soy protein concentrate substituting 25–100% FM protein did not differ significantly in moisture content of whole shrimp body. In the same study they observed that soy protein concentrate inclusion in shrimp diets lead to higher crude lipid and gross energy content in the whole body than for FM protein based diets. In the present study, crude lipid content of the whole shrimp body did not differ significantly among the three groups whereas highest gross energy content of the whole shrimp body was observed in the Control group, followed by JC_{25} and JC_{50} groups; all being significantly different. Higher gross energy content of whole body of shrimp in fish meal fed group (control) compared to DJKM fed groups appears to be due to significantly higher (P < 0.05) protein content. Detoxified Jatropha kernel meal based diets (JC_{25} and JC_{50}) contained bound phytate and most of the minerals bound to phytate. Consequently, lower availability of minerals in the body that leads to lower ash contents, as observed in JC_{25} and JC_{50} groups. On the other hand, Paripatananont et al. (2001) observed that soy protein concentrate substituting 25–100% FM protein did not differ significantly in ash content of the whole shrimp body.

Cholesterol is a vital component of cell membranes and is the precursor of bile acids, steroids, and molting hormones. It is reported to be an essential nutrient for growth and survival of all crustacean

species (Kanazawa et al., 1971). Experimental diets were supplemented with cholesterol, because shrimp, like other crustaceans, cannot synthesize cholesterol de novo (Teshima and Kanazawa, 1971). Previous researches have demonstrated that cholesterol supplementation in diets improves biological performance of prawns (*P. japonicus*; Teshima and Kanazawa, 1986), tiger shrimp (*P. monodon*; Sheen et al., 1994), and Pacific white shrimp (*L. vannamei*; Duerr and Walsh, 1996). Cholesterol levels in plasma of shrimp were affected by dietary treatments and lower (P > 0.05) cholesterol level was observed in JC_{25} and JC_{50} group (Figure 2). Detoxified Jatropha kernel meal reduced cholesterol level in plasma compared to the control group. The decrease in plasma cholesterol concentrations in shrimp fed diets with plant protein is in accordance with the results of Cheng and Hardy (2010). They observed that FM protein replaced by SBM reduced the plasma cholesterol in *L. vannamei*. In terrestrial animals, plant products are generally considered to have a hypocholesteromic effect, mainly due to the relatively high levels of estrogeno-mimetic isoflavones (Setchell and Cassidy, 1999). Cholesterol metabolism in animals, fish and shrimp could differ, and the hypocholesterolemia response to dietary plant protein supply in shrimp could be due to increased excretion of bile salts, inhibition of cholesterol intestinal absorption or just to the withdrawal of FM rather than to the direct effects of plant protein.
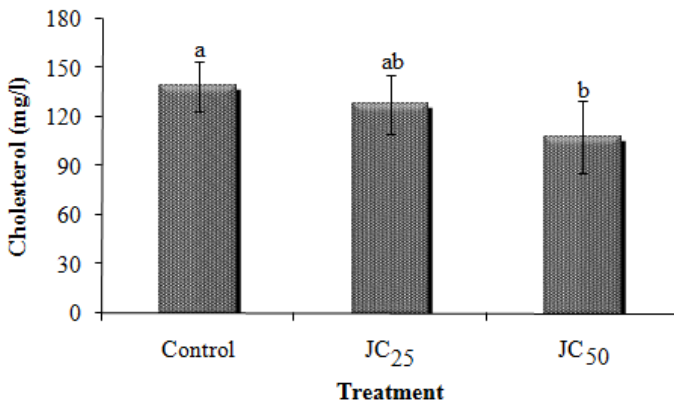
Figure 2 Cholesterol level in plasma of white leg shrimp (*Litopenaeus vannamei*) of different experimental groups

Table 4 Growth performance and nutrient utilization of white leg shrimp (*Litopenaeus vannamei*) fed with experimental diets for eight weeks

Treatment	IBM (g)	FBM (g)	BMG (%)	SGR (%)	MGR (g kg$^{0.8}$ day^{-1})	FCR	PER
Control	4.46 ± 0.60	10.54^b ± 3.17	138^b ± 29.23	1.54^b ± 0.21	5.51^b ± 0.70	3.18^a ± 0.37	1.01 ± 0.11
JC$_{25}$	4.47 ± 0.64	12.59^a ± 3.98	182^a ± 13.53	1.85^a ± 0.09	6.67^a ± 0.38	2.46^b ± 0.28	1.24 ± 0.21
JC$_{50}$	4.45 ± 0.69	13.60^a ± 3.18	209^a ± 40.88	2.00^a ± 0.24	7.22^a ± 0.75	2.28^b ±0.39	1.39 ± 0.23

IBM- Initial body mass, FBM- Final body mass, BMG - Body mass gain, SGR – Specific growth rate, MGR - Metabolic growth rate, FCR – Feed conversion ratio and PER – Protein efficiency ratio

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 5 Chemical composition of whole body of white leg shrimp (*Litopenaeus vannamei*) of different experimental groups (% wet basis weight ± SD)

Treatment	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)
Control	65.5^a ± 8.81	27.05^a ± 0.53	1.70^a ± 0.11	5.64^a ± 0.27	6.75^a ± 0.17
JC$_{25}$	67.2^a ± 3.82	25.62^b ± 0.60	1.77^a ± 0.13	4.92^b ± 0.20	6.26^b ± 0.13
JC$_{50}$	69.3^a ± 5.84	24.01^b ± 0.43	1.69^a ± 0.27	4.30^b ± 0.27	5.99^c ± 0.13

Values are mean (n = 4) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Conclusions

Detoxified Jatropha kernel meal could replace 50% FM protein in shrimp diets, without sacrificing growth and nutrient utilization parameters. Over all growth performance and nutrient utilization in white leg shrimp (*L. vannamei*) for DJKM fed groups were better than in fish meal fed groups indicating that shrimp can efficiently use DJKM and that DJKM is a good quality protein source. The results of this study enlarges the portfolio of plant protein sources that can be used in shrimp feeding, and opens new market opportunities for the use of a new feed resource. Additional studies with DJKM based diets at a larger scale and under commercial pond conditions are suggested.

Acknowledgements

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Chapter 8

Utilization of a byproduct from Jatropha biodiesel industry as a fish meal replacer in common carp *Cyprinus carpio* L. diet

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Utilization of a byproduct from Jatropha biodiesel industry as a fish meal replacer in common carp *Cyprinus carpio* L. diet

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Abstract

Jatropha curcas seeds are rich in oil and protein. The oil is used for biodiesel production. Jatropha kernel meal obtained after oil extraction is an excellent protein source; however, it is toxic. It was detoxified. Using *Cyprinus carpio* fingerlings, a 16 week experiment was conducted to evaluate the nutritional quality of detoxified Jatropha kernel meal (DJKM). Carp fingerlings (n=36, 22±0.12 g) were randomly distributed into three groups containing four replicates and fed iso-nitrogenous diets (crude protein 38%): $C_{control}$ (fishmeal based protein); J_{50} and $J_{62.5}$ (50% and 62.5% of fish meal protein replaced by DJKM). Growth performance and nutrient utilization parameters did not differ significantly (P > 0.05) among the groups. Nutrient digestibilities, digestive enzyme activities, and cholesterol in plasma were highest in $C_{control}$, followed by in J_{50} and $J_{62.5}$; all being significantly different; whereas, red blood cells count, hematocrit and creatinine in blood exhibited opposite trend. Metabolic enzymes (alkaline phosphatase and alanine transaminase) and other blood parameters did not differ significantly among the three groups and these values were within the normal ranges, suggesting no clinical toxicity. In conclusion, DJKM is a promising fishmeal replacer and it can replace 50% FM protein without compromising on health and growth as well in common carp.

Keywords: Common carp, *Jatropha curcas*, protein source, kernel meal, fish meal replacer

Introduction

In recent decades, global aquaculture has been the fastest growing food production sector. The most common aquaculture products are freshwater, omnivorous fish, most of which come from the Cyprinid family. Common carp is one of the widely cultured species, the production of which substantially contributes to the total aquaculture production. For the past several years, one of the main steps in improving fish feeds has been the search for protein source alternatives to fish meal (FM) and determining their nutritional suitability in diets (Mazurkiewicz, 2009). In the next decade demand for protein ingredients is expected to exceed annual world supply of FM, which will change the economic and nutritional paradigms of aquafeed industry. The challenge facing the aquaculture industry is to identify economically viable and environmental friendly alternatives to FM, and the challenge for the livestock industry is to find alternatives to conventional feed ingredients such as soybean meal (SBM), groundnut cake, rapeseed oil meal, maize and wheat. Studies on FM replacement in carp are sparse compared to other carnivore fishes. The use of oil seeds as a source of protein in formulating the diet of common carp was evaluated by several authors (Hasan et al., 1997; Hossain and Jauncey, 1989; Kumar et al., 2008; Mazurkiewicz, 2009).

The SBM can be substituted for FM in carp feed up to 50-60% (Kumar et al., 2008). The use of such a high percentage of these components is possible due to good nutritional (total protein content, amino acid composition) and palatable properties (Hasan et al., 1997). Presently most of the commercial feeds depend mostly on SBM as a FM replacer. However the over dependence will cause hike in price of SBM and therefore exploration of other inexpensive plant protein sources, particularly those that do not compete with human food is required.

Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. Its seeds have been extensively investigated as a source of oil. The seed kernel contains ~ 60% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel. Defatted kernel meal is an excellent source of nutrients and contains 58 to 62 % crude protein. The content of essential amino acids (except lysine) are higher in *Jatropha* kernel meal (290 g kg^{-1}) than SBM (211 g kg^{-1}) (Makkar and Becker, 2009). However the presences of high concentrations of antinutrients like trypsin inhibitor, lectin and phytate and the major toxic

components phorbol esters (PEs) (Makkar and Becker, 1997) restrict their use in fish feed. Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating. A method for detoxification of Jatropha kernel meal has been developed in our laboratory. It is based on extraction and destruction of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

The International Jatropha Organization has projected that in 2017 there will be around 32.72 million hectares of land cultivated worldwide with Jatropha producing 160 million tons of seeds and 95% of its total production will be concentrated in Asia (mostly in China and India) (Siang, 2009). Jatropha plant can yield up to 4 tons seed per year from one hectare of plantation, which can produce approximately one ton of kernel meal rich in protein. This implies that there is possibility of producing enough Jatropha kernel meal to meet growing aquaculture industry demand. Our previous short term (8-week) study (Kumar et al., 2008; Kumar et al., 2010) has shown that detoxified Jatropha kernel meal (DJKM) is a good protein source and better than SBM for common carp diet. The present paper is the second in a series, focusing on long term effects of DJKM on growth performance, and on biochemical, hematological and histological changes in common carp *Cyprinus carpio* L. The purpose of this long term study was to capture adverse effect, if any, of feeding a diet containing DJKM and to ascertain the complete detoxification of Jatropha kernel meal.

Material and methods

Preparation of the Jatropha meal

Jatropha seeds were purchased from India and deshelled and defatted in Germany. Organic solvents were used to detoxify defatted Jatropha kernel meal (patent application has been filed for the process of detoxification). After removal of PEs, the meal was autoclaved (121 ^{o}C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation, experimental system and animals

Fish meal was purchased from Kurt Becker GmbH, Bremen, Germany. Prior to feed formulation, the proximate composition of DJKM, wheat meal, wheat gluten and FM were determined (Table 1).

Table 1 Proximate composition and amino acid composition of feed ingredients

	Fish meal	Jatropha meal	Wheat gluten	Wheat meal
Proximate composition (g kg $^{-1}$)				
Dry matter	940	945	937	941
Crude protein	635	665	856	143
Crude lipid	88	11.4	13.4	16.3
Crude ash	142	82	8.7	14
Gross energy (MJ/kg)	21.1	18.3	21.1	18.7
Essential amino acids composition (g kg $^{-1}$)				
Arginine	35.3	69.7	41.1	5.4
Histidine	17.7	21.7	19.7	3.4
Iso leucine	22.8	26.7	42.8	4.2
Leucine	41.6	46.7	68.4	9.1
Lysine	40.9	23.3	16.9	3.3
Phenylalanine	21.8	30.4	30.7	6.5
Methionine	16	10.6	16.7	2
Threonine	23	22	23.2	3.7
Tryptophan	4.9	7.1	13.5	1.4
Valine	29.3	31.6	39.7	5.1
Non-essential amino acids composition (g kg $^{-1}$)				
Alanine	43.3	29.4	20.1	4.6
Asparginine	60.5	68.7	33.5	7.2
Cystine	4.3	2.3	17.1	2.9
Glycine	59.8	31.5	32.5	5.6
Glutamine	79.4	112.1	161.8	44.9
Proline	36.9	32.2	60.3	14.5
Serine	25.5	30.6	43.4	6.3
Tyrosine	14.8	18.8	28.2	3.3

Three isonitrogenous and isoenergetic diets were formulated. Experimental diets containing crude protein 38%, crude lipid 10%, vitamin premix 2%, mineral premix 2% and TiO$_2$ 1%. Titanium oxide (TiO$_2$) was added for digestibility measurement. Lysine was supplemented at the rate of 1.5% of DJKM inclusion in the diet. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF,

Ludwigshafen) per kg. The inclusion concentrations of the DJKM were as follows: Control diet (C_{ontrol}) was prepared with FM and wheat meal, without DJKM; J_{50}: 50% FM protein replaced by DJKM; and $J_{62.5}$: 62.5% FM protein replaced by DJKM. The final mixture of each diet was made into 2 mm diameter moist pellets and then freeze-dried (Table 2).

Table 2 Formulation of the experimental diets (g kg^{-1} feed) for common carp (*Cyprinus carpio* L.)

Ingredients	Experimental diets		
	C_{ontrol}	J_{50}	$J_{62.5}$
Fish meal	503	251	189
[1]Wheat meal	417	399	398
Jatropha meal	-	247	310
Wheat gluten	-	9	8
Sunflower oil	40	54	56
[2]Vitamin premix	20	20	20
[3]Mineral premix	20	20	20
Total	1000	1000	1000
TiO$_2$	10	10	10
Phytase (FTU/kg)	-	500	500
Lysine (g)	-	4.5	6.0

[1]Whole wheat meal.
[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.
[3]Mineral premix (g kg-1): CaCO$_3$, 336; KH$_2$PO$_4$, 50$_2$; MgSO$_4$. 7H$_2$O, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; MnSO$_4$. H$_2$O, 3.12; ZnSO$_4$. 7H$_2$O, 4.67;CuSO$_4$. 5H$_2$O, 0.62; KI, 0.16; CoCl$_2$. 6H$_2$O, 0.08; ammonium molybdate, 0.06; 3 NaSeO$_3$, 0.02.

Common carp (*Cyprinus carpio* L.) fingerlings from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. After acclimatisation, 36 fish were randomly distributed into three groups with four replicates; each replicate contained three fish (av. wt. 22 ± 0.12 g) in an aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 – 27.1°C, pH 7.0 – 7.5, dissolved oxygen 6.9 – 7.4 mg

l^{-1}, total NH_3 0.1– 0.2 mg l^{-1}, nitrite 0.07 – 0.1 mg l^{-1} and nitrate 1–3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass ($kg^{0.8}$) per day (equal to five times their maintenance requirement) and split into 5 equal rations per day (at 8:00, 10:30, 13:00, 15:30 and 18:00 h). The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day. During last two weeks of the experiment, fish were fed with a diet containing an inert marker (TiO_2) for digestibility measurement (Mamun, Focken & Becker 2007). The faeces collection was qualitative, as the experimental diets contained an inert marker (TiO2). During last two weeks of the experiment, faeces were collected daily. After each feeding the aquaria were controlled for remaining feed; generally, there were no feed residues left. Prior to the faeces collection, aquaria were siphoned out to clean any residues. Faeces subsequently excreted by the fish were collected in separate beakers for each aquarium by siphoning with a short small pipe (Mamun *et al.* 2007). The collected mixture of water and faeces was centrifuged at 4000 × g for 10 min, the supernatant discarded and the faeces were then stored at -20°C until analysis. At start of the experiment, six fish of the same population were also killed and preserved at −20 °C for analysis of the initial body composition.

The experiment was terminated after 16 weeks and the fish were killed. At the end of experiment, fish were anaesthetized by tricaine methanesulfonate (MS222) at 250 ppm in water. Blood was drawn near caudal peduncle from one fish from each group and transferred into a heparinized tube for hematological study. Blood was drawn from another fish from each group and divided into two equal part, one part was centrifuged at 1500×*g* for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at −20 °C for determination of cholesterol and triglycerides. Another part of blood was kept outside at room temperature for few minutes to collect serum. Serum was stored at −20 °C for lysozyme determination. One fish per group was carefully dissected to isolate intestine and stored in liquid nitrogen for digestive enzymes assay. Muscle was isolated from same fish and stored at −20 °C for determination of cholesterol and muscle lipid peroxides. One fish per group were stored at −20 °C for chemical composition analysis. Prior to determination of the proximate composition, the fish were autoclaved at 121 °C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and

freeze-dried. The University animal welfare committee (University of Hohenheim Germany) approved all experimental procedures involving common carp.

Proximate analysis and determination of phorbol esters, antinutrients and amino acid

Prior to analysis all samples were ground to a fine powder by using a coffee grinder (Severin, KM 3872, 230 V and 90 W, Severin Elektogerate GmbH, Sundern, Germany). The proximate composition of the feed ingredients, experimental feeds and of the fish carcasses was determined according to the official methods (AOAC, 1990), i.e. for moisture (oven-drying at 105 °C overnight), crude protein by CN analyser (Vario Max CN, Makro-Elementaranalysator, Element Analysensysteme GmbH, Hanau, Germany) (N X 6.25), fat [by extraction according to the method described by Smedes (1999) as modified by Schlechtriem, Schlechtriem, Focken and Becker (2003)] and ash (oven incineration at 480 °C overnight). Gross energy (GE) of feed ingredients, experimental feeds and fish bodies was determined with bomb calorimeter (IKA C7000, Janke and Kunkel, GmbH & Co. KG, Heitersheim, Germany) using benzoic acid as a standard.

Phorbol esters (PEs) were determined according to Makkar, Francis and Becker (2007), which was based on the method of Makkar et al. (1997). The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs by HPLC is 3 µg/g meal. Trypsin inhibitor activity was determined essentially according to Smith, VanMegen, Twaalfhoven and Hitchcook (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub & Lapteva 1988). Non-starch polysaccharides (NSP) were determined according to Englyst et al. (1994). Amino acid composition of FM, DJKM, wheat gluten and wheat meal was determined using an automated amino acid analyser after hydrolysing the samples with 6 N HCl at 110 °C for 24 h (Bassler and Buchholz 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. Tryptophan content of the above-mentioned samples was determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

Growth and nutrient utilization parameters

Growth performance and diet nutrient utilization were assessed in terms of body mass gain (BMG) = [(Final body mass - initial body mass) / Initial body mass] X 100; specific growth rate (SGR) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; metabolic growth rate (MGR) = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$}/2] / number of trial days; feed gain ratio (FGR) = dry feed fed (g)/body mass gain (g); protein efficiency ratio (PER) = body mass gain (g)/crude protein fed (g); protein productive value (PPV) = [(final fish body protein in g - initial fish body protein in g) / total protein consumed in g] X 100; lipid productive value (LPV) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid consumed in g] X 100 and energy productive value (EPV) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100.

Digestibility measurement and efficiency of digestible nutrients and gross energy

The percentage of apparent dry matter digestibility of diets was calculated according to Maynard et al. (1981). Apparent dry matter digestibility (%) = [1 – {(% TiO_2 in feed) / (% TiO_2 in faeces)}] X 100

Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets were calculated according to Maynard and Loosli (1969).

The nutrient and energy digestibility were obtained using the following formula:

Apparent nutrient digestibility (%) = [1 – {(% TiO_2 in feed) / (% TiO_2 in faeces) X (% Nutrient or energy in faeces) / (% Nutrient or energy in feed)}] X 100

Efficiency of digestible nutrients and gross energy = (Nutrient and energy retained in the whole body/Digestible nutrient and digestible energy) x 100

Digestible nutrients and energy = Total amount of nutrients and gross energy offered x digestibility coefficient.

Intestinal index (II), hepatosomatic index (HSI) and digestive enzymes assay

Intestinal index was measured and is expressed in relation to each animal weight and expressed in mm g^{-1}.

II and HIS are calculated as indicated below:

II = Intestine length (mm)/ body mass (g), HSI = Liver mass (g) X 100 / body mass (g).

The reducing sugars produced due to the action of glucoamylase and α- amylase on carbohydrate was estimated using dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as mmol of maltose released from starch per min at 37 °C. Protease activity was determined by the casein digestion method of Drapeau (1974), and one unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta0.001A_{280}$ per minute at 37 °C and pH 7.8. Lipase activity was assayed by the method of Cherry and Crandell (1932), and one unit of enzyme was the amount of enzyme that hydrolyses 1.0 microequivalent of fatty acid from a triglyceride in 24 h at pH 7.7 at 37 °C.

Determination of lipid peroxides, cholesterol and triglyceride

Lipid peroxides in fish muscle were determined using the procedure of Utley et al. (1967). The determinations of the plasma cholesterol and triglycerides were using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color intensity was determined photometrically and was directly proportional to the concentration of cholesterol and triglycerides.

Haematological parameters

Red blood cells and white blood cells were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The red blood cells lying inside the five small squares were counted under high power (40X) of light microscope.

Number of red blood cells /mm^3 = (N x dilution)/area counted x depth of fluid.

The haemoglobin content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Haematocrit was determined on the basis of sedimentation of blood. Heparinised blood (50 μl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value. From analyses of

haematocrit, haemoglobin and red blood cells, the following parameters were calculated: mean cell volume, MCV (fL) = (Haematocrit [in L/L] x 1000) ÷ (red blood cells count [in millions/µL]); mean corpuscular haemoglobin, MCH (pg) = (Haemoglobin [g/dL] x 10 ÷ (red blood cells count [in millions/µL]) and mean cell haemoglobin concentration, MCHC [in g/dL] = Haemoglobin [in g/dL] ÷ Haematocrit [in L/L]. Lysozyme activity of serum was measured by EnzChek Lysozyme Assay Kit (E-22013) Leiden, The Netherlands. VetScan Chemistry Analyzer (Scil Animal care company GmbH, Technischer Service, Germany) was used for determinations of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium (Ca^{++}), creatinine, globulin, glucose, phosphorus, potassium (K^+), sodium (Na^+), total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Duncan's multiple range test ($P<0.05$). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results and discussion

The crude protein contents of DJKM and FM are similar. Therefore, replacing FM by DJKM can achieve any desired level of FM protein replacement by DJKM protein in the diet on weight-to-weight basis. In this respect DJKM is unique, unlike other plant protein sources such as soybean meal, canola meal and lupin (used generally in fish diets) that have lower crude protein content. In the present study, lysine was added in the DJKM based diets and there was no other change except that the FM was replaced by DJKM; and hence the effects observed could be attributed to the lysine supplemented DJKM.

Phorbol esters and antinutrients content in defatted Jatropha kernel meal

Phorbol esters content in untreated defatted Jatropha kernel meal was 1.8 mg/g. However, PEs in DJKM was undetectable (< 3 µg/g). Trypsin inhibitor and lectins were also not detected in DJKM; whereas phyate and NSP levels in DJKM were 9.1% and 16% respectively.

Proximate and amino acid composition of feed ingredient and experimental diets

Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 1 and 3. Diets contained about 38% crude protein was isonitrogenous. Crude lipid and ash were in the range of 9.9–10.6% and 10.5–12.3% respectively. All experimental diets had almost similar amino acid composition. Essential amino acids were in all diets confirmed to the requirement of the common carp (NRC 1993).

Table 3 Proximate and amino acid composition of experimental diets (dry matter basis) of common carp (*Cyprinus carpio* L.)

	C_{ontrol}	J_{50}	$J_{62.5}$
Proximate (g kg^{-1})			
Dry matter	947	948	948
Crude protein	389	389	381
Crude lipid	106	104	99
Crude ash	123	106	105
Total carbohydrate	329	349	363
Gross energy (KJ/g)	19.9	19.1	18.9
Essential amino acids (g kg^{-1})			
Arginine	20.0	28.60	30.76
Histidine	10.3	11.34	11.58
Iso leucine	13.2	14.38	14.60
Leucine	24.7	26.22	26.51
Lysine	21.9	21.49	22.40
Phenylalanine	13.7	15.85	16.38
Methionine	8.9	7.58	7.24
Threonine	13.1	12.89	12.83
Tryptophan	3.0	3.66	3.79
Valine	16.9	17.55	17.68
Non-essential amino acids (g kg^{-1})			
Alanine	23.7	20.15	19.29
Asparginine	33.4	35.33	35.87
Cystine	3.4	2.96	2.82
Glycine	32.4	25.32	23.56
Glutamine	58.6	66.99	68.92
Proline	24.6	23.54	23.21
Serine	15.4	16.86	17.16
Tyrosine	8.8	9.93	10.16

Fish behaviour, feed intake and growth

Common carp is highly sensitive to toxins and can detect PEs at a level of 15 ppm in diet (Becker and Makkar, 1998). We did not find any phorbol ester peak in our HPLC method (detection limit 3 µg/g meal) this result designate that Jatropha kernel meal was detoxified. Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was normal. No left feed was observed in the aquaria. These results demonstrate that DJKM is a good source of protein for common carp diet. There was no mortality during the entire experimental period.

Growth performance and nutrient utilization parameters did not differ significantly (P > 0.05) among the three groups (Table 4). Similar to our earlier short term study on carp (Kumar et al., 2008; Kumar et al., 2010), we obtained encouraging results on replacement of FM with DJKM in the present study as well. The growth rate in the present study was relatively lower than our previous short term experiment (Kumar et al., 2008; Kumar et al., 2010). The initial size of fish and duration of the experiment has significant influence on growth rate. The present study was carried out for longer term (16 weeks) and the initial fish size was bigger (22 g versus 4 g). In the short term study, growth performance and nutrient utilization of carp fed DJKM based diet (50% FM protein replaced by DJKM) were similar to that of FM based diets. In addition, slight but significantly lower (P < 0.05) performance of fish fed the diet in which 75% of FM protein was replaced by DJKM suggested that the capacity of DJKM to fully sustain growth at this level of incorporation was slightly lower compared to the diet based on FM only (control diet).

Chemical composition of whole body of fish

Whole body chemical composition is shown in Table 5. Highest whole body lipid was observed in $C_{control}$ group, which was statistically not different (P > 0.05) from that in J_{50} group and was lowest in J_{62} group; whereas, crude protein in whole body had reverse trend (Table 5). In our study whole body moisture content does not show any inverse relation with whole body lipid but Hasan et al. (1997) found inverse relation of moisture with lipid content. Lower whole body lipid content on replacing more than 50% FM protein by DJKM, which resulted in decreased accretion of lipid in liver appear to lower the hepatosomatic index in $J_{62.5}$ group (Table 5). Hasan et al. (1997) and Mazurkiewicz (2009) observed opposite trend. In their study common carp

fed with plant proteins such as mustard, sesame, linseed, copra, groundnut oil cakes and legume-rapeseed mixture substituting 17-75% FM protein, significantly higher ($P < 0.05$) deposition of crude lipid in whole body has been observed. Gross energy and ash content of the whole body did not differ significantly ($P > 0.05$) among the groups.

Efficient protein synthesis requires sufficient availability of all essential amino acids. Unbalanced amino acid concentrations in diet resulted in increased protein degradation, and thereby increased protein turnover (Martin et al., 2003). Cheng et al. (2003) reported that the plant protein (SBM) based diets lower nitrogen retention in fish because these diets have less digestible energy and an amino acid profile that is suboptimal for muscle growth. Interestingly in our study whole body protein content was higher in DJKM fed groups. Similarly Hasan et al. (1997) and Mazurkiewicz (2009) also found that the body protein content increased significantly ($P < 0.05$) when plant protein replaced FM in common carp diet. The similar profile of amino acid content in DJKM (Table 3) and FM along with supplementation of lysine in test diets might resulted in the increased protein accretion in test groups J_{50} and $J_{62.5}$ compared to control group. In addition, proper combination of FM along with plant protein efficiently increased protein retention in carp (Jahan et al., 2003). This indicates that DJKM containing diets contained optimum digestible energy and balanced amino acid profile optimal for carp growth.

Digestibility measurement and efficiency of digestible nutrients and energy

Apparent digestibility coefficient of dry matter, protein, lipid and energy were highest in C_{ontrol} group, followed by J_{50} and $J_{62.5}$ groups; all being significantly different (Table 6). Growth performance and nutrient utilization parameters were not statistically different ($P > 0.05$) amongst the groups although a numerically descending trend was noticed as DJKM increased in the carp diets. It is possible that this lack of statistical difference was because of high variability of replicates within the treatment groups. The decrease in digestibility coefficients in J_{50} and $J_{62.5}$ might have contributed to decrease in growth performance and nutrient utilization. Generally, oil seed meal proteins have digestibilities of 80-95% for fish (Jauncey and Ross, 1982) and carp are also reported to be able to digest the plant proteins well, generally slightly better than monogastric animals (National Research Council, 1983). The protein digestibility coefficient is a key factor in the evaluation of the quality of the diet for fish and the potential of the diet for the synthesis of new tissues.

Detoxified Jatropha kernel meal in combination with FM protein showed excellent dry matter, crude protein, lipid and energy digestibility in the present study. Dry matter, protein, lipid and energy digestibility of experimental diets were 70-75%, 79-86%, 80-90% and 73-82% respectively, which indicate excellent utilization of feed ingredients. Hasan et al. (1994) reported that apparent protein digestibility values ranged between 68.3-72.9% for carp fed plant protein (leucaena leaf meal) based diets. In the present study, crude protein digestibility of DJKM diets were high (above 79%) in common carp suggested DJKM to be an excellent protein source for carp diet. Energy digestibility of DJKM protein based diets was considerably lower than protein digestibility due to their high carbohydrate content (Gouveia et al., 1993). The lower lipid digestibility of the fish fed the DJKM based diets may be associated with the increase in the NSP content, which reduces fat absorption by disturbing micelle formation in the gastro intestinal tract (Øverland et al., 2009).

Efficiency values of digestible nutrients and energy indicate retained nutrients and energy in whole body relative to the total digestible nutrients and digestible energy ingested. Efficiency of digestible nutrients and energy of diets did not differ significantly among the three groups, indicating that common carp has utilized DJKM well and retained nutrients in the body maximally and similar to control group. The value for the efficiency of digestible protein, lipid and energy were in the range of 28-31%, 37-50% and 18-25% respectively (Table 6).

Digestive enzyme activities and intestinal index

The results of digestive enzymes revealed that the activities are altered significantly due to replacement of FM by DJKM (Table 6). Digestive enzyme (amylase, protease and lipase) activities were highest in C_{ontrol} group, followed by J_{50} and $J_{62.5}$ groups; all being significantly different. Heat labile antinutrients (trypsin inhibitors and lectins) were not detected in the DJKM, whereas heat stable enzyme inhibitor (phytate) was present in DJKM. Phytate inhibits activities of digestive enzymes such as pepsin, trypsin and alpha-amylase (Alarcon, Moyano & Diaz 1999), or form complexes with minerals (Sugiura et al., 1999) and proteins (Moyano et al., 1999), thereby modifying digestion processes and this could impair intestinal absorption. Carp showed a significant decrease in protease, amylase and lipase activities in intestine on inclusion of plant proteins in the diet.

Table 4 Growth performance and nutrient utilization in common carp (*Cyprinus carpio* L.) juveniles

Treat	IBM (g)	FBM (g)	BMG (%)	SGR (% day⁻¹)	MGR (g kg⁰·⁸ day⁻¹)	FGR	PER	PPV (%)	LPV (%)	EPV (%)
C$_{control}$	21.5 ± 0.74	148 ± 29.4	588 ± 120	1.7 ± 0.35	8.5 ± 0.76	1.7 ± 0.26	1.6 ± 0.16	26.5 ± 4.26	43.0 ± 14.99	20.1 ± 4.56
J$_{50}$	21.6 ± 1.04	137 ± 24.6	531 ± 82	1.6 ± 0.26	7.9 ± 0.15	1.8 ± 0.05	1.4 ± 0.02	26.1 ± 0.29	36.5 ± 6.34	17.6 ± 4.04
J$_{62.5}$	21.8 ± 1.03	122 ± 36.5	460 ± 139	1.4 ± 0.42	6.8 ± 1.32	2.1 ± 0.48	1.2 ± 0.26	21.9 ± 3.88	29.4 ± 4.04	15.4 ± 5.69
SEM	0.24	5.95	43.56	0.07	0.36	0.13	0.08	1.22	3.42	1.27

IBM- Initial body mass, FBM- Final body mass, BMG - Body mass gain, SGR – Specific growth rate and MGR - Metabolic growth rate; FGR – Feed conversion ratio, PER - Protein efficiency ratio, PPV - Protein productive value, LPV - Lipid productive value and EPV - Energy productive value. Values are mean (n = 4) ± standard deviation. For all parameters, mean values in the same column were not significantly different (P < 0.05).

Table 5 Chemical composition of whole body (% wet basis ± SD) at the start and at the end of the experiment and hepatosomatic index (HSI) of carp

Treatment	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)	HSI
Initial fish	78.1 ± 0.41	14.5 ± 0.08	3.6 ± 0.21	3.4 ± 0.14	4.4 ± 0.11	
C$_{control}$	75.2 ± 0.65	16.3[b] ± 0.34	5.8[a] ± 1.13	2.4 ± 0.24	5.6 ± 0.39	1.4 ± 0.12
J$_{50}$	75.8 ± 2.08	17.3[a] ± 0.26	5.1[a] ± 1.57	2.5 ± 0.36	5.2 ± 0.82	1.3 ± 0.17
J$_{62.5}$	76.1 ± 0.87	17.5[a] ± 0.58	3.7[b] ± 0.17	3.0 ± 0.21	4.8 ± 0.19	1.1 ± 0.03
SEM	0.41	0.22	0.42	0.12	0.19	0.06

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 6 Effects of experimental diets on the dry matter, nutrient and energy digestibility (%); digestive enzymes activities (U/mg protein); and utilization of digestible protein, lipid and energy in common carp (*Cyprinus carpio* L.) fingerlings

Treat	Dry matter digestibility	Protein digestibility	Lipid digestibility	Energy digestibility	Amylase	Protease	Lipase	DPE (%)	DLE (%)	DEE (%)
C$_{control}$	74.9[a] ± 0.98	85.9[a] ± 0.98	89.6[a] ± 0.51	82.0[a] ± 1.62	17.4[a] ±1.32	36.5[a] ± 1.31	6.8[a] ± 0.29	30.8 ± 4.95	40.8 ± 7.11	24.5 ± 2.85
J$_{50}$	71.5[b] ± 0.64	83.2[b] ± 0.80	86.2[b] ± 0.76	77.7[b] ± 1.83	13.6[b] ± 0.83	28.1[b] ± 0.83	5.3[b] ± 0.32	31.4 ± 0.41	49.8 ± 17.3	22.7 ± 4.38
J$_{62.5}$	69.6[b] ± 1.32	78.6[c] ± 0.37	80.1[c] ± 1.65	73.4[c] ± 0.49	11.0[c] ± 0.76	20.7[c] ± 1.24	4.3[c] ± 0.22	27.8 ± 4.92	36.6 ± 4.32	18.3 ± 3.74
SEM	0.83	1.09	1.43	1.35	0.84	1.96	0.31	1.29	3.75	1.42

DPE: digestible protein efficiency; DLE: digestible lipid efficiency; DEE: digestible energy efficiency. Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Lower protease activities, the level of which decreased with increase in DJKM in common carp diets, corresponded to decrease in protein availability from DJKM. Similar results were observed by Santigosa et al. (2008). They found that protein digesting enzyme (trypsin) activity decreases as plant protein inclusion increases in trout diet and they concluded that trypsin is highly sensitive to plant antinutrients. Escaffre et al. (1997) observed that increasing levels of dietary soya protein concentrate induced a significant decline in trypsin activity in common carp. The decrease in protease activity at higher inclusion level of DJKM might be caused by the presence of phytate (Kumar et al., 2009; Kumar et al., 2010). The lower activity of digestive enzymes in DJKM fed groups was correlated with lower nutrient digestibility.

It is known that carnivorous and omnivorous fish require longer time to digest plant protein based diets (Buddington et al., 1997). Direct relationship between the amount of dietary plant protein and intestinal index has been reported earlier in fish (Kramer and Bryant, 1995, Kumar et al., 2009; Kumar et al., 2010). In carp, DJKM based diets exhibited higher intestinal index than the control group (Table 7). Intestinal index value increases as the plant protein inclusion increases in the common carp and trout diets (Santigoga et al., 2008; Kumar et al., 2009; Kumar et al., 2010). From a physiological view point, a longer intestinal index would facilitate an increase in retention time and digestibility by enhancing contact time of the digestive enzymes and the feed components, resulting in increase in their digestion and absorption. Omnivorous fish like common carp species showed compensation mechanisms, such as an increase in intestinal index and as a result increase in digestive activity, to achieve a digestive balance and growth rates similar to those observed for FM fed group.

Cholesterol and triglycerides, muscle lipid peroxide; and blood glucose level

Cholesterol and triglyceride levels in plasma, and muscle cholesterol concentration were highest in C_{ontrol} group, followed by J_{50} and $J_{62.5}$ groups; all being significantly different whereas muscle lipid peroxide value did not differ significantly ($P > 0.05$) among the three groups (Table 7). Detoxified jatropha kernel meal reduced cholesterol level in plasma and muscle as compared to control group. The decrease in plasma cholesterol concentrations in fish fed diets with plant proteins is in accordance with the results of Yamamoto et al. (2007) and Kumar et al. (2008). In terrestrial animals, plant products are generally considered to have a hypocholesteromic effect, mainly due to the relatively high levels of

estrogeno-mimetic isoflavones (Setchell and Cassidy, 1999). In humans, different plant constituents have been reported to lower plasma cholesterol concentration (Wester, 2000). Although cholesterol metabolism in animals and fish could differ, the fish hypocholesterolemia in response to dietary plant protein supply could be due either to an increased excretion of bile salts, to an inhibition of cholesterol intestinal absorption, or just to the withdrawal of FM rather than to the direct effects of plant protein (Kaushik et al., 2004). Serum triglycerides act as a short-term indicator of feeding or nutritional status (Bucolo and David, 1973). The decrease in whole body fat content in plant protein fed group ($J_{62.5}$) along with the decrease in plasma triglyceride concentrations also suggest lipid mobilisation in these groups. Shimeno et al. (1993) observed similar trend in the yellowtail. Blood glucose level was not affected by dietary treatments. The blood glucose concentrations were similar in all groups (Table 9). Similar trend was shown in fish fed diets containing yellow lupin as a substitute for FM (Glencross et al., 2004). On the other hand, Kikuchi (1999) observed that dietary inclusion of SBM and corn gluten in fish diet increased blood glucose level.

Blood chemistry

The white blood cells counts and haemoglobin content did not differ significantly (P > 0.05) among the three groups (Table 8). Red blood cells counts were higher in DJKM fed groups whereas MCV, MCH, MCHC exhibited opposite trend (Table 8). As the DJKM content increased, an increase in the red blood cells count was observed. Plant ingredients may cause early release of immature erythrocytes (Hemre et al., 2005), increasing the red blood cells count. Consequently, MCV value was changed at the same time. Lower MCV was observed in DJKM protein fed groups than control group (Table 8). Similarly, significant reduction of MCV on increase in the content of plant proteins in salmon diet was observed (Hemre et al., 2005). As this observation appeared to coincide with increased spleen size (Hemre et al., 2005), it was suggested that some of the plant ingredients might cause early release of immature erythrocytes. The haemoglobin and haematocrit assays are normally used as general indicators of fish health (NRC, 1981). Hematocrit level in all groups was within the normal range and did not differ significantly (P > 0.05) among the groups (Sun et al., 1995). In the present study higher haematocrit content was observed in DJKM fed groups, which is in contrast with the observation of Soltan et al. (2008), who observed that FM protein replaced by mixture of plant proteins in Nile tilapia diets that led to lower haematocrit content. This was attributed to the

binding of phytate to minerals (iron) and/or amine group of amino acids causing their low availabilities in the body and increase in erythrocyte fragility.The concentration of total protein in blood is used as a basic index for health and nutrititional status in fish (Martinez, 1976). Among the blood proteins, albumin and globulin are the major proteins that play a significant role in the immune response. Albumin is used as an indicator of liver impairment (Silverman et al., 1986). Blood protein did not differ significantly (P > 0.05) among the three groups (Table 8), indicating that there are no nutritional deficiencies and no impaired protein metabolism in the liver. Lysozyme has an important role in nonspecific immune response and it is found in mucus, serum and ova of fish. Innate immunity due to lysozyme is caused by lysis of bacterial cell wall and this stimulates the phagocytosis of bacteria. The suppression of the non-specific immune capacity by high concentrations of dietary soybean proteins has been reported in rainbow trout (Burrells et al., 1999).

However, other reports wherein SBM was fed to rainbow trout and Atlantic salmon or alginate to Atlantic salmon, increased values of different non-specific immune parameters have been reported, which have been interpreted as immunostimulating effects of plant protein sources (Krogdahl et al., 2000). In the present study lysozyme activity was statistically not different (P > 0.05) amongst the groups (Table 8) but numerically it was higher in DJKM fed groups, indicating immunostimulating effect of DJKM in common carp.

Table 7 Cholesterol and triglyceride (mg/dl) level in plasma; and muscle cholesterol (mg/100 g) level and muscle lipid peroxide (nMol MDA (malondialdehyde) /100g tissue) level and intestinal index (mm g^{-1}) of common carp (*Cyprinus carpio* L.) fingerlings

Treatment	Plasma cholesterol	Plasma triglycerides	Muscle cholesterol	Muscle lipid peroxide	Intestinal index
Control	152[a] ± 5.4	102[a] ± 4.5	141[a] ± 22	1.98 ± 0.89	2.24[c] ± 0.07
J$_{50}$	131[b] ± 6.4	88[b] ± 5.2	112[b] ± 14	2.21 ± 1.13	2.78 [b] ± 0.10
J$_{62.5}$	108[c] ± 7.2	72[c] ± 6.9	86[c] ± 18	2.56 ± 1.12	3.17[a] ± 0.10
SEM	2.41	2.55	6.34	0.84	0.17

Values are mean (n = 4) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 8 Effects of experimental diets on the haematological parameters (red blood cells (RBC, 10^6 cells/mm^3), white blood cells (WBC, 10^3 cells/mm^3), haemoglobin (Hb, g/dl), haematocrit (Hct, %), MCV (fL), MCH (pg), MCHC (g/dl), albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood and lysozyme activity (LA, IU/ml) in the serum of common carp (*Cyprinus carpio* L.) fingerlings

Treat	RBC	WBC	Hb	Hct	MCV	MCH	MCHC	Albumin	Globulin	Total protein	LA
C[control]	1.32[c] ± 0.02	0.78 ± 0.04	5.0 ± 0.00	32[b] ± 5.10	380[a] ± 6.3	37.9[a] ± 0.63	17.7[a] ± 1.70	2.25 ± 0.48	0.53 ± 0.10	2.78 ± 0.39	404 ± 98
J[50]	1.41[b] ± 0.06	0.79 ± 0.06	5.2 ± 0.50	40[ab] ± 12.31	372[a] ± 28.3	37.2[a] ± 2.83	13.6[ab] ± 4.34	2.05 ± 0.71	0.78 ± 0.26	2.83 ± 0.50	505 ± 122
J[62.5]	1.52[a] ± 0.06	0.77 ± 0.06	5.0 ± 0.00	45[a] ± 4.57	330[b] ± 12.8	33.0[b] ± 1.28	11.3[b] ± 1.18	2.13 ± 0.05	0.75 ± 0.10	2.88 ± 0.13	500 ± 54
SEM	0.03	0.01	0.10	2.81	11.41	0.81	1.08	0.18	0.06	0.10	26

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05)

MCV: Mean cell volume (fL); MCH: Mean corpuscular hemoglobin (pg); MCHC: Mean corpuscular hemoglobin concentration (g/dl)

IU- The amount of enzyme required producing a change in the absorbance at 450 nm of 0.001 units per minute at pH 6.24 and 25^{0}C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

Table 9 Effects of experimental diets on alkaline phosphatase (ALP, U/l), alanine transaminase (ALT, U/l), glucose (mg/dl), total bilirubin (TBIL, mg/dl), blood urea nitrogen (BUN, mg/dl) and creatinine (mg/dl) in blood, blood ions (calcium (mg/dl), phosphorus mg/dl, sodium (mmol) and potassium (mmol) of common carp (*Cyprinus carpio* L.) fingerlings

Treat	ALP	ALT	Glucose	TBIL	BUN	Creatinine	Calcium	Phosphorus	Sodium	Potassium
C[control]	61 ± 6.0	80 ± 17.2	115 ± 14	0.30 ± 0.00	3.75 ± 0.50	1.25[a] ± .42	10.15 ± 0.93	8.03 ± 4.15	131 ± 2.63	5.9 ± 2.86
J[50]	65 ± 29.6	63 ± 16.1	118 ± 31	0.28 ± 0.05	4.75 ± 0.96	0.68[b] ± 0.15	10.15 ± 0.45	9.03 ± 2.23	132 ± 4.57	3.6 ± 2.29
J[62.5]	85 ± 24.4	65 ± 13.1	127 ± 33	0.28 ± 0.05	3.75 ± 0.96	0.56[b] ± 0.00	10.38 ± 1.16	11.38 ± 1.44	131 ± 2.38	2.3 ± 1.65
SEM	6.64	4.66	7.29	0.01	0.06	0.25	0.24	0.85	0.89	0.75

Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute).

Metabolic enzymes and blood ions

Alkaline phosphatase and ALT are released into blood during organ damage (Racicot et al., 1975). Alkaline phosphatase level rises during bile duct obstruction, and in intrahepatic infiltrative diseases of the liver. Alanine transaminase also called serum glutamic pyruvate transaminase is an enzyme present in hepatocytes (liver cells). When liver cell is damaged, it leaks into the blood. Alanine transaminase rises dramatically in acute liver damage (Racicot et al., 1975). Thus, detection of blood level of ALP and ALT gives information on the damage of organs and in particular of liver cells. Concentrations of ALP and ALT were similar in all the diets (Table 9), indicating normal organ function on feeding of DJKM. Hemre et al. (2005) and Sanden et al. (2006) also reported similar results on feeding SBM containing diets to fish.

Blood urea nitrogen concentrations were in the normal ranges (Wedemeyer, 1996). Blood urea nitrogen concentrations are thought to be associated with liver or gill dysfunction (Stoskopf, 1993), as these are the sites of urea production and excretion, respectively. Blood urea nitrogen concentration did not differ significantly ($P > 0.05$) among the three groups (Table 9), suggesting that DJKM fed groups was normal and healthy. Total bilirubin is an indicator of liver dysfunction (Tietz, 1986). Total bilirubin concentration in the blood did not differ significantly among the three groups. Creatinine used as an indicator of kidney damage or malfunction (Tietz, 1986). Creatinine is a degraded product of creatine, which is involved in muscle energy metabolism. Blood creatinine is normally quite stable. Its level in the blood becomes elevated if kidney function is impaired. Creatinine was highest in control group but it was within the normal range (Tietz, 1986). Creatinine is a metabolite of animal protein and its highest level in control is due to highest content of FM in this group (Table 9). DJKM based diets were supplemented with phytase that leads to increased release of phosphorus, calcium and other ions from feed and making them available for common carp. Similar blood ions were observed in DJKM fed groups and control group (Table 9). Our preliminary histological findings demonstrate that common carp did not show any abnormal changes in intestine and liver.

Conclusions

The results of the long term feeding study showed that the detoxified Jatropha kernel meal fed groups exhibited good growth performance

(almost five times increased in fish body mass after 16 weeks). Also biochemical and hematological parameters were within the normal ranges and similar to those in control group (fish meal fed group). The current study demonstrates that the DJKM can be used as one of the promising fish meal replacers in the diets of common carp. Although non-significant, numerically, growth performance and nutrient utilization parameters were much lower in J_{62} group (62.5% fish meal protein replaced by DJKM) than J_{50} group (50% fish meal protein replaced by DJKM) therefore we would recommend to replace 50% fish meal protein by DJKM in common carp diet without sacrificing fish yield.

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Chapter 9

Jatropha platyphylla, a new non-toxic Jatropha species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds

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Jatropha platyphylla, a new non-toxic Jatropha species: Physical properties and chemical constituents including toxic and antinutritional factors of seeds

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Abstract

Local communities in Mexico consume *Jatropha platyphylla* seeds after roasting. The kernels of *J. platyphylla* contained *ca* 60% oil and were free of phorbol esters. The kernel meal of this Jatropha species contained trypsin inhibitor, lectins and phytate. However, trypsin inhibitor and lectins are heat labile so this explains why the local people can eat roasted seeds without ill effects. Heat treated *J. platyphylla* kernel meal (JPKM) was free of trypsin inhibitor and lectin activities. Crude protein content of JPKM was 75%. Heated JPKM and soybean meal were included in a standard diet (crude protein 36%) for Nile tilapia (*Oreochromis niloticus)* to replace 50% of the fish meal protein. The growth of fish in all the three groups was statistically similar and the blood biochemical parameters that serve as biomarkers for toxicity were within the normal ranges. This is the first study that confirms the non-toxic nature of *J. platyphylla*.

Introduction

The genus name *Jatropha* is derived from the Greek words *iatros* (doctor) and *trophe* (food). It belongs to the Euphorbiaceous family of plants and is well known for its toxicity. Jatropha species for which the toxicity has been widely studied are *J. curcas, J. elliptica, J. glauca, J. gossypifolea, J. aceroides, J. tanoresisi, J. macarantha J. integerrima, J. glandulifera, J. podagrica and J. multifida*

(Devappa et al., 2010a). Some of these species are known to contain a lethal cocktail of toxins including phorbol esters (Siam et al., 2009). Curcin, another possible toxin is found in *J. curcas seeds.* It belongs to a group of proteins called ribosome-inactivating proteins (RIPs), which inactivate ribosomes and finally halt protein synthesis. Type 1 RIPs are single-chain (~30 kDa) with the enzymatic activity and can inhibit cell-free protein synthesis, but are relatively nontoxic to cells and animals (Devappa et al., 2010b). Curcin is not responsible for causing Jatropha toxicity and also it is heat labile (Makkar and Becker, 2009). Although almost all the Jatropha species so far studied have been found to be toxic, presence of a non-toxic genotype of *J. curcas* has been recorded, which to the best of out knowledge exists only in Mexico (Makkar and Becker, 2009). *Jatropha curcas* (toxic genotype) is currently being widely promoted as a source of biofuel. Its seeds contain 25 to 35% oil that can be used as fuel directly or as a substitute to diesel in the transesterified form. However, these seeds contain a highly toxic group of compounds called phorbol esters and antinutritional factors such as trypsin inhibitor, lectins and phytates (Makkar and Becker, 2009).

This communication is the first report of a species of Jatropha that has been proved by modern scientific methods to be non-toxic and follows a visit by the first author to a community in the state of Sinaloa, Mexico whose members are descended from the Lacapaxa tribes. The people of this community eat the seed kernels of *Jatropha platyphylla* Müell after roasting them in a manner similar to that used for peanuts. The authors found the kernels (shape and size similar to Macadamia nuts) from the roasted seeds tasted delicious. The kernels are also used for the preparation of traditional dishes. The species is restricted to warm areas (average temperature 20–29 °C) on the pacific coast from Sinaloa to Michoacán including the Nayarit and Jalisco states in Mexico and is usually found in or around deciduous forests. It is a drought resistant shrub or tree 2 to 5 m high and almost glabrous. The peltate leaves are 15 to 35 cm across with broad, rounded lobes.

This communication reports physical characteristics of seeds, and chemical composition of seeds and kernel meal including the toxins and antinutritional factors of *J. platyphylla* Müell. It was established that seeds are free of the toxins, phorbol esters. In addition, fish (Nile tilapia) based bioassay was used, in which 50% fish meal protein was replaced by *J. platyphylla* kernel meal and a 12 week feeding experiment was conducted to establish the non-toxic nature of the kernels.

Materials and methods

Collection of material

The seeds were collected from three trees located in wild (this Jatropha species is not yet cultivated) in La Chilla, Culiacán, Sinaloa, Mexico. The coordinates of the place are N 24o-24´-18.5´´ and W 107 o -05´-39.9´´.

Physical parameters

Fifteen seeds from each tree were cracked to separate shells and kernel. The weights of shells and kernels were individually recorded and used for determination of shell and kernel mass.

Determination of oil and protein in kernel

The kernels were ground using a coffee grinder. Oil content was determined using a Soxhlet apparatus and protein content as (N x 6.25) using Kjeldahl method (AOAC, 1990). Completely defatted kernel meal (designated as kernel meal subsequently) after treatment of the kernel with petroleum ether (b.p. 40-60 oC) in Soxhlet apparatus was collected and used for various analyses. The kernel was the white inner portion of the seed after removal of shells.

Determination of chemical composition

The moisture content of the samples was determined by oven-drying to a constant weight at 105o C. Crude protein, lipid, and ash content were determined in accordance with the standard methods of AOAC (1990).

Heat treatment of kernel meal

Ten ml distilled water was added to 5 g of the kernel meal to bring the moisture level to approximately 66%. The samples were heated at 121o C for 20 min, and then freeze dried. This heat treatment is the optimized condition for inactivation of trypsin inhibitor and lectin activity in *J. curcas* kernel meal (Aderibigbe et al., 1997). Along with the unheated kernel meal, the heated kernel meal was taken for determination of phorbol esters, trypsin inhibitor and lectin activities, phytate, protein digestibility and available lysine. The heated kernel meal was used for fish feeding studies.

Extraction and estimation of phorbol esters by HPLC

The samples (kernel and kernel meal) were extracted in methanol and phorbol esters were determined on a reverse phase C18 (LiChrospher 100, endcapped 5 lm) 250 x 4 mm I.D. column protected with a guard column containing the same material as the main column according to the procedure outlined by Makkar et al. (2007), which was based on the method of Makkar and Becker (1997). The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of phorbol esters was 3 µg/g sample.

Determination of amino acids and fatty acids

An amino acid composition of kernel meal was determined by the use of an automated amino acid analyser. The sample was hydrolysed with 6 M HCl at 110 ºC for 24 h (Bassler and Buchholz, 1993). Tryptophan content of the samples was determined spectro-photometrically by the Pinter-Szakacs and Molnar-Perl (1990) method.

Fatty acids profile of oil was determined. Fatty acid methyl esters (FAME) were prepared by the boron trifluoride method (AOAC 1990; Schlechtriem et al., 2004). FAME were analysed in a Shimadzu GC-14A gas chromatograph equipped with a DB Wax-fused silica capillary column (50 m × 0.25 mm i.d., film thickness: 0.2 μm; Chrompac CP7723) using nitrogen as carrier gas (1.3 kg cm^{-2}). The oven thermal gradient increased from an initial 160 to 198°C at 2.5°C min^{-1}, from 198 to 218°C at 2°C min^{-1} from 218 to 240°C at 1.5°C min^{-1} and from 240 to 250°C at 1°C min^{-1}. Temperature was maintained for 5, 15, 10 and 2 min at 198°C, 218°C, 240°C and the final temperature, respectively. Individual FAME were identified by comparison with a known standard mixture (Sigma 47885-U) and quantified by means of a Shimadzu C-R4AX integrator. Separation and identification of FAME were carried out in duplicate.

Determination of antinutritional factors

Trypsin inhibitor activity was determined essentially according to Smith et al. (1980) except that the enzyme was added last, as suggested by Liu and Markakis (1989). In brief, about 0.25 g defatted sample was Ultra-turraxed for 30 seconds in 12.5 ml of 0.01 M NAOH. The pH of this mixture was adjusted in the range of 9.4-9.6 with 1M NaOH or 1M HCl and was centrifuged at the rate of 3000 *g* for 15 min. The supernatant was taken for the assay using Benzoyl-dl-arginine-p-nitroanilide (BAPA) as the substrate.

Analysis of the lectin content was conducted by hemagglutination assay in round-bottomed wells of microtitre plates using 1% (v/v) trypsinised cattle blood erythrocytes suspension in saline phosphate buffer, pH 7.0 (Makkar and Becker, 1997). The hemagglutination activity was expressed as the minimum amount of the material (in mg per ml of the assay medium), which produced agglutination. The minimum amount was the material per ml of the assay medium in the highest dilution that was positive for agglutination. Phytate content of the sample was determined by a spectrophotometric procedure described by Vaintraub and Lapteva (1988). Results are expressed as g/100 g phytic acid, using standard phytic acid (sodium salt; Sigma, St. Louis, MO, USA). Non-starch polysaccharides (NSPs) were estimated according to Englyst et al. (1994). Total saponin content was determined using a spectrophotometric method, described by Hiai et al. (1976). The concentration of saponins was read off from a standard curve of different concentrations of diosgenin in 80% aqueous methanol and expressed as diosgenin equivalents.

Amylase inhibitor activity was determined against porcine pancreatic alpha-amylase (Boehringer GmbH, Mannheim, Germany, catalogue number 102806). The activity of amylase was determined by the method of Bemfeld (1955) at 30°C. Glucosinolate and cyanogens were determined by methods based on the reduction of picrate and reduction of ferricyanide respectively, as described in Makkar et al. (2007).

In vitro protein digestibility and available lysine

Protein digestibility in kernel meals was determined by digestion with pepsin, followed by trypsin, according to the method of Saunders et al. (1972). The available lysine was determined according to the procedure of Tsao et al. (1978) using the trinitrobenzene sulfonic acid (TNBS) reagent.

Fish based bioassay

A total of three experimental diets were formulated which were isonitrogenous and isocaloric in terms of crude protein (36 %) and gross energy (18 MJ/kg). Fish meal (FM) was obtained from Kurt Becker GmbH, Bremen and wheat meal from a local market in Stuttgart, Germany. The defatted soybean meal (SBM) was obtained from Institute for Animal Nutrition system (450a), University of Hohenheim, Germany. The control diet was prepared with FM as the primary source of dietary protein. The second experimental diet (Jatropha) was prepared with heated *J. platyphylla*

kernel meal replacing 50 % FM protein; while, third experimental diet (Soybean) was prepared with SBM replacing 50% FM protein. Lysine monohydrochloride (lysine 80% in this salt; Reinst; Merck KGaA, Darmstadt) was supplemented at the rate of 2.14% of *J. platyphylla* kernel meal and 0.05% of SBM inclusion in the diets. Jatropha and Soybean diets were also supplemented with phytase enzyme at a level of 500 FTU/kg (NATUPHOS 5000G, BASF, Ludwigshafen). The ingredients were thoroughly mixed, made into pellets and oven dried at 40 ℃. The feed was preserved at 4 ℃ during the course of experiment. The composition and proximate composition of the experimental diets are given in Table 1.

Table 1 Composition (g kg^{-1} feed) and proximate composition (g kg^{-1} dry matter basis) of the experimental diets for Nile tilapia (*Oreochromis niloticus*) fingerlings

Ingredients	Experimental diets		
	Control	Jatropha	Soybean
Fish Meal	437	218	218
Soybean meal	-	-	308
[1]Wheat meal	483	451	347
Jatropha meal	-	218	-
Wheat gluten	-	5	22
Lysine monohydrochloride	-	8	5
Sunflower oil	40	60	60
[2]Vitamin premix	20	20	20
[3]Mineral premix	20	20	20
Total	1000	1000	1000
Phytase (FTU/kg)	-	500	500
Proximate composition (g kg^{-1} dry matter basis)			
Dry matter	954	943	944
Crude protein	357	361	356
Crude lipid	82	80	79
Ash	105	87	87
Gross energy (MJ/kg)	18.5	18.1	17.8

[1]Whole wheat meal.

[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[3]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 50$_2$; $MgSO_4 \cdot 7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4 \cdot H_2O$, 3.12; $ZnSO_4 \cdot 7H_2O$, 4.67; $CuSO_4 \cdot 5H_2O$, 0.62; KI, 0.16; $CoCl_2 \cdot 6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

All male Nile tilapia (*Oreochromis niloticus*) were obtained from the bio-lab, University of Hohenheim, Germany. The fish were acclimated for 12 days in 45 litre aquaria and fed at maintenance energy requirements in the Fish laboratory, University of Hohenheim, Germany. At the beginning of the experiment, 30 fish were selected randomly and distributed into 30 aquaria with one fish in each to avoid dominance and cannibalism at 10 replicates for each treatment. The average weight of the fish selected was between 5–6 g. The aquaria were supplied with fresh water at a flow to keep the oxygen saturation above 80 % and water temperature was kept at 28° C ± 2 in a recirculating system. The system was subjected to a photoperiod of 12 h light and 12 h darkness. Water quality parameter was monitored throughout the period of the experiment. The fish were fed five times their body maintenance requirement ($5 \times 3.2 \times$ (fish wt(g)/1000)$^{0.8}$) daily. The feed required for each day was divided into five equal parts and were given five times a day (9.00, 11.00, 13.00, 15.00 and 17.00 h) using auto-feeders. Feed intake was observed and aquaria were checked for probable leftover. Fish were weighed every week in order to adjust the feeding level for subsequent week. The fish were not fed on the weighing day. At the start of the experiment, 15 fish of the same population were killed and preserved at −20° C for the analysis of the initial body composition.

The experiment was terminated after 12 weeks and the fish were sacrificed. At the end of the experiment, fish were anesthesized by tricaine methane sulfonate (MS222, Sigma Chemical Co., USA) at 250 ppm in water. Five fish per group were used for hematological study. Five fish per group were stored at −20 °C for chemical composition analysis. Prior to the determination of the proximate composition, the fish were autoclaved at 121°C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and freeze-dried. Water quality was observed throughout the period of the experiment. All water quality parameters were maintained at optimum range.

Samples of the diets and fish bodies that have been homogenized and lyophilized were analysed for the dry matter, ash, crude protein and lipid (AOAC, 1990). The gross energy for the samples of the diets and fish bodies was determined with bomb calorimetry (IKA C7000) using benzoic acid as a standard.

Growth performance, body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), apparent lipid conversion (ALC) and energy retention (ER) were measured as follows:

BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100

SGR = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100 MGR = (Body mass gain in g) / [{(initial body mass in g / 1000)^0.8 + (final body mass in g / 1000)^0.8}/2] / number of trial days

FCR = dry feed fed (g)/body mass gain (g)

PER = fresh body mass gain (g)/crude protein fed (g)

PPV (%) = [(final fish body protein, g - initial fish body protein, g) / total protein consumed, g] x 100

ALC (%) = [(final fish body lipid, g - initial fish body lipid, g) / total crude lipid consumed, g] x 100

ER (%) = [(final fish body energy - initial fish body energy)/ (gross energy intake)] * 100

Blood collection

Blood samples from the fish that were anaesthetised with tricaine methane sulfonate were taken with 2 ml heparinized syringe and 21 swg needles from the caudal vein of a set of five fish from each treatment and put separately in 2 ml heparinised tubes and taken to the laboratory for determination of hematocrit (Hct), and haemoglobin (Hb). A comprehensive diagnostic of the blood composition was done .

Total erythrocyte count (RBC) and total leucocyte count (WBC)

RBC and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope.

The following formula was used to calculate the number of RBC per mm^3 of the blood sample:

Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid

Haemoglobin and hematocrit content

The haemoglobin content of the blood was analysed using Reflotron Haemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hematocrit was determined on the basis of sedimentation of blood. Heparinised blood (50 µl) from the hematocrit capillary (Na-heparinised) was centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Haematocrit [in L/L] x 1000) ÷ (RBC count [in millions/µL]); mean corpuscular haemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/µL]) and mean cell haemoglobin concentration, MCHC [in g/dL] = Haemoglobin [in g/dL] ÷ Haematocrit [in L/L].

Blood analytes

VetScan Chemistry Analyzer (Scil Animal care company GmbH, Technischer service, Germany) was used for quantitative determination of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium (Ca^{2+}), creatinine, globulin, glucose, phosphorus, potassium (K^+), sodium (Na^+), total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinised whole blood.

Statistical analysis

The data were analyzed for ANOVA using Sigma Stat – Statistical Software Version 2.0 (Jandel Scientific, Sausalito, CA, USA). The significance of the differences between the means was tested using Tukey's test ($p<0.05$). The values are expressed as means ± standard deviation (SD). Chemical constituent data are reported on dry matter (DM) basis.

Results and discussion

Jatropha curcas is the most widely available Jatropha species. It is also being extensively planted in various parts of the tropical world as a biofuel plant. As mentioned in the introduction section, we have reported a non-toxic genotype of *J. curcas*. Therefore, most of the parameters for *J. platyphylla* investigated in the present study have been compared with that of the toxic and non-toxic genotypes of *J. curcas* taken from our previous publications (Makkar and Becker, 2009; Aderibigbe et al., 1997; Makkar and Becker, 1997; Makkar et al., 1998a; Makkar et al., 1998b; Martinez-Herrera et al., 2006).

Physical parameters of seeds

The seeds of *J. platyphylla* were almost circular with a diameter of 15.54 mm ± 1.01; while that of the toxic and non-toxic genotypes of *J. curcas* were elliptical (length 17.0 mm ± 0.96, width 8.4 mm ± 0.50) (Figure 1). The

average seed, shell and kernel mass of *J. platyphylla* were approximately 2.3-, 3- and 2-folds respectively higher than those of the toxic and non-toxic genotypes of *J. curcas* (Table 2). Shells of *J. platyphylla* form slightly more than 50% of the total weight of the seeds, whereas for *J. curcas* this value was much lower, 32 to 39%. These results suggest that on per unit weight basis, *J. platyphylla* would yield lower amount of kernel, which is the main source of nutrients; and on the other hand, the shell yield would be higher. Shells generally are used as energy source. The gross energy and lignin contents of shells of *J. platyphylla* and of the two genotypes of *J. curcas* were almost similar (gross energy 19.6 MJ/kg and lignin 42%). The gross energy of these shells is almost similar to that of wood (19 MJ/kg dry matter; Fuwape, 1993), showing that the shells can be used for burning and higher amount of energy can be generated per unit weight of seeds of *J. platyphylla* than of *J. curcas.* Furthermore, the shells are also a good source for lignin isolation, which could be used for various industrial applications (Gosselink et al., 2004).

Fig. 1. Seeds of **(A)** *Jatropha platyphylla* and **(B)** *Jatropha curcas.*

Oil and protein contents

Oil, crude protein and ash contents in kernels did not differ significantly (Table 3). High oil content in *J. platyphylla* (*ca* 60%) makes this species a valuable source for oil production. Since the proportion of kernels in *J. platyphylla* seeds is lower than that in *J. curcas*, per unit weight of the seeds from the former would yield lower oil content; and same would be for crude protein content. The crude protein and ash contents in *J. platyphylla* and *J. curcas* kernel meals were also similar (Table 3).

Table 2 Seed, shell and kernel mass (g) of *Jatropha plytaphylla* and of toxic and non-toxic genotypes of *Jatropha curcas.*

	Jatropha plytaphylla	*Jatropha curcas*[x]	
		Toxic	Non-toxic
Seed	$1.80^a \pm 0.15$	$0.80^b \pm 0.08$	$0.73^b \pm 0.09$
Shell	$0.92^a \pm 0.01$	$0.31^b \pm 0.05$	$0.26^c \pm 0.03$
Kernel	$0.85^a \pm 0.13$	$0.49^b \pm 0.0.7$	$0.47^b \pm 0.07$

Means (n=3) with different superscripts within a row differ significantly (P<0.05)
[x] Compiled data from our previous studies

Table 3 Composition (% in DM) of kernels (with oil) and defatted kernel meal from *Jatropha plytaphylla* and from toxic and non-toxic genotypes of *Jatropha curcas*

	Jatropha plytaphylla[x]	*Jatropha curcas*[y]	
		Toxic	Non-toxic
Kernel			
Crude protein	27.1 ± 2.0	26.6 ± 1.12	26.8 ± 1.25
Oil	60.3 ± 3.54	57.4 ± 0.50	57.5 ± 0.69
Ash	3.9 ± 0.09	4.0 ± 0.67	4.5 ± 0.56
Defatted kernel meal			
Crude protein	66.4 ± 2.0	63.7 ± 1.11	62.4 ± 2.65
Ash	9.0 ± 0.58	9.4 ± 1.01	9.1 ± 1.04

None of the values for the three groups differed significantly (P>0.05)
[x] n = 3, [y] Compiled data from our previous studies

Amino acid composition

The amino acid composition of *J. platyphylla* kernel meal is almost similar to that in the toxic and non-toxic genotypes of *J. curcas* (Table 4). The levels of all essential amino acids except lysine are comparable with, or higher than, the FAO reference protein for a growing child of 2 to 5 years of age. A comparison between the amino acid composition of Jatropha meals and soybean meal reveal an almost similar pattern for all essential amino acids, except lysine and sulphur amino acids which were lower and higher respectively in Jatropha meals. The levels of essential amino acids in the Jatropha meals are higher than or similar to those in castor bean meal (Makkar et al., 1998a). From the crude protein content and summation of amino acid content, the non-protein nitrogen in *J. platyphylla* meal was < 5.0 % of the total nitrogen, suggesting the presence of high levels of true protein.

Table 4 Amino acid composition (g/16 g nitrogen) of kernel meals from *Jatropha plytaphylla,* toxic and non-toxic genotypes of *Jatropha curcas*, soybean meal and FAO reference protein

Aminoacids	*Jatropha platyphylla*	*Jatropha curcas*[x]		Soybean meal	FAO Reference protein[†]
		Toxic	Non-toxic		
Essential					
Methionine	1.58	1.56-1.91	1.38-1.76	1.32	2.50*
Cystine	1.55	1.77-2.24	1.58-1.81	1.38	
Valine	6.91	4.35-5.19	3.79-5.30	4.50	3.50
Isoleucine	4.10	3.93-4.53	3.08-4.85	4.16	2.80
Leucine	6.68	6.55-6.94	5.92-7.50	7.58	6.60
Phenylalanine	4.71	4.08-4.34	3.93-4.89	5.16	6.30**
Tyrosine	2.69	2.45-2.99	2.62-3.78	3.35	
Histidine	2.66	2.81-3.30	2.65-3.08	3.06	1.90
Lysine	3.16	3.63-4.28	3.40-3.49	6.18	5.80
Threonine	3.64	3.33-3.96	3.15-3.59	3.78	3.40
Tryptophan	1.06	1.31	ND	1.36	1.10
Non-essential					
Serine	5.05	4.67-4.80	4.59-4.91	5.18	--
Arginine	12.46	11.8-12.2	11.4-12.90	7.64	--
Glutamic acid	16.21	14.68-16.7	15.91-16.50	19.92	--
Aspartic acid	9.33	9.49-11.8	9.92-11.7	14.14	--
Proline	5.16	4.13-4.96	3.80-4.21	5.99	--
Glycine	4.56	4.40-4.92	4.18-4.61	4.52	--
Alanine	4.04	4.36-5.21	4.26-4.94	4.54	--

ND, Not determined; * Methionine plus cystine; ** Phenylalanine plus tyrosine. [x] Compiled data from our previous studies. [†] 2–5 year old children

Table 5 Pepsin plus trypsin digestibilities and available lysine of heated kernel meals from *Jatropha plytaphylla* and from toxic and non-toxic genotypes of *Jatropha curcas.*

	Jatropha plytaphylla	*Jatropha curcas*	
		Toxic	Non-toxic
Pepsin plus trypsin digestibility (% of total nitrogen)	97.1	93.7	92.1
Available lysine (mg/100 mg sample)	3.29	3.10	3.16
Available lysine (g/16 g N)*	4.95	4.87	5.06

* Calculated using crude protein content in the sample

Protein digestibility and available lysine

Pepsin plus trypsin digestibility simulate monogastric digestion. For all the heated kernel meals, pepsin plus trypsin digestibility values were similar and very high (Table 5). Likewise, the available lysine content was also similar (Table 5). It may be noted that the available lysine content when expressed as g/16 N (Table 5) was higher than the lysine content observed using amino acid analyzer (Table 4). In our earlier studies on Jatropha protein isolate (Makkar et al., 2008) and on landraces of rice (our unpublished results), we found a similar trend. It appears that the TNBS method for the available lysine determination overestimates lysine. The TNBS method could be a good method for evaluation of the effect of a treatment or processing condition, for example of heating on the available lysine content but would not correctly represent the absolute available lysine in a product. Nevertheless, it is evident from the results of this study that the contents of available lysine in kernel meals of *J. platyphylla* and the non-toxic genotype of *J. curcas* are similar, and the protein digestibility is very high.

Table 6 Fatty acid composition of oil from *Jatropha plytaphylla* and from toxic and non-toxic genotypes of *Jatropha curcas*.

Fatty acid composition (%)	*Jatropha plytaphylla*	*Jatropha curcas*[x]	
		Non-toxic	Toxic
Myristic, 14:0	0.2	0.2	0.1
Palmitic, 16:0	13.2	13.4	15.3
Heptadecanoic, 17:0	traces	0.1	0.1
Stearic, 18:0	7.5	6.4	6.6
Arachidic, 20:0	0.2	0.2	0.2
Behenic, 22:0	Not detected	Traces	Traces
Lignoceric, 24:0	Not detected	Traces	0.1
Total saturated	21.1	20.3	22.3
Palmitoleic, 16:1n-7	0.7	0.8	0.9
Oleic, 18:1n-9	23.1	36.5	41.0
Oleic, 18:1n-7	1.0	Not detected	Not detected
Eicosenoic, 20:1n-9	0-0.2	0.1	0.1
Total monosaturated	25.0	37.3	42.0
Linoleic, 18:2n-6	53.7	42.1	35.3
α-Linolenic, 18:3n-3	0.1	0.2	0.3
Total polyunsaturated	52.8	42.3	35.7

[x] Compiled data from our previous studies

Fatty acid composition

The saturated fatty acid levels in *J. platyphylla* oil were almost similar to that in the toxic and non-toxic genotypes of *J. curcas* (Table 6). The results showed that the oil is composed mainly of unsaturated fatty acids (oleic and linoleic acid). It is interesting to note higher linoleic acid level in *J. platyphylla* oil from the point of view of its beneficial effects on human health. The oil was free of the toxin, phorbol esters (see below; Table 7) and the seeds are consumed by communities in Mexico for centuries. The seeds therefore, could potentially be a source of edible oil.

Toxic and antinutritional factors

The main toxin present in *J. curcas* and other Jatropha species is phorbol esters, and the antinutritional factors present are trypsin inhibitor, lectins and phytate (Makkar and Becker, 2009). These along with other antinutritional factors were determined in *J. platyphylla* kernel meal.

Phorbol esters were absent in kernels of *J. platyphylla* and the non-toxic genotype of *J. curcas*, but were present in high concentration in the kernels of the toxic genotype (Table 7). In our earlier studies, this level of phorbol esters in the kernels (*ca* 2.80 mg/g) has been found to be toxic (Goel et al., 2007). The oil from *J. platyphylla* seeds was also free of phorbol esters and the same was the case for the non-toxic genotype of *J. curcas* (results not shown in table), while oil from the toxic genotype of *J. curcas* contained from 2 to 8 mg phorbol esters/g oil, depending on the provenances (data from our laboratory). Phorbol esters are diterpenes of phorbol type and they cause severe toxic symptoms in livestock. These are reported to mimic the action of diacyl glycerol, activator of protein kinase C, which regulates different signal transduction pathways. Interference with the activity of protein kinase C affects processes such as phospholipid and protein synthesis, enzyme activities, DNA synthesis, phosphorylation of proteins, cell differentiation and gene expression. They are also co-carcinogens and have purgative and skin-irritant activities. In humans accidental poisoning by Jatropha seeds has been reported to elicit giddiness, vomiting and diarrhoea. Mortality has also been reported in a number of animal species, both monogastrics and ruminants (Makkar and Becker, 1998; Makkar and Becker, 1999).

Trypsin inhibitor activity in *J. platyphylla* kernel meal was almost similar to that in the toxic and non-toxic genotypes of *J. curcas* (Table 7) and these values are similar to that in raw soybean meal (Aderibigbe et al., 1997).

Lectin activities, as determined by haemagglutintation assay, in the kernel meals from both the Jatropha species were almost similar. The same was the case for phytate content, which was very high (ca 9%). Phytate is known to decrease absorption of minerals, particularly calcium, zinc and iron. Therefore, the addition of phytase enzyme should be considered for feeds containing kernel meal from *J. platyphylla*, to mitigate adverse effects of phytate and to enhance mineral and other nutrient availability. Tannins, cyanogens, glucosinolates and amylase inhibitors were not detected in any of the Jatropha meals. Saponins were present in kernel meal of both the Jatropha species (1.94 to 3.4 %); however, these saponins did not possess haemolytic activity.

Non starch polysaccharide content was higher in the *J. plytaphylla* kernel meal compared to *J. curcas* or soybean meals (Table 8). These NSPs do not appear to be deleterious since inclusion of a substantial amount of the kernel meal (up to 75% replacement of fish meal protein) incorporation in fish diet did not elicit adverse effects (see below; section 'Fish Feeding Studies', subsection 'Growth performance and feed utilization').

The above results demonstrate the absence of the main toxin, phorbol esters in *J. platyphylla* kernel meal and the presence of three antinutritional factors, which need attention. These are trypsin inhibitor, lectins and phytate; and same is the situation for the non-toxic genotype of *J. curcas*, the heated kernel meal of which has been found be efficiently utilized in rats and fish (Makkar and Becker, 2009). The trypsin inhibitor and lectins are heat labile and can be destroyed by moist heating (Aderibigbe et al., 1997). The seeds of *J. platyphylla* and the non-toxic genotype of *J. curcas* are roasted and kernels consumed by local communities in certain regions of Mexico. Consumption of unroasted seeds of the non-toxic *J. curcas* is known to produce discomfort in humans and this could be due to the presence of trypsin inhibitor and lectins. In our earlier study on the non-toxic genotype of *J. curcas*, the roasting treatment has been found to reduce the level of trypsin inhibitor completely, while decrease in lectin activity was approximately 50 % and phytate level remains unchanged (Makkar et al., 1998a). Consumption of large amounts of kernels from the roasted seeds might produce discomfort due to the remaining lectin activity. In the present study, heated kernel meal of *J. platyphylla* was free of trypsin inhibitor and lectin, however phytate level was not affected. It may also be noted that phorbol ester level in the heated kernel meal from the toxic genotype of *J. curcas* was lower by only 5 % from that in the unheated kernel meal (results not shown in table).

Table 7 Levels of toxic and antinutritional factors in unheated kernel meals of *Jatropha plytaphylla* and of toxic and non-toxic genotypes of *Jatropha curcas*.

Component	*Jatropha plytaphylla*[a]	*Jatropha curcas*[x]	
		Toxic	Non-toxic
Phorbol esters (mg/g kernel)[b]	ND	2.79	ND
Total phenols (% tannic acid equivalent)	0.33 $\pm$ 0.01	0.36	0.22
Tannins (% tannic acid equivalent)	0.17 $\pm$ 0.02	0.04	0.02
Condensed tannins (% leucocyanidin equivalent)	ND	ND	ND
Phytates (% dry matter)	8.66 $\pm$ 0.93	9.40	8.90
Saponins (% diosgenin equivalent)	1.94 $\pm$ 0.44	2.60	3.40
Trypsin inhibitor (mg trypsin inhibited per g sample)	20.81 $\pm$ 0.78	21.31	26.54
Lectin activity (1/mg of meal that produced haemagglutination per ml of assay medium)	51–102	51–102	51–102
Glucosinolates	ND	ND	ND
Cyanogens	ND	ND	ND
Amylase inhibitor	ND	ND	ND

ND, Not detected

[a] n = 3

[b] As phorbol-12-myristate 13-acetate equivalent

[x] Compiled data from our previous studies

The protein efficiency ratio, weight growth and intake for rats fed diets containing raw kernel meal from the non-toxic genotype of *J. curcas*, devoid of phorbol esters, was significantly lower than for the diet containing the heated kernel meal. The heat treatment inactivated trypsin inhibitor and lectins leading to better performance of rats on the heat-treated kernel meal. However, when raw and heat treated Jatropha kernel meal from the non-toxic genotype was fed to carp (*C. carpio*), both groups grew to an almost similar extent (Makkar and Becker, 1998; Makkar and Becker, 1999). On the other hand, a diet containing kernel meal from the toxic *J. curcas,* at levels lower than *J. plytaphylla* kernel meal used in the present study, when fed to carp decreased body mass in 4 days. The fish refused the diet on the second day of feeding and abundance mucus was seen in the aquarium, but no fish died. Similar effects were observed when purified phorbol esters from Jatropha were mixed in standard fish feed at a level of 2 mg/g (Becker and Makkar, 1998). It would be interesting to study the effects of feeding heated *J. platyphylla* kernel meal, free of phorbol esters and trypsin and lectin

activities to fish, to ascertain its non-toxic nature. The results of this study are discussed below.

Table 8 Levels of constituent sugars of non-starch polysaccharides (NSP) in kernel meals (% in DM) of *Jatropha plytaphylla* and of toxic and non-toxic genotypes of *Jatropha curcas.*

	Rha	Fuc	Ara	Xyl	Man	Gal	Glu	Glc-A	GalA	Total-NSP
J. plytaphylla	0.3	0.1	3.1	2.0	0.5	1.4	5.7	0	3.0	16.0
J. curcas (Toxic)[x]	0.2	0.1	2.5	1.2	0.3	1.2	4.7	0.9	2.6	12.7
J. curcas (Non-toxic)[x]	0.2	0.1	2.7	1.4	0.3	1.2	4.7	0.0	3.0	13.6
Soybean meal	0	0	2.4	1.1	0.6	4.2	3.2	0	2.4	13.9

Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose, Glu, glucose; GlcA, Glucuronic acid; GalA, Galacturonic acid
[x]Compiled data from our previous studies

Fish Feeding Studies

Fish behaviour and feed intake

Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was normal. No left feed was observed in the aquaria. There was no mortality during the entire experimental period.

Growth performance and feed utilization

Nile tilapia diets containing heated *J. platyphylla* kernel meal, SBM and FM showed good growth performance. The body mass gain, specific growth rate, metabolic growth rate, feed conversion ratio, protein efficiency ratio, protein productive value, apparent lipid conversion and energy retention did not differ significantly ($P>0.05$) among the three groups (Table 9 -10). Presence of antinutrients such as phytate and NSPs, which are present in high amounts in the Jatropha kernel meal can adversely affects the feed utilization but in the current study they did not elicit any detrimental effects. In the plant protein based feeds, phytase was added at a level of 500 FTU per kg, which could have mitigated the potential adverse effects of phytate. The NSPs present in the *J. platyphylla* kernel meal do not appear be antinutrient.

Although, NSPs in *J. platyphylla* kernel meal is slightly higher than in soybean meal or in kernel meal of the non-toxic genotype of *J. curcas*, 75% replacement of fishmeal protein in the diet by heated kernel meal from the non-toxic genotype of *J. curcas* in common carp (*Cyprinus carpio*) and Nile tilapia did not decrease growth compared to the fish fed 100% fishmeal protein diet (our unpublished observations). As observed by us for the heated *J. platyphylla* kernel meal, Jackson et al. (1982) reported no negative effect on the feed intake and growth performance in tilapia fed diets in which wherein plant protein such as groundnut meal and SBM substituted 25% and 44% of FM protein respectively.

Table 9 Initial body mass (IBM) and growth performance of Nile tilapia (*Oreochromis niloticus*) fingerlings fed with experimental diets for 12 weeks

Treatment	IBM (g)	FBM (g)	BMG	SGR	MGR
Control	5.4 ± 0.24	27.1 ± 5.3[a]	403 ± 103[a]	1.9 ± 0.22[a]	7.20 ± 0.80[a]
Jatropha	5.4 ± 0.23	31.7 ± 7.31[a]	486 ± 132[a]	2.08 ± 0.26[a]	7.86 ± 0.98[a]
Soybean	5.4 ± 0.22	28.9 ± 7.81[a]	439 ± 143[a]	1.97 ± 0.28[a]	7.45 ± 1.04[a]
SEM	0.04	1.3	23.3	0.05	0.17

FBM (g) – Final body mass, BMG (%) - Body mass gain, SGR (%.day^{-1}) – Specific growth rate and MGR (gkg$^{0.8}$ day^{-1}) - Metabolic growth rate.
Mean values in the same column with different superscript differ significantly ($P < 0.05$).
Values are mean (n = 5) ± standard deviation.

Table 10 Nutrient utilisation of Nile tilapia (*Oreochromis niloticus*) fingerlings fed with experimental diets for 12 weeks.

Treatment	FCR	PER	PPV	ALC	ER
Control	2.08 ±0.20[a]	1.36 ± 0.13[a]	22.9 ± 3.24[a]	38.7 ± 4.99[a]	16.4 ± 1.93[a]
Jatropha	1.96 ± 0.18[a]	1.42 ± 0.14[a]	24.4 ± 3.99[a]	44.2 ± 14.33[a]	18.4 ± 3.95[a]
Soybean	2.07 ± 0.23[a]	1.37 ± 0.18[a]	25.0 ± 4.64[a]	33.8 ± 10.82[a]	16.3 ± 3.69[a]
SEM	0.04	0.03	0.98	2.81	0.83

Mean values in the same column with different superscript differ significantly ($P < 0.05$).
Values are mean (n = 5) ± standard deviation.
FCR – Feed conversion ratio, PER – Protein efficiency ratio, PPV (%) – Protein productive value and ER (%) – Energy retention, ALC (%) – Apparent lipid conversion

Hematological parameters

White blood cell (WBC) counts; Hb level, MCV, MCH and MCHC did not differ significantly among the groups (Table 11) and their ranges were in the normal range reported by Soltan et al. (2008) for Nile tilapia. Plant protein fed groups had significantly higher RBC count than control group. Plant ingredients may cause early release of immature erythrocytes, increasing the

RBC count. The hematocrit assay is normally used as a general indicator of fish health (NRC, 1981). Hematocrit level in all groups was also within the normal range (Soltan et al., 2008). The higher hematocrit levels observed in plant protein fed groups could be because of higher RBC count in these groups. Hct is sedimentation part of RBC. Hct and RBC count are directly related.

Calcium phosphorus, sodium and potassium ions in the blood

The blood ions of different experimental groups are presented in Table 13. Potassium ion in blood was higher in the control group than the two plant protein fed groups; whereas, phosphorus ion in blood exhibited a reverse trend. Calcium and Sodium ions did not differ significantly among the three groups. All these levels of ions were within the normal ranges for tilapia (Baruah et al., 2007). The higher phosphorus in plant based groups could be attributed to the increased availability of phytate-phosphorus by added phytase.

Metabolic enzymes activities in the blood

Alkaline phosphatase (ALP) and ALT are released into blood during organ damage (Racicot et al., 1975). Alkaline phosphatase level rises during bile duct obstruction, and in intrahepatic infiltrative diseases of the liver (Goel et al., 1984). Alkaline phosphatase is present in hepatocytes (liver cells). When liver cells are damaged, it leaks into the blood. Alkaline phosphatase rises dramatically in acute liver damage (Racicot et al., 1975). Thus, blood level of ALP and ALT gives information on the damage of organs and in particular of liver cells. Levels of ALP and ALT were similar in all the diets (Table 13), indicating normal organ function on feeding of *J. platyphylla* kernel meal. Sanden et al. (2006) also reported similar results on feeding SBM containing diets to Atlantic salmon.

Albumin, globulin and total protein concentration in the blood

The concentration of total protein in blood is used as a basic index for health and nutrititional status in fish (Martinez, 1976). Higher total protein, albumin and globulin were observed in the plant protein fed groups (Table 12). Globulin was higher in *J. platyphylla* kernel meal fed group, suggesting its immuno stimulating effect on the fish.

Table 11 Effects of experimental diets on the haematological parameters (red blood cells, RBC, 10^6 cells/mm^3); while blood cells, WBC, 10^5 cells/mm^3; hemoglobin, Hb, g/dl; hematocrit, Hct, %; mean cell volume, MCV (fL); mean corpuscular volume, MCH (pg); and Mean corpuscular hemoglobin concentration, MCHC (g/dl) of Nile tilapia (*Oreochromis niloticus*) fingerlings.

Treatment	RBC	WBC	Hb	Hct	MCV	MCH	MCHC
Control	1.26 ± 0.03[a]	2.9± 0.13[a]	5.16 ± 0.26[a]	28.32 ± 3.00[a]	408 ±13.2[a]	40.8 ± 1.32[a]	15.37± 1.68[a]
Jatropha	1.31 ± 0.02[b]	3.04 ± 0.61[a]	5.334 ± 0.50[a]	32.00 ± 2.00[b]	384.0 ±34.9[a]	38.4 ± 3.49[a]	16.70± 1.64[a]
Soybean	1.37[a] ± 0.02[b]	3.06 ± 0.07[a]	5.32 ± 0.28[a]	49.2 ± 5.36[b]	386± 16.9[a]	38.6± 1.69[a]	10.90 ± 1.08[b]
SEM	0.03	0.13	0.09	0.13	6.36	0.64	0.75

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 12 Albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood of Nile tilapia (*Oreochromis niloticus*) fingerlings.

Treatment	Albumin	Globulin	Total protein
Control	1.18 ± 0.18[b]	2.16 ± 0.11[b]	3.41± 0.34[b]
Jatropha	1.52 ± 0.20[a]	2.36 ± 0.19[a]	3.88 ± 0.29[a]
Soyabean	1.50 ± 0.23[a]	2.24 ± 0.05[ab]	3.66 ± 0.30[ab]
SEM	0.06	0.04	0.10

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 13 Effects of experimental diets on alkaline phosphatase (ALP, U/l), alanine transaminase (ALT, U/l), total   bilirubin (TBIL, mg/dl), blood urea nitrogen (BUN, mg/dl) and creatinine (mg/dl) in blood, blood ions (calcium (mg/dl), phosphorus mg/dl, sodium (mmol) and potassium (mmol) of (*Oreochromis niloticus*) fingerlings.

Treatment	ALP	ALT	TBIL	BUN	Creatinine	Calcium	Phosphorus	Sodium	Potassium
Control	52 ± 9.14[a]	95 ± 21[a]	0.42 ± 0.04[a]	2.00 ± 0.00[a]	0.6 ± 0.1[a]	13.1 ± 0.7[a]	8.8 ± 2.8[b]	153 ± 2.3[a]	4.2 ± 1.2[a]
Jatropha	52 ± 17.8[a]	114 ± 25[a]	0.34 ± 0.05[a]	2.20 ± 0.45[a]	0.3 ± 0.1[b]	15.2 ± 2.9[a]	13.5±3.0[a]	156 ± 4.1[a]	3.4 ± 1.3[ab]
Soybean	47 ± 6.8[a]	96 ± 37[a]	0.36 ± 0.15[a]	2.20 ± 0.45[a]	0.3 ± 0.1[b]	14.6 ± 3.1[a]	14.3±3.6[a]	156 ± 4.1[a]	2.2 ± 0.6[b]
SEM	2.98	32.43	0.02	0.09	0.1	0.6	1.0	1.1	0.3

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mMol of substrate per minute.

Total bilirubin, blood urea nitrogen, creatinine level in blood

Glucose level, total   bilirubin (TBIL), blood urea nitrogen (BUN) and creatinine in blood of different experimental groups are presented in Table 13. Blood urea nitrogen concentrations were in the normal ranges (Wedemeyer, 1996). Blood urea nitrogen concentrations are thought to be associated with liver or gill dysfunction (Stoskopf, 1993), as these are the sites of urea production and excretion, respectively. Blood urea nitrogen concentration did not differ significantly among the three groups, suggesting that *J. platyphylla* kernel meal fed groups was normal and healthy. Total bilirubin, an indicator of liver dysfunction (Tietz, 1986), was also similar for all groups. Creatinine is a degraded product of creatine, which is involved in muscle energy metabolism. Blood creatinine is normally quite stable. Its level in the blood becomes elevated if kidney function is impaired (Tietz, 1986); and it was highest in control group but was within the normal range. Creatinine is a metabolite of animal protein and its highest level in control is due to highest content of FM in this group.

Conclusions

The results of the fish studies showed that the feeding of heated *J. platyphylla* kernel meal exhibit good growth performance, and all biochemical and hematological parameters that are markers of toxicity are within the normal ranges and similar to those in control group (fish meal fed group). These observations confirm the non-toxic nature of the heated *J. platyphylla* kernel meal.

The kernels are rich in protein and energy and kernel meal is rich in protein. Furthermore, the quality of protein and oil is good. This species could help alleviate protein and energy deficiency in malnourished children in the lower mountain skirts of pacific coastal areas of Mexico. It could also be used to produce new cultivars with desirable traits using conventional inter species cross breeding and molecular breeding techniques

This study is a good example of the marriage of ethnic wisdom and science. It has presented a new, non-toxic Jatropha species to the world by putting traditional knowledge in a scientific context. *J. platyphylla* is another gift from Mexico to the World and may be added to the long list of edible plants to have come from that region.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Chapter 10

Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed a diet containing *Jatropha platyphylla* kernel meal as a protein source

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Growth performance and metabolic efficiency in Nile tilapia (*Oreochromis niloticus* L.) fed a diet containing *Jatropha platyphylla* kernel meal as a protein source

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Abstract

Jatropha platyphylla is available on the pacific coast from Sinaloa to Michoacán including the Nayarit and Jalisco states in Mexico. The seeds of *J. platyphylla* are rich in oil and protein, and the kernel meal (JPKM) prepared after oil extraction contains 70-75% crude protein. Contents of essential amino acids (except lysine) are higher in JPKM than in soybean meal (SBM). Phorbol-esters, the main toxin present in most Jatropha species is absent in *J. platyphylla*. Heat treated JPKM (H-JPKM)was evaluated as a protein supplement in tilapia feed and compared with that of SBM and fishmeal (FM). Nile tilapia (*Oreochromis niloticus* L.) fingerlings (15; av. body mass 13.9±0.17 g) were randomly distributed in three groups with five replicates each. A 12-week experiment was conducted in a respirometer system to evaluate the growth performance, nutrient utilization and energy budget. Nile tilapia fingerlings were fed three iso-nitrogenous diets (36% crude protein): Control containing fishmeal (FM), and Jatropha and Soybean diets in which 62.5% of FM protein was replaced by SBM and H-JPKM respectively. The growth performance, feed-conversion-ratio, protein-efficiency-ratio, apparent lipid conversion and energy-retention did not differ significantly among the three groups. Higher protein productive value was observed in plant protein fed groups. Average metabolic rate, energy expenditure per g protein fed and retained in the body did not differ significantly among the three groups. Conclusively, Nile tilapia fed plant protein (heated JPKM and SBM) and FM protein based diets exhibited equal average metabolic rate which indicate that JPKM can be used as a protein source in aqua feed.

Keywords: Nile tilapia, *Jatropha platyphylla*, kernel meal, growth, metabolic efficiency

Introduction

Despite the fact that fish meal (FM) is the single most expensive major ingredient in aquaculture feeds, it is widely used as the main source of dietary protein for most commercially farmed fish species (Tacon, 1993; Cavalheiro et al., 2007; Davis et al., 2010). In the mean time, the shortage in world production of FM, coupled with increased demand and competition with terrestrial domestic animals, has further increased FM prices (FAO, 2007; Cavalheiro et al., 2007). In recent years the rapid expansion of fish culture is demanding the development of nutritious fish feeds as well as their better, mainly due to the fact that increasing feed cost may increase the cost of fish production by 50–80% (Cavalheiro et al., 2007). Tilapia is one of the oldest and most rapidly growing cultured finfish species in modern times. Based on species, tilapia represents the third largest group of farmed finfish species after the cyprinids and salmonids (FAO, 2007). Among the major commercial finfish species, tilapias have become a major source of protein around the world, primarily because of their outstanding adaptability under a wide range of environmental conditions and excellent growth on a variety of natural and prepared diets (Lim and Webster, 2006; Davis et al., 2010).

In the production of compound feed for the growing aquaculture industry, there is a constant search for feed ingredients that maximize production of fish while requiring less energy for metabolic activities. Measured oxygen consumption can yield important information on the metabolic rate. Metabolism can be used to calculate the dietary energy requirement, which is an important economic parameter in modern aquaculture. Many factors have an effect on metabolism, e.g. age of fish, time of day, body size, activity of fish, breeding season, light, temperature, feeding frequency, and feed composition (Yager and Summerfelt, 1993). Little information is available on the impact of nutrient source on the metabolism of fishes (Tandler and Beamish, 1981, Cai and Summerfelt, 1992; Forsberg and Summerfelt, 1992). The partition of ingested energy into growth, metabolism, excretion and feces may vary among fish species depending on factors such as diet composition (Pandian, 1987; Cui et al., 1992), activity level (Pérez-Pinzón and Lutz, 1991; Shulman and Love, 1999; Wuenschel et al., 2004; Han et al., 2004). A balanced energy budget is a tool for bioenergetics modeling in aquaculture (Jobling, 1993). A number of reports on metabolism of or oxygen consumption in tilapia have been published (Meyer-Burgodorff et al., 1989; Becker and Fiscelson, 1990; Yamamoto, 1992; Fernandes and Ratin, 1994; Mamun et al., 2007). Most of

these studies report metabolism in relation to body size, swimming speed and salinity; and some report fasting metabolism. Also studies have been carried out on the relationship of dietary energy and protein content to fish growth (Ogino et al., 1976; Schwarz et al., 1983; Gongnet et al., 1987; Lupatsch et al., 1998; Peres and Oliva-Teles, 2001; Gauquelin et al., 2007), while others deal with the nutritional energetics related to the effect of diets on energy budget (Cui et al., 1992; Helland and Helland, 1998; Francis et al., 2002; Azevedo et al., 2005; Mamun et al., 2007). However, little work has been done to examine the effects of dietary plant proteins on energy allocation in fish (Refstie and Tiestra, 2003).

In our previous study (Makkar et al., 2011), we observed that 50% replacement of FM protein by heated *Jatropha platyphylla* kernel meal (H-JPKM) in Nile tilapia (*Oreochromis niloticus* L.) diet exhibited similar growth performance and nutrient utilization as control group (FM as protein source) therefore in this study we decided to replace 62.5 % FM protein by H-JPKM. Our previous work describes the effects of heated H-JPKM (62.5 % FM protein replaced) on growth performance, biochemical and hematological changes in Nile tilapia (Akinleye et al., 2010). The present paper is the second in a series, focusing on the effects of dietary H-JPKM on growth performance, feed utilization and interrelationships among the major components of the energy budget of Nile tilapia, *Oreochromis niloticus* (L.), and on comparison of these parameters with those obtained on dietary inclusion of soybean meal (SBM) and FM.

Materials and Method

Diet Formulation

Fish meal and wheat meal were purchased from Kurt Becker GmbH, Bremen, Germany, and a German local supermarket respectively. Defatted and roasted SBM was obtained from Institute of Animal Nutrition (450), University of Hohenheim, Germany. Deshelled Jatropha seeds were supplied by Centro de Investigación en Alimentación y Desarollo (CIAD), Hermosillo, Sonora, Mexico and defatted in our institute. The defatted kernel meal was heat treated (121 ºC, 20 min, 66% moisture) to inactivate lectins and trypsin inhibitor; and is designated as H-JPKM, hereafter. Prior to feed formulation, the proximate composition of defatted Jatropha meal, wheat meal, SBM and FM were determined.

A total of three isonitrogenous and isoenergetic diets were formulated. Control diet was prepared with FM and wheat meal, without

any H-JPKM and SBM included. Jatropha diet was prepared by replacing 62.5% FM protein with H-JPKM, and Soybean diet was prepared by replacing 62.5% FM protein with SBM. Composition of the experimental diets is shown in Table 1 of our previous published article (Akinleye et al., 2010). The final mixture of each diet was made into 2 mm diameter moist pellets and then freeze-dried. Experimental diets containing crude protein 36%, crude lipid 8%, vitamin premix 2% and mineral premix 2%. Lysine monohydrochloride (lysine 80% in this salt) was supplemented at the rate of 2.14% and 0.2% of H-JPKM and SBM inclusion in the diets respectively. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg.

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets		
	Control	Jatropha	Soybean
FM	425	159	159
Soybean meal	-	-	347.4
Wheat meal	495	487.4	367
Jatropha meal	-	248	-
Wheat gluten	-	1	22
Sunflower oil	40	64.6	64.6
Vitamin premix[1]	20	20	20
Mineral premix[2]	20	20	20
Total	1000	1000	1000
Lysine monohydrochloride	-	5.5	0.6
Phytase (FTU/kg)	-	500	500

[1]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[2]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 502; $MgSO_4. 7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4. H_2O$, 3.12; $ZnSO_4. 7H_2O$, 4.67; $CuSO_4. 5H_2O$, 0.62; KI, 0.16; $CoCl_2. 6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

Experimental animal and experimental set-up

Male tilapia (*Oreochromis niloticus*) fingerlings (12.0 - 15.0 g) were selected from our Aquaculture laboratory, University of Hohenheim, Stuttgart, Germany. Fish were kept individually in the respirometric chamber (5 l) for acclimatization period of one week during which they were fed Hohenheim standard fish diet containing 35%   protein, 8% lipid, 10% ash and with

a gross energy content of 20 kJ g^{-1} dry matter. The experiment was performed in a respirometric system (see next section).

Fingerlings 15 fish (13.9 ± 0.17 g) were randomly distributed in three treatment groups with five replicates. Duration of the experiment was 12 weeks. The system was subjected to a photoperiod of 12 h light: 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 - 27.1°C, pH 7.0 - 7.5, dissolved oxygen 6.9 – 7.4 mg l^{-1}, total NH_3 0.1 - 0.2 mg l^{-1}, nitrite 0.07 - 0.1 mg l^{-1} and nitrate 1 - 3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80 %.

Respirometric System

Fish were kept in individually in respiration chambers of 5 l water capacity, fully automated and computer controlled as described by Focken et al. (1994). After the acclimatization period, fish were starved for a day and then the three experimental diets were fed. The feeding schedule is given in the next section. Thirty-two measurements of oxygen consumption per individual fish were made every 24 h and recorded on the hard disk of the computer. The system was lit with fluorescent tubes to give a day length of 12 h. The water flow rate through the respirometer chambers (controlled by electronic flow meters connected to each chamber) was adjusted between 0.5 and 0.8 l min^{-1} on average, water in the chamber was replaced 10 times per day. Once a week, the fish were weighed, the chambers cleaned and the oxygen electrode calibrated. During the experimental period, all water quality parameters were maintained at optimum range.

Feeding schedule

The three experimental diets namely Control, Soybean and Jatropha were assigned to experimental animals in a random manner. Fish were fed 5 times their maintenance requirement (3.2*5*(fish wt (g)/1000)$^{0.8}$), split into five equal rations per day (at 8.00, 11.00, 14.00, 17.00 and 20.00 h) daily. The feeds were dispensed using automatic feeders. Fish were individually weighed at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feeding level for subsequent week.

Pre and post experimental activities

Before the experiment commenced, nine fish from same population were

killed and preserved at −20 °C for the analysis of the initial body composition. The experiment lasted 12 weeks and fish were sampled thereafter. At the end of experiment, fish were killed by a blow to the head with metal rod and then stored at −20 °C for chemical composition analysis. Prior to determination of the proximate composition, the fish were autoclaved at 121 °C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and freeze-dried.

The University animal welfare committee (University of Hohenheim Germany) approved all experimental procedures involving tilapia.

Biochemical analyses

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Experimental diets and animals were analyzed for dry matter (DM), ash, crude protein (CP), and lipid. Gross energy (GE) determination was done using bomb calorimeter (IKA C7000) and benzoic acid was used as a standard.

Calculation of growth performance and nutrient utilization parameters

All calculations were performed for each fish individually. Growth performance and nutrient utilization were assessed in terms of body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), energy retention (ER) and apparent lipid conversion (ALC).

BMG = Final body mass - initial body mass, BMG (%) = [(Final body mass - Initial body mass) / Initial body mass]*100, SGR (% day^{-1}) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days]*100, MGR (gkg$^{0.8}$ day^{-1}) = (Body mass gain, g) / [{(initial body mass, g / 1000)$^{\wedge 0.8}$ + (final body mass,g / 1000)$^{\wedge 0.8}$}/2] / duration of the trial days, FCR = dry feed fed (g)/body mass gain (g), PPV (%) = [(final fish body protein, g - initial fish body protein, g) / total protein consumed, g]*100, ALC (%) = [(final fish body lipid, g - initial fish body lipid, g) / total crude lipid consumed, g]*100, ER (%) = [(final fish body energy - initial fish body energy)/ (gross energy intake)]*100 and PER = fresh body mass gain (g)/crude protein fed (g)

Calculation of oxygen consumption and energy budget

All calculations were performed for each fish individually. Energy budget

was measured in the respirometric system in terms of routine metabolic rate (RMR), energy retention (ER), energy expenditure (EE), metabolizable energy (ME), apparent unmetabolized energy (AUE), efficiency of energy retention (ER/EE). The under listed formulae were used.

- The oxygen consumption was calculated based on the data gathered from the respirometric system.
- RMR = Oxygen consumed (mg/h)/[fish wt (g)]$^{\wedge 0.8}$
- The heat dissipation or energy expenditure (EE) of fish during the whole experimental period was calculated as:

  EE (kJ) = total oxygen consumption (g) X Q_{oxy});

  Where, Q_{oxy}, the oxyenergetic equivalent for growth, was 14.85 kJ for each gram of oxygen consumed by the fish (Huisman 1976).
- The total gross energy of fish at stocking time (E_{ini}) and at the end (E_{fin}) of the experiment, which were calculated as follows:

  E_{ini} (kJ) = (initial dry weight of fish (g) X gross energy (kJ g^{-1}) of dried fish)

  E_{fin} (kJ) = (final dry weight of fish (g) X gross energy kJg^{-1}) of dried fish);
- The total energy contained in the consumed feed (based on the amount of feed offered): E_{feed} (kJ) = F X $GE_{feed;}$ where F in g (DM), is the total amount of feed dispensed and GE_{feed} in kJ g^{-1} is the gross energy content of the feed.
- The energy retained in the fish body (tissue) was calculated as: ER = E_{fin} - E_{ini}.
- Heat released (kJ kg$^{\wedge 0.8}$ d^{-1}) = (AMR X 24 X 14.85)/1000
- Metabolizable energy (ME) intake (kJ) = EE (kJ) + ER (kJ)
- AUE (kJ) = E_{feed} (kJ) – (EE + ER).

Statistical analysis

All data collected were subjected to one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Duncan's multiple range test ($P<0.05$). SAS Version 9.1 (Statsoft Inc., Tulsa, USA) was used for determining the level of significance and values are expressed as means ± standard deviation.

Results

We observed that palatability and acceptability of feed was good and the behaviour of fish was normal during the whole experimental period of time. We did not observe any residual feed in the aquaria.

Whole body composition parameters such as moisture content,

crude protein, crude lipid and crude ash did not differ significantly among the three groups (Table 2). Highest gross energy content of the whole body was observed in soybean group, which was statistically not different (P > 0.05) to that in Jatropha group but was statistically higher (P < 0.05) than that in control group.

Weekly body mass developments of different groups are presented in Figure 1. Growth performance and nutrient utilization parameters are shown in Table 3. Body mass gain, SGR, MGR, PER, ER and ALC did not differ significantly among the three groups. Highest PPV was observed in soybean group, which was statistically not different (P > 0.05) to that in Jatropha group but was statistically higher than that in control group (Table 3).
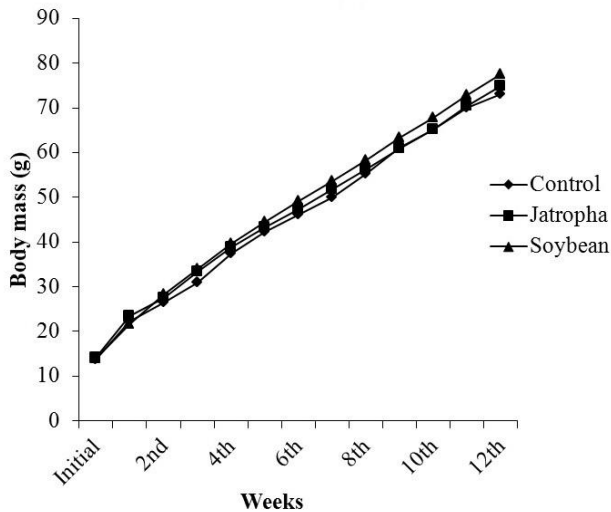
Fig. 1 Weekly body mass development (g) of Nile tilapia (*Oreochromis niloticus*) juveniles fed experimental diets for 12 weeks.

Routine metabolic rate, energy expenditure per g protein fed and energy expenditure per g protein retained in the body did not differ significantly among the three groups (Table 4). Highest gross energy uptake was observed in soybean group, which was statistically not different (P > 0.05) to that in Jatropha group but was statistically higher than that in control group (Table 5). Each component of energy budget (energy expenditure, energy retention, metabolisable energy ingested and apparently unmetabolised energy) did not differ significantly among the three groups (Table 5).

Table 2 Chemical composition of body tissue of Nile tilapia (*Oreochromis niloticus*) juveniles at the start and at the end of the experiment (%, wet basis ± SD)

Treatment	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)
Initial fish	73.6 ± 2.0	17.3 ± 0.50	5.6 ± 1.20	3.0 ± 1.10	5.9 ± 1.50
Control	72.6 ± 1.49[a]	16.1 ± 0.49[a]	6.8 ± 0.56[a]	3.8 ± 0.18[a]	4.8 ± 0.41[b]
Jatropha	72.9 ± 1.77[a]	16.4 ± 0.87[a]	6.8 ± 0.87[a]	3.9 ± 0.28[a]	4.9 ± 0.60[ab]
Soybean	72.1 ± 2.26[a]	16.9 ± 0.41[a]	7.0 ± 0.55[a]	4.0 ± 0.17[a]	5.5 ± 0.48[a]
SEM	0.66	0.17	0.16	0.05	0.15

Mean values in the same column with different superscript differ significantly (P < 0.05). Values are mean (n = 5) ± standard deviation.

Table 3 Growth performance and nutrient utilization in Nile tilapia (*Oreochromis niloticus*) juveniles fed the experimental diets for 12 weeks

Treatment	IBM (g)	FBM (g)	BMG (%)	SGR (%)	MGR (g kg$^{0.8}$ day^{-1})	FCR	PER	PPV (%)	ALC (%)	ER (%)
Control	13.8 ± 0.14[a]	73.0 ± 2.78[b]	430 ± 25.2[a]	2.0 ± 0.15[a]	9.1 ± 0.21[a]	1.7 ± 0.06[a]	1.6 ± 0.06[a]	24.3 ± 1.38[b]	53.6 ± 4.31[a]	15.1 ± 1.62[a]
Jatropha	14.1 ± 0.23[a]	74.8 ± 1.13[ab]	431 ± 26.1[a]	2.0 ± 0.1[a]	9.1 ± 0.16[a]	1.7 ± 0.03[a]	1.6 ± 0.03[a]	25.5 ± 2.10[ab]	48.7 ± 6.60[a]	14.9 ± 1.99[a]
Soybean	13.9 ± 0.11[a]	77.4 ± 1.28[a]	456 ± 29.3[a]	2.0 ± 0.12[a]	9.3 ± 0.19[a]	1.7 ± 0.06[a]	1.7 ± 0.06[a]	26.9 ± 1.62[a]	49.6 ± 4.19[a]	16.7 ± 1.70[a]
SEM	0.03	0.66	4.58	0.01	0.05	0.02	0.02	0.5	1.36	0.48

IBM- Initial body mass, FBM- Final body mass, BMG - Body mass gain, SGR – Specific growth rate and MGR - Metabolic growth rate; FGR – Feed conversion ratio, PER - Protein efficiency ratio, PPV - Protein productive value, ER (%)- Energy retention, ALC (%)- Apparent lipid conversion
Values are mean (n = 4) ± standard deviation.
For all parameters, mean values in the same column were not significantly different (P < 0.05).

Table 4 Routine metabolic rate and energy expenditure per g protein fed and retained in Nile Tilapia (*Oreochromis niloticus*)

	Control	Jatropha	Soybean	SEM
Routine metabolic rate (mgO$_2$kg$^{\wedge 0.8}$ h^{-1})	318 ± 18.91 [a]	303 ± 18.79 [a]	304 ± 24.26 [a]	5.28
Energy expenditure (EE)/g protein fed (kJ)	12.4 ± 0.65 [a]	11.9 ± 0.65 [a]	11.6 ± 0.83 [a]	0.19
Energy expenditure (EE)/g protein retained (kJ)	35.2 ± 1.66 [a]	35.1 ± 0.87 [a]	36.3 ± 1.54 [a]	0.37

Mean values in the same row with different superscript differ significantly (P < 0.05). Values are mean (n = 5) ± standard deviation.
Metabolic rate = Oxygen consumed (mg/h)/[fish wt (g)]$^{\wedge 0.8}$

Table 5 Energy budget of Nile tilapia (*Oreochromis niloticus*) in different experimental groups

	Control	Jatropha	Soybean	SEM
Initial GE content of carcass (kJ)	52.6 ± 0.55	53.9 ± 0.89	53.2 ± 0.41	0.21
Final GE content of carcass (kJ)	348 ± 20.9 [b]	370 ±48.3 [b]	426 ± 33.48 [a]	12.32
Feed GE uptake (kJ)	1798 ± 56.1[b]	1856± 22.1[ab]	1889 ± 49.67[a]	19.46
Energy expenditure (kJ)	758 ± 56.9[a]	731 ± 24.3[a]	776 ± 33.99[a]	16.06
Energy expenditure (EE; % of GE fed)	42.2 ± 3.91[a]	39.4 ± 2.4[a]	41.1 ± 2.17[a]	1.73
Energy retention (kJ)	602 ± 22.1[a]	638 ± 47.6[a]	619 ± 43.39[a]	15.78
Energy retention (ER; % of GE fed)	33.5 ± 1.54[a]	34.4 ± 2.4 [a]	32.8 ± 2.17 [a]	1.39
Metabolizable energy (ME) ingested (kJ)	1360 ± 62.6[a]	1369 ± 56.7[a]	1395 ± 36.37[a]	23.60
Metabolizability (ME % of diet)	75.7 ± 5.23 [a]	73.8 ± 4.7[a]	73.9 ± 3.37[a]	2.08
Apparently unmetabolised energy (AUE) (kJ)	437 ± 63.31[a]	486 ± 43.7[a]	493 ± 50.09[a]	12.84
Apparently unmetabolised energy (AUE; % of GE fed)	24.3 ± 2.23[a]	26.2 ± 2.7[a]	26.1 ± 1.37[a]	2.23

Mean values in the same row with different superscript differ significantly (P < 0.05). Values are mean (n = 5) ± standard deviation.

Discussion

The results of the present investigation demonstrate that H-JPKM is a good dietary protein source for Nile tilapia feed because growth performance and feed utilization of Jatropha group was similar to control group (FM fed group) and Soybean meal group.

No significant difference in growth performance (BMG, SGR and MGR) and feed utilization parameters (FCR, PER, ALC and ER) show that digestion and absorption of nutrients from H-JPKM and FM were similar. Nutrient utilization values observed in this study signify excellent utilization of the diets. Growth performance of H-JPKM fed group was similar to FM group indicating that nutrients and energy availability from the H-JPKM for the protein synthesis were similar to animal protein (FM). In the present study SGR was about 2.0 which is also indicative of good growth performance. Ogunji and Wirth (2000) observed SGR of 3.3 when *Oreochromis niloticus* fingerlings were fed FM based diets. Feed utilization could have been adversely affected by the presence of high amount of antinutrients such as phytate in the H-JPKM (Makkar et al., 2010, 2011) but surprisingly, in the present study it did not induce any unfavorable effects. Phytase were supplemented at the rate of 500 FTU per kg in plant protein based feeds and that could have eased the anti-nutritive effects of phytate. We did not observe any harmful effects of non starch polysaccharides (NSPs) in the present study; the level of NSPs in H-JPKM is almost similar to SBM. Jackson et al. (1982) reported that substitution of FM protein by 25 and 44% plant proteins by groundnut meal and SBM respectively did not adversely affect the feed intake and growth performance in tilapia; and in our study 62.5% of FM protein replacement by plant proteins showed similar growth performance and feed utilization. A number of studies have been conducted using processed SBM as a FM replacer in tilapia feeds. In general the studies have shown that between 67% to 100% of the dietary protein can be supplied in the form of SBM; the inclusion level depending upon a variety of different factors, including fish species and size, SBM source and processing method, aqua feed processing and manufacturing method, and culture system employed. For example, prepressed solvent extracted or full-fat SBM, with or without methionine supplementation successfully replaced up to 75% of FM in the diets of *O. niloticus* fry (Pantha, 1982; Tacon et al., 1983), *O. mossambicus* (Jackson et al., 1982), and tilapia hybrids (Shiau et al., 1989). Many authors (De Silva and Anderson, 1995; Ogunji and Wirth, 2000) have reported that FCR ranges from 1.2 - 1.5, whereas in the present study we

observed FCR was about 1.7. It is evident that H-JPKM is a promising protein source for incorporation in Nile tilapia feed from the point of view of growth performance and feed utilization parameters.

A comparison was made of the growth rate and energy budget in Nile tilapia fed different experimental diets. Throughout the experiment, no difference in oxygen consumption of fish in FM, H-JPKM and SBM fed groups indicate similar metabolic activity, from start to end of the experiment, among the groups. Similar results have been reported by Suárez et al. (2009), wherein for white shrimp (*Litopenaeus vannamei*) juveniles fed soybean-canola meal (at an 80% substitution level) energy budget did not differ significantly for plant protein and FM protein fed groups. Kumar et al. (2010a) also observed that energy budget did not differ significantly when common carp are fed with plant protein (detoxified Jatropha kernel and SBM) and FM containing diets.

In this study, routine metabolic rate was not significantly affected by dietary treatments. The routine metabolic rate is attributed to a multitude of factors associated with feed utilization; it is generally accepted that its magnitude is mainly due to deamination of ingested amino acids (Cho and Kaushik, 1990). However, while some authors observed a change in the routine metabolic rate with different types of protein sources (Jobling and Davies, 1980; Helland et al., 2006; Hatlen et al., 2007), surprisingly we were not able to find any effect of H-JPKM protein on this parameter compared to FM and SBM. In the present study routine metabolic rate did not differ significantly among the three groups and this value were in the range of 303 - 318 $mgO_2kg^{0.8} h^{-1}$. Becker and Fishelson (1990), Schreiber et al. (1998), Focken et al. (2000) and Francis et al. (2001) have also found similar values of routine metabolic rate for Nile tilapia under similar feeding conditions. However, Mamun et al. (2004, 2007), found lower (145 - 156 $mgO_2kg^{0.8} h^{-1}$) value of routine metabolic rate in conventional, genetically improved and genetically male Nile tilapia, *Oreochromis niloticus* (L.).

The metabolizable energy observed in the present study was higher than what has been observed in studies of other fish species in which estimates are usually between 60% and 70% (Carter and Brafield, 1991; Schwarz and Kirchgessner, 1995; Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999; Booth and Allan, 2003; Lupatsch et al., 2003; Lupatsch and Kissil, 2005). Our value was similar to those found in European eel (*Anguilla anguilla*) (76%, Owen et al., 1998), and Atlantic salmon (80 – 81%, Helland et al., 2006; Hatlen et al., 2007). Our data suggest that the Nile tilapia is relatively efficient in utilising dietary FM and plant proteins for growth. The diets used in the present study were based on high quality fish meal, JPKM

and SBM as the major protein source and had a well-balanced amino acid profile and high digestible protein and energy.

Cui and Liu (1990) constructed average energy budgets for six teleost species fed *ad libitum* and found that heat loss was always the largest component, 50 - 69% of consumed energy. Whereas the energy used for growth was much smaller, 21 – 35%. In the present study, for all the experimental diets, the energy retained for growth was between 32.8 and 34.4%, whereas energy expenditure was between 39.4 and 42.2%. Metabolizable energy of the diet ranged from 74 – 76%. Conclusively, Nile tilapia fed plant protein (H-JPKM and SBM) and FM protein based diets exhibited similar average metabolic rate.

In the current study the moisture, protein, lipid and ash content in whole body of fish did not differ significantly among the three groups. Higher ($P < 0.05$) gross energy content in whole body was observed in plant protein fed groups than FM fed group. Similar trend has been observed by Kumar et al. (2010b). They found that detoxified Jatropha kernel meal and soybean meal based diets increases the lipid and gross energy content in common carp (*Cyprinus carpio* L.). The replacement of FM by plant protein sources such as corn gluten meal and soy protein concentrates has been shown to increase hepatic lipogenic enzyme activities in seabass (Dias, 1999; Kaushik et al., 2004), which could lead to higher whole body lipid and higher value of gross energy content of whole body. Crude protein content of fish was similar in all groups. Cheng et al. (2003) reported that the plant protein (soy protein) based diets decrease nitrogen retention in salmon and trout because these diets have lower digestible energy content and an amino acid profile that is sub optimal for muscle growth. Interestingly in our study, crude protein content of fish fed plant protein was similar to those fed FM. The protein accretion in the test groups (Jatropha and Soybean), equivalent to that in control group, could be attributed to almost similar amino acid contents in H-JPKM, SBM and FM, and the supplementation of lysine in the diets (Jatropha and Soybean). In addition, combination of H-JPKM or SBM with FM in the diets would have also contributed to this equivalent protein accretion. Jahan et al. (2003) have shown that proper combination of FM and plant proteins increases protein retention in carp. These results suggest that H-JPKM and SBM containing diets have optimal digestible energy and a balanced amino acid profile for optimum growth of Nile tilapia.

Conclusion

The results of current study showed that the heated *J. platyphylla* kernel meal (H-JPKM) fed group exhibited good growth performance (almost five to six times increased in fish body mass within 12 weeks). Overall, growth performance, feed utilization and energy budget of fish fed H-JPKM and fish meal were similar, demonstrating that H-JPKM has a potential for use in aquaculture diets. It can be used as a promising fish meal replacers in the diets of Nile tilapia.

Acknowledgement

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Chapter 11

Jatropha platyphylla kernel meal as feed ingredient for Nile tilapia (*Oreochromis niloticus* L.): Growth, nutrient utilization and blood parameters

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Abstract

Jatropha platyphylla is a multipurpose and drought-resistant shrub, available in Mexico, locally known as "sangregrado" and belonging to the family *Euphorbiaceae*. Its seeds are rich in oil and protein and do not contain the main toxin, phorbol esters present in other Jatropha species. *Jatropha platyphylla* kernel meal (JPKM) obtained after oil extraction contained 70-75% crude-protein; however, it contained phytate, lectin and trypsin-inhibitor. The levels of essential amino acids (except lysine) were higher in JPKM than in soybean meal (SBM). Using Nile tilapia (*Oreochromis niloticus*) fingerlings a 12-week experiment was conducted to evaluate the nutritional quality of the heated JPKM and compare with that of SBM and fishmeal. Fingerlings 15 fish; average weight 13.7±0.21 g were randomly distributed in three treatment-groups with five-replicates. Fish were fed three iso-nitrogenous diets (crude-protein 36%) control diet containing fishmeal based protein and two other diets replacing 62.5% fismeal protein with JPKM (Jatropha group) and SBM (Soybean group). The growth performance, feed-conversion-ratio, protein-efficiency-ratio and energy-retention did not differ significantly among the three groups. Higher protein productive value was observed in plant protein fed group than control group whereas apparent lipid conversion exhibited reverse trend. RBC count, hematocrit and blood glucose contents were higher in plant-protein fed groups than control group, other hematological-parameters (WBC count, haemoglobulin, mean-cell-volume; calcium and sodium ions, total-bilirubin and urea-nitrogen in the blood) and metabolic enzymes (alkaline-phosphatase and alanine-transaminase) activities in blood did not differ significantly among the three groups. The results from the present study established that JPKM is a promising and good quality protein source for Nile tilapia feed.

Keywords: Nile tilapia, *Jatropha platyphylla*, growth, nutrient utilization, hematology

Introduction

Aquaculture is growing rapidly at an average rate of 8.9% per year, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems (FAO, 2007). This rapid growth is because of the intensification of the culture systems. Nile tilapia, an important food fish in developing countries is widely used in commercial farming systems for intensive aquaculture (Fitzsimmons, 2000). The high level of intensification requires improved production technology, including efficient low-cost and low-pollution feeding systems. Fish meal (FM) is still a preferred protein source for fish diets corresponding to its high protein quality (NRC, 1993). However, due to high cost and limited availability in many countries (Naylor et al., 2000), the replacement of fish meal by plant protein sources is of great interest (Mabahinzireki et al., 2001). Our previous studies demonstrate that plant protein (*Moringa oleifera* leaf meal, *Sesbania aculeate* seed meal, detoxified Jatropha kernel meal) could partially replace FM in the diet of tilapia, *Oreochromis niloticus* and common carp, *Cyprinus carpio* (Hossain et al., 2001; Richter et al., 2003; Dongmeza et al., 2006, Makkar et al., 2009; Kumar et al., 2008, 2010a,b,c).

Soybean meal (SBM) is currently the most commonly used plant protein source in fish feeds and amounts to 50% of the diet of freshwater omnivorous fish species (Yue and Zhou, 2009). However this over dependence on SBM and its other food and feed uses are increasing its price. In addition, SBM competes with human food and hence there is a need to explore other inexpensive plant protein source for use in fish diet.

Jatropha platyphylla (locally called "sangregrado") is a drought-resistant shrub or tree, 2 to 5 m high, almost glaborous and belong to the family *Euphorbiaceae.* The species is restricted to warm areas (average temperature 20–29 °C) on the pacific coast from Sinaloa to Michoacán including the Nayarit and Jalisco states in Mexico and is usually found in and around deciduous forests. It has thick succulent branches, large peltate glaborous leaves (25-35 cm) on long petioles, and white urceolate flowers that is held on long and branched florescence (Deghan, 1982). The seed kernel contains about 50 - 60% oil which can be used as edible oil or can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar et al., 2010). The kernel meal obtained after oil extraction is an excellent source of nutrients and contains 70 to 75 % crude protein (Makkar et al., 2010). The levels of essential amino acids (except lysine) are higher in defatted *J. platyphylla* kernel meal than SBM

(Makkar et al., 2010). In addition, it is free of phorbol esters, the main toxin which is present in most Jatropha species (Makkar et al., 2010). However, antinutrients like trypsin inhibitor, lectin and phytate are present in the meal at high levels (Makkar et al., 2010). Heat labile antinutrients, like protease inhibitors, and lectins are easy to inactivate by moist heating, and phytase could be incorporated into the diet for degradation of phytate.

Main aim of the present study was to determine the growth performance, nutrient utilization and physiological responses when Nile tilapia fed with heated *J. platyphylla* kernel meal (JPKM) and compare it with that of SBM and FM.

Materials and Method

Diet Formulation

Fish meal and wheat meal were purchased from Kurt Becker GmbH, Bremen, Germany, and a German local supermarket respectively. Defatted and roasted SBM was obtained from Institute of Animal Nutrition (450), University of Hohenheim, Germany. Deshelled Jatropha seeds were supplied by Centro de Investigación en Alimentación y Desarollo (CIAD), Hermosillo, Sonora, Mexico and defatted in our institute. The defatted kernel meal was heat treated (121 ^{o}C, 20 min, 66% moisture) to inactivate lectins and trypsin inhibitor; and is designated as H-JPKM, hereafter. Prior to feed formulation, the proximate composition of JPKM, wheat meal, SBM and FM were determined.

A total of three isonitrogenous and isoenergetic diets were formulated. Control diet was prepared with FM and wheat meal, without any H-JPKM and SBM included. Jatropha diet was prepared by replacing 62.5% FM protein with H-JPKM, and Soybean diet was prepared by replacing 62.5% FM protein with SBM (Table 1). The final mixture of each diet was made into 2 mm diameter moist pellets and then freeze-dried. Experimental diets containing crude protein 36%, crude lipid 8%, vitamin premix 2% and mineral premix 2%. Lysine monohydrochloride (lysine 80% in this salt) was supplemented at the rate of 2.14% and 0.2% of H-JPKM and SBM inclusion in the diets respectively. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen) per kg.

Experimental animal and experimental set-up

Male tilapia (*Oreochromis niloticus*) fingerlings (12.0 - 15.0 g) were selected from our Aquaculture laboratory, University of Hohenheim, Stuttgart, Germany. Fish were kept individually in the recirculatory system aquaria (45 l) for acclimatization period of one week during which they were fed Hohenheim standard fish diet containing 35% protein, 8% lipid, 10% ash and with a gross energy content of 20 kJ g^{-1} dry matter.

Fingerlings (15 fish; av. wt. 13.7 ± 0.21 g) were randomly distributed in three treatment groups with five replicates in aquaria (45 l) of recirculatory system. Duration of the experiment was for 12 weeks.

The system was subjected to a photoperiod of 12 h light: 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 - 27.1°C, pH 7.0 - 7.5, dissolved oxygen 6.9 – 7.4 mg l^{-1}, total NH_3 0.1 - 0.2 mg l^{-1}, nitrite 0.07 - 0.1 mg l^{-1} and nitrate 1 - 3 mg l^{-1}). Water flow was adjusted to keep the oxygen saturation above 80 %.

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets		
	Control	Jatropha	Soybean
FM	425	159	159
Soybean meal	-	-	347.4
Wheat meal	495	487.4	367
Jatropha meal	-	248	-
Wheat gluten	-	1	22
Sunflower oil	40	64.6	64.6
Vitamin premix[1]	20	20	20
Mineral premix[2]	20	20	20
Total	1000	1000	1000
Lysine monohydrochloride	-	5.5	0.6
Phytase (FTU/kg)	-	500	500

[1]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.
[2]Mineral premix (g kg-1): $CaCO_3$, 336; KH_2PO_4, 502; $MgSO_4$. $7H_2O$, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; $MnSO_4$. H_2O, 3.12; $ZnSO_4$. $7H_2O$, 4.67; $CuSO_4$. $5H_2O$, 0.62; KI, 0.16; $CoCl_2$. $6H_2O$, 0.08; ammonium molybdate, 0.06; $NaSeO_3$, 0.02.

Feeding schedule

The same feeding schedule was followed for both experimental set up. The three experimental diets namely Control, Soybean and Jatropha were assigned to experimental animals in a random manner. Fish were starved a day before the experiment commenced and were fed 5 times their

maintenance requirement (3.2*5*(fish wt (g)/1000)$^{0.8}$), split into five equal rations per day (at 8.00, 11.00, 14.00, 17.00 and 20.00 h) daily. The feeds were dispensed using automatic feeders (Graesslin, Rondomatic 400, Graesslin GmbH, St. Georgen/Schwarzw, Germany).

Fish were individually weighed at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feeding level for subsequent week.

Pre and post experimental activities

Before the experiment commenced, 9 fish from same population were killed and preserved at −20 °C for the analysis of the initial body composition.

At the end of experiment, fish were anaesthetized by tricaine methanesulfonate (MS222) at 250 ppm in water. Blood was drawn near caudal peduncle from two fish from each replicate and transferred into a heparinized tube. Blood from one fish was used for haematological study and the other was centrifuged at 1500×g for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at –20 °C for determination of cholesterol and triglycerides.

The University animal welfare committee (University of Hohenheim Germany) approved all experimental procedures involving Nile tilapia.

Biochemical analyses

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Experimental diets and animals were analyzed for dry matter (DM), ash, crude protein (CP), and lipid. Gross energy (GE) determination was done using bomb calorimeter (IKA C7000) and benzoic acid was used as a standard.

Amino acid analysis, determination of phorbol esters and antinutrients

Amino acid compositions of FM, H-JPKM, SBM, wheat gluten and wheat meal were determined by the use of an automated amino acid analyser. The sample was hydrolysed with 6 M HCl at 110 °C for 24 h (Bassler and Buchholz, 1993). Tryptophan content of the samples was determined spectro-photometrically by the Pinter-Szakacs and Molnar-Perl (1990) method. Phorbol esters (PEs) were determined according to Makkar et al. (2007), which was based on the method described in Makkar et al. (1997).

The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of PEs by HPLC was 3 µg/g kernel meals. Trypsin inhibitor activity was determined essentially according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Non-starch polysaccharides (NSP) were determined according to Englyst et al. (1994).

Growth and nutrient utilization

Growth performance and nutrient utilization were assessed in terms of body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed conversion ratio (FCR), protein efficiency ratio (PER), protein productive value (PPV), apparent lipid conversion (ALC) and energy retention (ER).

BMG = Final body mass - initial body mass, BMG (%) = [(Final body mass - Initial body mass) / Initial body mass]*100, SGR (% day^{-1}) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days]*100, MGR ($gkg^{0.8}$ day^{-1}) = (Body mass gain, g) / [{(initial body mass, g / $1000)^{0.8}$ + (final body mass,g / $1000)^{0.8}$}/2] / duration of the trial days, FCR = dry feed fed (g)/body mass gain (g), PPV (%) = [(final fish body protein, g - initial fish body protein, g) / total protein consumed, g]*100, ALC (%) = [(final fish body lipid, g - initial fish body lipid, g) / total crude lipid consumed, g]*100, ER (%) = [(final fish body energy - initial fish body energy)/ (gross energy intake)]*100 and PER = fresh body mass gain (g)/crude protein fed (g).

Cholesterol and triglyceride estimation

The plasma cholesterol and triglycerides were determined by the use of enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color intensity was determined spectro-photometrically and was directly proportional to the concentration of cholesterol and triglycerides in the plasma sample.

Hemato-immunological parameter evaluation

RBC and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope. The following formula was used to calculate the number of RBC per mm^3 of the blood sample:

Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid

N= Number of cells counted

Hemoglobin (Hb) and hematocrit (Hct) content

The Hb content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hematocrit was determined on the basis of sedimentation of blood. Heparinised blood (50 µl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Hematocrit [%] x 1000) ÷ (RBC count [millions/µL]); mean cell hemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/µL]) and mean cell hemoglobin concentration, MCHC [g/dL] = Hemoglobin [g/dL] ÷ Hematocrit [%].

Nobiflow cholesterin (kit lot number 60041889; Hitado Diagnostic system) and Nobiflow triglyceride-GPO (kit lot number 60040710) were used to determine the plasma cholesterol and triglycerides respectively. The color intensity was measured spectro-photometrically and it was directly proportional to the concentration of cholesterol and triglycerides in the plasma sample.

Blood parameters analysis by Vet Scan

VetScan Chemistry Analyzer (Scil Animal care company GmBh, Technischer service, Germany) was used for determination of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium, creatinine, globulin, glucose, phosphorus, potassium, sodium, total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Statistical analysis

All data collected were subjected to one-way analysis of variance (ANOVA) and the significance of the differences between means was tested using Duncan's multiple range test (P<0.05). SAS Version 9.1 (Statsoft Inc., Tulsa, USA) was used for determining the level of significance and values are expressed as means ± standard deviation.

Results

Phorbol esters and antinutrients content in Jatropha kernel meal

Phorbol ester was not detected in the H-JPKM. Trypsin inhibitor and lectins were also not detected in H-JPKM, but phytate and NSP levels in H-JPKM were 9.3 % and 16% respectively (Table 2). Phytate and NSP levels in SBM were 2.5% and 14.0% respectively (Table 2).

Table 2 Proximate composition, amino acid composition and anti nutrient content of feed ingredients

	FM	JPKM	SBM	Wheat meal	Wheat gluten
Proximate composition (g kg $^{-1}$)					
Dry matter	940	945	955	941	937
Crude protein	635	665	471	143	856
Crude lipid	88	11.4	11.7	16.3	13.4
Crude ash	142	137	21.4	14	8.7
Gross energy (kJ/g)	21.1	18.3	18.2	18.7	21.4
Essential amino acids composition (g kg $^{-1}$)					
Arginine	35.3	86.7	37.6	5.4	41.1
Histidine	17.7	18.5	13.6	3.4	19.7
Iso leucine	22.8	28.5	22.8	4.2	42.8
Leucine	41.6	46.5	39.1	9.1	68.4
Lysine	40.9	22.0	32.1	3.3	16.9
Phenylalanine	21.8	32.8	26.9	6.5	30.7
Methionine	16.0	11.0	7.1	2.0	16.7
Threonine	23.0	25.3	20.3	3.7	23.2
Tryptophan	4.9	7.4	6.4	1.4	13.5
Valine	29.3	48.1	28.4	5.1	39.7
Non-essential amino acids composition (g kg $^{-1}$)					

Table 2 continued

Alanine	43.0	28.0	19.6	4.6	20.1
Asparaginsaüre	60.5	64.9	60.2	7.2	33.5
Cystine	4.3	10.8	7.3	2.9	17.1
Glycine	59.8	31.7	22.1	5.6	32.5
Glutamine	79.4	112.8	100.8	44.9	161.8
Proline	36.9	35.9	31.1	14.5	60.3
Serine	25.5	35.1	26.9	6.3	43.4
Tyrosine	14.8	18.7	16.7	3.3	28.2
Anti-nutrients					
Trypsin inhibitor (mg trypsin inhibited per g sample)	ND	ND	ND	-	-
Phytate (% dry matter)	-	9.3	2.41	-	-
Lectin	-	ND	ND	-	-
Non-starch polysaccharides (NSP) (g kg $^{-1}$)					
Rhamnose	-	3	0	-	-
Fucose	-	1	0	-	-
arabinose	-	31	24	-	-
Xylose	-	20	11	-	-
Mannose	-	5	6	-	-
Galactose	-	14	42	-	-
Glucose	-	57	32	-	-
Glucuronic acid	-	0	0	-	-
Galacturonic acid	-	30	24	-	-
Total	-	160	140	-	-

ND- Not detected,

FM- Fish meal, JPKM - *Jatropha platyphilla* kernel meal, SBM – Soybean meal.

Proximate and amino acid composition of experimental diets

Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 2 and 3. Diets contained about 36% crude protein and 19.0 kJ/g gross energy and were isonitrogenous and isoenergetic. Crude lipid and ash contents were in the range of 9.2 - 9.8 % and 10.3 - 11.0 % respectively. All experimental diets had almost similar amino acid composition (Table 3). All the diets containing essential amino acid are essentially as per the requirement of Nile tilapia (NRC, 1993).

Table 3 Proximate and amino acid composition of the experimental diets in (g kg^{-1} feed)

	Control	Jatropha	Soybean
Proximate composition (g kg^{-1})			
Dry matter	958	964	959
Crude protein	36.2	36.1	36.0
Crude lipid	94	98	92
Crude ash	108	103	111
Gross energy (kJ/g)	19.1	19.4	19.0
Essential amino acid (g kg^{-1})			
Arginine	20.1	32.1	26.2
Histidine	10.4	11.5	11.1
Iso leucine	13.3	14.3	15.0
Leucine	24.8	26.0	27.6
Lysine	22.1	18.7	22.4
Phenylalanine	13.7	16.5	17.9
Methionine	8.9	6.7	6.0
Threonine	13.2	12.4	13.8
Tryptophan	3.1	3.8	4.5
Valine	16.9	17.3	16.8
Non-essential amino acid (g kg^{-1})			
Alanine	23.8	18.1	18.7
Asparginine	33.6	35.9	47.2
Cystine	3.4	2.5	4.8
Glycine	32.6	21.3	20.8
Glutamine	58.4	68.7	73.0
Proline	24.6	22.1	23.7
Serine	15.5	16.9	18.5
Tyrosine	8.8	10.1	11.7

Amino acid composition of the experimental diets were calculated from amino acid profile of individual feed ingredients

Growth performance and feed utilization

Average weekly body mass gain in the entire group is presented in Figure 1. Growth performance and nutrient utilization parameters (except ALC) did not differ significantly among the three groups (Table 4) expect in ALC where plant protein fed group were lower value was observed compared to control group (Table 4).
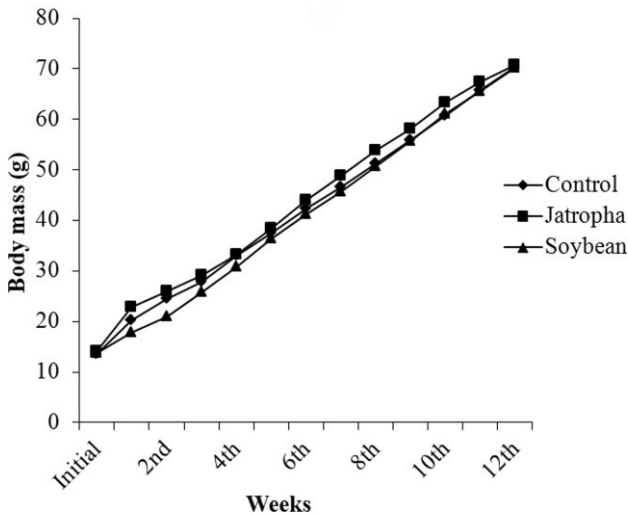
Fig. 1 Weekly body mass development (g) of Nile tilapia (*Oreochromis niloticus*) juveniles fed experimental diets for 12 weeks.

Chemical composition of whole body of fish

Moisture, crude lipid, and crude protein and gross energy content of the whole body did not differ significantly among the three groups (Table 5). Ash deposition in H-JPKM fed group was statistically not different (P > 0.05) to that in SBM fed groups, but was statistically higher than that in control group (Table 5).

Cholesterol and triglyceride levels

Cholesterol and triglycerides levels in plasma of different experimental groups are shown in Table 5. Cholesterol level in plasma was statistically higher in control group than in plant protein fed groups.

Table 4 Growth performance and nutrient utilization in Nile tilapia (*Oreochromis niloticus*) juveniles fed with experimental diets for 12 weeks

Treatment	IBM (g)	FBM (g)	BMG (%)	SGR (%)	MGR (g kg$^{0.8}$ day^{-1})	FCR	PER	PPV (%)	ALC (%)	ER (%)
Control	13.6 ± 0.21 [a]	70.4 ± 1.45 [a]	418.3 ± 19 [a]	2.0 ± 0.06 [a]	8.9 ± 0.09 [a]	1.7 ± 0.06 [a]	1.6 ± 0.06 [a]	27.2 ± 2.02 [a]	40.5 ± 9.25 [a]	14.9 ± 2.53 [a]
Jatropha	13.8 ± 0.25 [a]	70.8 ± 1.14 [a]	402.1 ± 16 [a]	1.9 ± 0.05 [a]	8.8 ± 0.07 [a]	1.7 ± 0.05 [a]	1.6 ± 0.04 [a]	29.3 ±1.89 [a]	29.8 ± 1.89 [b]	13.7 ± 1.67 [a]
Soybean	13.7 ± 0.19 [a]	70.2 ± 2.23 [a]	414.2 ± 10 [a]	2.0 ± 0.04 [a]	8.9 ± 0.13 [a]	1.7 ± 0.07 [a]	1.7 ± 0.07 [a]	27.6 ± 4.87 [a]	31.2 ± 8.86 [b]	15.0 ± 2.97 [a]
SEM	0.02	0.4	2.76	0.01	0.03	0.02	0.02	0.81	2.71	0.61

IBM- Initial body mass, FBM- Final body mass, BMG - Body mass gain, SGR – Specific growth rate and MGR - Metabolic growth rate; FGR – Feed conversion ratio, PER - Protein efficiency ratio, PPV - Protein productive value, ER (%)- Energy retention, ALC (%)- Apparent lipid conversion. Values are mean (n = 4) ± standard deviation. For all parameters, mean values in the same column were not significantly different (P < 0.05).

Table 5 Proximate body composition of Nile tilapia (*Oreochromis niloticus*) juveniles in recirculatory set up at the start and at the end of the experiment (%, wet basis ± SD); cholesterol and triglyceride (mg/dl) level in plasma in Nile tilapia (*Oreochromis niloticus*) fingerlings

Treatment	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)	Gross energy (kJ/g)	Plasma cholesterol	Plasma triglycerides
Initial fish	73.6 ± 2.0	17.3 ± 0.50	5.6 ± 1.20	3.0 ± 1.10	5.9 ± 1.50		
Control	73.1 ± 1.72[a]	16.9 ± 0.74 [a]	5.2 ± 1.05 [a]	4.2 ± 0.23 [b]	4.6 ± 0.65 [a]	112[a] ± 4.1	71[b] ± 6.5
Jatropha	73.4 ± 0.92 [a]	16.7 ± 1.02 [a]	4.6 ± 0.69 [a]	4.9 ± 0.47 [a]	4.65 ± 0.45 [a]	91[b] ± 4.7	82[a] ± 4.2
Soybean	73.0 ± 1.41 [a]	16.5 ± 2.09 [a]	5.5 ± 1.08 [a]	4.4± 0.47 [ab]	4.97 ± 0.95 [a]	78[b] ± 8.4	88[a] ± 6.1
SEM	0.34	0.38	0.29	0.13	0.18	2.23	2.34

Values are mean (n = 5) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 6 Effects of experimental diets on the haematological parameters (RBC (10^6 cells/mm³), WBC (10^3 cells/mm³), Hb (g/dl), Hct (%), MCV (fL), MCH (pg), MCHC (g/dl) of Nile tilapia (*Oreochromis niloticus*) juveniles

Treatment	RBC	WBC	Hb	Hct	MCV	MCH	MCHC	Albumin	Globulin	Total protein
Control	1.2 ± 0.92 [b]	30.7 ± 1.47 [a]	5.0 ± 0.0 [a]	27.4 ± 8.02 [b]	224.5 ± 59 [a]	41.3 ± 3.18 [a]	19.3 ± 4.65 [a]	1.6 ± 0.48 [a]	2.2 ± 0.27 [a]	3.8 ± 0.54 [a]
Jatropha	1.4 ± 0.87 [a]	32.5 ± 1.95 [a]	5.0 ± 0.09 [a]	38.8 ± 6.50 [a]	212.3 ± 55 [a]	35.8 ± 2.38 [b]	17.7 ± 4.40 [ab]	1.6 ± 0.42 [a]	2.3 ± 0.16 [a]	3.9 ± 0.52 [a]
Soybean	1.5 ± 0.91 [a]	32.7 ± 1.67 [a]	5.0 ± 0.09 [a]	39.2 ± 5.59 [a]	267.8 ± 27 [a]	34.7 ± 2.70 [b]	13.1 ± 2.43 [b]	1.4 ± 0.16 [a]	2.5 ± 0.23 [a]	3.9 ± 0.21 [a]
SEM	0.35	0.47	0.02	2.12	13.34	1.01	1.11	0.09	0.06	0.12

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
MCV. Mean corpuscular volume (pg), MCH. Mean corpuscular hemoglobin (pg) and MCHC. Mean corpuscular hemoglobin concentration (g/dl)

Table 7 Effects of experimental diets on alkaline phosphatase (U/l), alanine transaminase (U/l), glucose (mg/dl) Total bilirubin (mg/dl), blood urea nitrogen (mg/dl), creatinine (mg/dl); blood ions [(calcium (mg/dl), phosphorus (mg/dl), sodium (mmol/l) and potassium (mmol/l)] in the blood of Nile tilapia (*Oreochromis niloticus*) juveniles

Treat	ALP	ALT	Glucose	TBIL	BUN	Creatinine	Calcium	Phosphorus	Sodium	Potassium
Control	46.6 ± 9.40[a]	62.6 ± 16.94[a]	50.6 ± 1.82[b]	0.3 ± 0.07[a]	2.4 ± 0.89[a]	0.92 ± 0.11[a]	15.9 ± 3.77[a]	13.1 ± 3.16[b]	153 ± 1.82[a]	3.3 ± 1.67[b]
Jatropha	55 ± 9.54[a]	95.2 ± 38.51[a]	65.4 ± 8.56[a]	0.24 ± 0.05[a]	2.8 ± 1.64[a]	0.22 ± 0.04[b]	14.9 ± 2.93[a]	16.7 ± 3.99[a]	156 ± 4.06[a]	3.4 ± 1.12[a]
Soybean	50.4 ± 13.28[a]	62.6 ± 31.41[a]	62.4 ± 4.64 [a]	0.26 ± 0.05[a]	2.6 ± 1.34[a]	0.24 ± 0.05[b]	13.8 ± 0.48[a]	16.7 ± 4.75[a]	157 ± 2.30[a]	5.5 ± 1.08[ab]
SEM	2.76	8.33	2.91	0.01	0.43	0.01	0.78	1.12	0.88	0.42

Values are mean (n = 5) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute). ALP - alkaline phosphatase, ALT - Alanine transaminase, TBIL – Total bilirubin and BUN - Blood urea nitrogen

Hematological parameters and proteins in blood

White Blood cell (WBC), hemoglobulin content and mean cell volume (MCV) did not differ significantly among the three groups. Statistically higher RBC count and hematocrit level was observed in plant protein fed groups whereas MCH and MCHC exhibited opposite trend (Table 6). Albumin, globulin and total protein in blood did not differ significantly among the three groups (Table 6).

Metabolic enzymes activities, blood glucose and blood ions

Alanine transaminase and ALP activities, TBIL and BUN level in blood did not differ significantly among the three groups (Table 7). Statistically higher blood glucose level was observed in plant protein fed groups than FM fed group, whereas creatinine level in blood exhibited opposite trend (Table 7).

The blood ion (calcium, phosphorus, sodium and potassium) of the experimental animals in each group are presented in Table 7. Calcium and sodium ions in blood did not differ significantly among the three groups. Phosphorus and potassium ions in blood were statistically similar for Jatropha and soybean groups but were significantly higher ($P < 0.05$) than those for control group.

Discussion

Fish behaviour and feed intake

Based on the visual observation during feeding time, palatability and acceptability of feed was good and the behaviour of fish was normal. No left over in the aquaria and no loss of fish throughout the experimental period was observed.

Growth performance and nutrient utilizations

In the present study an excellent growth was observed. The fish body mass increased four to five times within 12 weeks; and growth performance and feed utilization of Jatropha and soybean groups were similar to control group. Feed conversion ratio of about 1.7 was reported, although generally it ranges between 1.2 and 1.5 (De Silva and Anderson, 1995; Ogunji and Wirth,

2000). Ogunji and Wirth (2000) observed SGR of 3.3 when *Oreochromis niloticus* fingerlings were fed FM based diets.

No difference in growth performance and feed utilization parameters such as FCR, PER and ER suggest that digestion and absorption of nutrients from H-JPKM and FM were similar. Nutrient utilization values found in this study signify very good utilization of the diets. Growth performance of H-JPKM fed group was similar to FM fed group which indicate that protein and energy availability from the H-JPKM for the protein synthesis was similar to fish meal. Presence of antinutrients such as phytate, which is present in high amounts in the H-JPKM, could have adversely affected the feed utilization (Makkar et al., 2010). In our study it did not elicit any detrimental effects. In the plant protein based feeds, phytase has been added in the feed at a level of 500 FTU per kg to mitigate the phytate-mediated anti-nutritive effects. This phytase level (500 FTU/kg diet) appears to be optimum for the diet. The NSPs level in H-JPKM is of the similar order as present in SBM (Table 2) and is high; however, the H-JPKM NSPs do not appear to be deleterious.

Our result of growth performance and feed utilization are similar to those obtained by Jackson et al. (1982) for tilapia, even with our substitution at 62.5%. These workers reported that substitution of FM protein by 25 and 44% plant proteins such as groundnut meal and SBM respectively did not adversely affect the feed intake and growth performance. It is evident that H-JPKM is a promising protein source for incorporation in Nile tilapia feed from the point of view of growth performance and feed utilization parameters.

Chemical composition of whole body of fish

Moisture, protein and lipid content did not differ significantly among the three groups. Higher ($P < 0.05$) gross energy content in whole body was observed in plant protein fed groups than control group. The replacement of FM by plant protein sources such as corn gluten meal and soy protein concentrates has been shown to increase hepatic lipogenic enzyme activities in seabass (Dias, 1999; Kaushik et al., 2004) that could lead to higher whole body lipid and higher value of gross energy content of whole body.

Crude protein content of fish was similar in all groups. Cheng et al., (2003) reported that the plant protein (soy protein) based diets decrease nitrogen retention in salmon and trout because these diets have lower digestible energy content and an amino acid profile that is sub optimal for

muscle growth. Interestingly in our study, crude protein content fish fed plant protein was similar to those of FM fed groups. The similarity in amino acid content observed in all group have shown that proper combination of FM and plant proteins increases protein retention in fish (Jahan et al., 2003). This observation indicates that H-JPKM and SBM containing diets have optimum digestible energy and a balanced amino acid profile for optimum growth of Nile tilapia.

Plant protein based diets (H-JPKM and SBM) were supplemented with phytase and that could have released minerals bound to phytate. Consequently, leading to increase in body minerals contents, as observed in H-JPKM and SBM fed groups.

Cholesterol, triglyceride and blood glucose level

Fish fed plant protein containing diets had lower cholesterol level in plasma as compared to fish fed FM. The decrease in plasma cholesterol concentrations in fish fed diets with plant proteins is in accordance with the results of Yamamoto et al. (2007), Kumar et al. (2008, 2010c) and Makkar et al. (2009). The fish hypocholesterolemia in response to dietary plant protein supply could be either due to an increased excretion of bile salts, an inhibition of cholesterol intestinal absorption, or just to the withdrawal of FM rather than to the direct effects of plant protein (Kaushik et al., 2004). Serum triglycerides act as a short-term indicator of feeding or nutritional status (Bucolo and David, 1973). In the present study plasma triglycerides increased in concentrations with increased dietary DJKM level. Whereas Shimeno et al. (1993) observed opposite trend in the yellowtail (*Seriola dumerilii*).

Blood glucose concentration was affected by dietary treatments. Higher ($P < 0.05$) blood glucose concentration was observed in plant protein fed groups than in control group. Plant protein based diets contain higher amount of carbohydrates. Usually, breakdown of carbohydrates into smaller sugar compounds could contribute to higher glucose level in blood. Similar trends have been shown in fish fed diets containing SBM and corn gluten (Kikuchi et al., 1994; Kikuchi, 1999). Contrarily, Glencross et al. (2004) observed that dietary inclusion of yellow lupin in fish diet did not affect blood glucose level.

Blood Chemistry

The WBC counts, Hb content and MCV did not differ significantly among the three groups. RBC counts and Hct content were higher in plant protein fed groups whereas MCH and MCHC exhibited opposite trend. Plant ingredients may cause early release of immature erythrocytes (Hemre et al., 2005), increasing the RBC count. Consequently, MCH and MCHC values were change at the same time. Lower MCH and MCHC values were observed in plant protein fed groups. Similarly, significant reduction of MCH and MCHC on increase in the content of plant proteins in salmon diet was observed (Hemre et al., 2005). The Hb and Hct assays are normally used as general indicators of fish health (NRC, 1993). Hematocrit level in all groups was within the normal range and did not differ significantly among the groups. In the present study higher Hct content observed in plant protein fed groups, is in contrast with the observation of Siddiqui et al. (1988), who observed that FM protein replaced by mixtures of plant proteins in Nile tilapia diets led to lower Hct content. This was attributed to the binding of phytate to minerals (iron) and/or amine group of amino acids causing their low availabilities in the body and increase in erythrocyte fragility. In our study phytase was added, which would have prevented this to occur.

The concentration of total protein in blood is used as a basic index for health and nutrititional status in fish (Martinez, 1976). Among the blood proteins, albumin and globulin are the major proteins that play a significant role in the immune response. Albumin is used as an indicator of liver impairment (Silverman et al., 1986). Blood protein did not differ significantly among the three groups, indicating that there are no nutritional deficiencies and no impaired protein metabolism in the liver.

Metabolic enzymes and blood ions

Alkaline phosphatase and ALT are released into blood during organ damage (Racicot et al., 1975). Thus, detection of high levels of ALP and ALT in blood gives information on the damage of organs and in particular of liver cells. Levels of ALP and ALT were similar for all the diets, indicating normal organ function on feeding of H-JPKM and SBM. Hemre et al. (2005) also reported similar results on feeding SBM containing diets to Atlantic salmon.

High blood urea nitrogen levels are associated with liver or gill dysfunction (Stoskopf, 1993), and in our study these levels were in the normal ranges (Witters, 1986; Wedemeyer, 1996). Furthermore, these values did not differ significantly among the groups. These results show that H-JPKM fed group was normal and healthy. Total bilirubin, an indicator of

liver dysfunction (Tietz, 1986) was similar for all the groups. Creatinine was highest in control group. Creatinine is a metabolite of animal protein degradation and its highest level in control group is due to highest content of FM in the diet. Creatinine level reflects kidney dysfunction, and its level in all the experimental group were in the normal range. Plant protein based diets were supplemented with phytase and higher phosphorus and other ions in blood observed in plant protein fed fish could be due to increased release of phosphorus and potassium from feed and making them available for Nile tilapia. All these levels of ions were within the normal ranges for tilapia (Baruah et al., 2007).

Conclusion

The heated *J. platyphylla* kernel meal (H-JPKM) can replace FM protein up to 62.5% in the diet of Nile tilapia without any unfavorable effects on the growth performance, nutrient utilization and biochemical activities in the fish and it can be utilized in tilapia diet as a good quality of protein source. Further research should be conducted to examine the possibility of increasing the inclusion of H-JPKM beyond 62.5% fish meal protein replacement in the diet of Nile tilapia. Also studies on the utilization of H-JPKM in other fish species are required.

Acknowledgement

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Chapter 12

Evaluations of the nutritional value of *Jatropha curcas* protein isolate in common carp (*Cyprinus carpio* L.)

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Abstract

Jatropha curcas seeds are rich in oil and protein. The oil is used for biodiesel production. Jatropha-seed-cake (JSC) obtained after oil extraction is rich in protein, however, it is toxic (phorbol esters content 1.3 mg/g) and consists of 50-60% shells, which are indigestible. The principle of isoelectric precipitation was used to obtain Jatropha protein isolate (JPI) from JSC and it was detoxified (DJPI). Carp (n = 45, 20.3 ± 0.13g) were randomly distributed into five-groups with three-replicates and for 12-weeks fed iso-nitrogenous diets (crude-protein 38%): Control (fishmeal based protein); J_{50} and J_{75} (50% and 75% of fishmeal protein replaced by DJPI); S_{50} and S_{75} (50% and 75% of fishmeal-protein replaced by soy-protein-isolate). Growth performance and nutrient utilization parameters were highest in S_{75} group and not significantly different to those in J_{50} and S_{50} groups but were significantly higher than those for all other groups. Similar trend was observed for protein and energy digestibilities of experimental diets, whereas opposite trend was observed for the feed to gain ratio. Activities of intestinal digestive enzymes did not different significantly among the five groups. In conclusion, DJPI is a good quality protein source for carp.

Introduction

Fish meal (FM) is a major protein source in compound feeds for intensive fish farming. In an effort to reduce reliance on FM as the primary protein source, most modern nutrient-dense aquaculture diets now use some plant protein ingredients. Modern nutrient-dense diets for aquatic species have little formulation flexibility to accommodate large amounts of indigestible material (neutral detergent fibre). Because of this, many plant protein sources are not viable alternatives for FM, despite having reasonable protein or energy digestibility (Glencross et al., 2005). In addition, many plant materials contain antinutritional factors. Modern processing technologies have overcome many of these obstacles, not only by denaturing anti-nutritional factors and solvent extracting much of the unsuitable lipid, but also by removing a large part of the undesirable carbohydrate and other components (Oliva-Teles et al., 1994). The resulting protein concentrate or protein isolate products contain high levels of protein, which often have digestibility similar or higher than that of FM protein (Xie and Jokumsen, 1998). Techniques for production of protein concentrates and isolates from legumes are relatively well known. Among these are processes such as dehulling, air classifying, solvent extraction and solubilised extraction (Lasztity et al., 2001), all of which have some commercial application. A range of such products produced from soybean already exists in the market and have previously been assessed in common carp, rainbow trout and Atlantic salmon (Kaushik et al., 1995; Escaffre et al., 1997; Refstie et al., 1998).

Jatropha curcas (L.) (physic nut) is a multipurpose and drought resistant tree, widespread throughout the tropics and subtropics. It is a hardy plant, thrives on degraded land and requires limited amounts of nutrients and water. The International Jatropha Organization has projected that in 2017 there will be around 32.72 million hectares of land cultivated worldwide with *J. curcas,* producing 160 million tons of seeds and 95% of the total production will be in Asia. Jatropha seeds have been extensively investigated as a source of oil. The seed contains about 30-35% oil that can be converted into biodiesel of high quality upon transesterification and used as a substitute for diesel fuel (Makkar and Becker, 2009). The seed cake left after mechanical pressing of oil contains 50-60% shells which are indigestible. The protein isolate obtained from the seed cake, using the principle of isoelectric precipitation, is an excellent source of nutrients and contains 80 to 85 % crude protein. However, the presence of high levels of antinutrients such as trypsin inhibitor, lectin and phytate and the major toxic components phorbol esters (PE$_S$) (Makkar et al., 2007) restrict their use in fish feed. Heat labile

antinutrients such as protease inhibitors, and lectins are easy to inactivate by moist heating (Makkar and Becker, 2009). A method for detoxification of Jatropha protein isolate has been developed in our laboratory. It is based on extraction of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment. Improved Jatropha plant can yield 4 to 5 tons seed per year from one hectare of plantation, which can give approximately 0.3 to 0.4 ton of the protein isolate (Makkar and Becker, 2009). This means that there is a possibility of producing enough Jatropha protein isolates to meet growing aquaculture industry demand. Our previous studies have shown that detoxified Jatropha kernel meal (kernel meal is the product obtained after removal of oil by solvent extraction of the shell-free kernels) is a good protein source for common carp (Kumar et al., 2008, 2010a; Makkar et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Makkar et al., 2009; Kumar et al., 2010b), Nile tilapia (*Oreochromis niloticus*) and white leg shrimp (*Penaeus vannamei*) (Harter et al., 2011) diets. Here we report nutritional value of the protein isolate prepared from *Jatropha curcas* seed cake and compare it with soy protein isolate (SPI) and FM in common carp.

Materials and methods

Preparation of the Jatropha protein isolates

Jatropha seed cake obtained using a mechanical screw press (German screw press type Komet D85-1G, Germany) was used for preparation of protein isolate. The chemical composition of the seed cake was: crude protein 23.6 %, oil 9.3 %, and ash 5.8%; all on dry matter basis).

Isoelectric precipitation principle was used to obtain protein isolate from the seed cake. To 500 g of defatted seed cake (in triplicate) was added 5000 ml of distilled water adjusted to pH 11 using 10 mol L^{-1} NaOH. The mixture was stirred for 1 h at room temperature (20 ^{o}C). Every 15 min the pH was checked and adjusted to 11 using 10 mol L^{-1} NaOH. The contents were centrifuged at 3000 × g for 20 min and the supernatant was collected. The supernatant was brought to pH 4 using 6 mol L^{-1} HCl, stirred for 10 min and kept at 4 ^{o}C for 1 h to precipitate proteins. The contents were centrifuge at 3000 × g for 20 min to obtain the protein isolate. The protein isolate was freeze-dried. Organic solvents were used to detoxify the protein isolate (patent application has been filed for the process of detoxification). After removal of PEs, the protein isolate was autoclaved (121 ^{o}C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation, experimental system and animals

Five isonitrogenous diets were prepared contained 38% crude protein. Lysine monohydrochloride (lysine 80% in this salt) was supplemented at the rate of 2.71% of detoxified Jatropha protein isolate (DJPI) inclusion in the diet. The inclusion level of the DJPI and SPI were as follows: Control diet (Control) was prepared with FM and wheat meal, without DJPI and SPI; J_{50} and J_{75} (50% and 75% of FM protein replaced by DJPI), and S_{50} and S_{75} (50% and 75% of FM protein replaced by SPI). The final mixture of each diet was made into 2 mm diameter moist pellets using a Bosch, Type UM60ST 2-M pelletizer (Robert Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then freeze-dried (Table 1).

Common carp (*Cyprinus carpio* L.) fingerlings obtained from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 L capacity tanks for acclimatisation. At start of the experiment, six fish of the same population were killed and preserved at −20 °C for analysis of the initial body composition. After acclimatisation, 45 fish were randomly distributed into five groups with three replicates; each replicate contained three fish (av. wt. 20.3 ± 0.13 g) in an aquarium (45 L capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 – 27.1°C, pH 7.0 – 7.5, dissolved oxygen 6.9 – 7.4 mg l^{-1}, total NH_3 0.1– 0.2 mg L^{-1}, nitrite 0.07 – 0.1 mg L^{-1} and nitrate 1–3 mg L^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. Before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass (kg$^{0.8}$) per day (equal to five times their maintenance requirement) and split into 5 equal rations per day (at 8:00, 10:30, 13:00, 15:30 and 18:00 h). The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day. During last two weeks of the experiment, fish were fed with a diet containing a marker (TiO_2) for digestibility measurement and faeces were collected daily according to Mamun et al. (2007). The collected faeces were centrifuged at 4000 × g for 10 min, the supernatant discarded and the faeces were then stored at −20°C until analysis.

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets[*]				
	Control	J$_{50}$	J$_{75}$	S$_{50}$	S$_{75}$
[1]Fish meal	484	242	121	242	121
[2]Wheat meal	436	435	435	435	435
Jatropha protein isolate	-	196	295	-	-
[3]Soya protein isolate	-	-	-	172	258
Cellulose	-	23	33	47	70
Sunflower oil	40	64	76	64	76
[4]Vitamin premix	20	20	20	20	20
[5]Mineral premix	20	20	20	20	20
Total	1000	1000	1000	1000	1000
[6]Lysine monohydrochloride	-	5.4	8.0	-	-
[7]Phytase (FTU/kg)	-	500	500	500	500
TiO$_2$	10	10	10	10	10

[*]Experimental diets
Control: Fish meal and wheat meal, without any Jatropha protein isolate and soy protein isolate
J$_{50}$: 50% of fish meal proteins replaced by Jatropha protein isolate
J$_{75}$: 75% of fish meal protein replaced by Jatropha protein isolate
S$_{50}$: 50% of fish meal protein replaced by soy protein isolate
S$_{75}$: 75% of fish meal protein replaced by soy protein isolate
[1]Source: Kurt Becker GmbH, Bremen, Germany
[2]Whole wheat meal.
[3]Source: SUPRO® 500E IP, Solae Europe S.A., 2, Chemin du Pavillon, CH-1218 Le Grand-Saconnex, Geneva, Switzerland
[4]Source: Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.
[5]Source: Altromin Spezialfutter GmbH & Co. KG, Lage, Germany Mineral premix (g kg-1): CaCO$_3$, 336; KH$_2$PO$_4$, 502; MgSO$_4$. 7H$_2$O, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; MnSO$_4$. H$_2$O, 3.12; ZnSO$_4$. 7H$_2$O, 4.67; CuSO$_4$. 5H$_2$O, 0.62; KI, 0.16; CoCl$_2$. 6H$_2$O, 0.08; ammonium molybdate, 0.06; NaSeO$_3$, 0.02.
[6]Source: Merck KGaA, 64271 Darmstadt, Germany.
[7]Source: Natuphos 5000 G (EU) 3-phytase (EC 3.1.8) BASF Ludwigshafen, Germany.

The experiment lasted 12 weeks and fish were sampled thereafter. At the end of experiment, one fish per group was anaesthetized with tricaine methanesulfonate (MS222; 250 mg/L). Anaesthetized fish were carefully dissected to isolate intestine and intestinal samples, which were stored in liquid nitrogen for determination of activities of digestive enzymes. One fish per group was killed by a blow to the head with metal rod and then stored at −20 °C for chemical composition analysis. Prior to determination of the proximate composition, the fish were autoclaved at 121 °C for 20 min, thoroughly homogenised using an Ultra-Turrax T25, frozen overnight and freeze-dried.

The University of Hohenheim Animal Welfare Committee approved all the experimental procedures involving in keeping, feeding and sampling of common carp.

Proximate analysis and determination of phorbol esters, antinutrients and amino acid

All samples were ground to a fine powder by using a coffee grinder (Severin, KM 3872, 230 V and 90 W, Severin Elektogerate GmbH, Sundern, Germany) prior to analyses. The proximate composition of the experimental feeds and of the fish carcasses was determined according to the official methods (AOAC 1990), i.e. for moisture (oven-drying at 105 ^{0}C overnight), crude protein by CN analyser (Vario Max CN, Makro-Elementaranalysator, Element analysensysteme GmbH, Hanau, Germany) (N X 6.25), fat [by extraction according to the method described by Smedes (1999) as modified by Schlechtriem et al. (2003)] and ash (oven incineration at 480 ^{0}C overnight). Phorbol esters (PEs) were determined according to Makkar et al. (2007) based on the method of Makkar et al. (1997). The results were expressed as equivalents to a standard phorbol-12-myristate 13-acetate (Sigma, Saint Louis, USA). Detection limit of PEs by HPLC was 3 µg/g protein isolates. Trypsin inhibitor activity was determined according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997). Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Non-starch polysaccharides (NSP) were determined according to Englyst et al. (1994). Amino acid composition of FM, DJPI, SPI and wheat meal was determined using an automated amino acid analyser after hydrolysing the samples with 6M HCl at 110 ^{0}C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. Tryptophan content of the above-mentioned samples was determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

Growth and nutrient utilization parameters

Growth performance and diet nutrient utilization were assessed in terms of body mass gain (BMG) = [(Final body mass - initial body mass) / Initial body mass] X 100; specific growth rate (SGR) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; metabolic growth rate (MGR)

= (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$}/2] / number of trial days; feed gain ratio (FGR) = dry feed fed (g)/body mass gain (g); protein efficiency ratio (PER) = body mass gain (g)/crude protein fed (g); protein productive value (PPV) = [(final fish body protein in g - initial fish body protein in g) / total protein consumed in g] X 100; lipid productive value (LPV) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid consumed in g] X 100 and energy productive value (EPV) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100.

Digestibility measurement and efficiency of digestible nutrients and gross energy

Titanium dioxide in the feed and faeces was determined according to the method described by Richter et al. (2003). The percentage of apparent dry matter digestibility of diets was calculated according to Maynard et al. (1981). Apparent dry matter digestibility (%) = [1 – {(% TiO$_2$ in feed) / (% TiO$_2$ in faeces)}] X 100

Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets were calculated according to Maynard and Loosli (1969).

The nutrient and energy digestibility were obtained using the following formula:

Apparent nutrient digestibility (%) = [1 – {(% TiO$_2$ in feed) / (% TiO$_2$ in faeces) X (% Nutrient or energy in faeces) / (% Nutrient or energy in feed)}] X 100

Digestible nutrients and gross energy retained (%) = (Nutrient and energy retained in the whole body/Digestible nutrient and digestible energy) X100

Digestible nutrients and energy = Total offered of nutrients and gross energy through feed X digestibility coefficient.

Hepatosomatic index (HSI), intestinal somatic index (ISI) and digestive enzymes assay

Hepatosomatic index, and ISI are calculated as indicated below:

HSI = Liver mass (g) X 100 / body mass (g) and ISI = Intestinal mass (g) X 100 / body mass (g).

Amylase activity was estimated using dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as mmol of maltose released from starch per min at 37 $^{\circ}$C. Protease activity was

determined by the casein digestion method of Drapeau (1974), and one unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to $\Delta 0.001A_{280}$ per minute at 37 ^{O}C and pH 7.8. Lipase activity was assayed by the method of Cherry and Crandall (1932), and one unit of enzyme was the amount of enzyme that hydrolyses 1.0 micro equivalent of fatty acid from a triglyceride in 24 h at pH 7.7 at 37 ^{0}C.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test ($P < 0.05$). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results and discussion

The use of plant protein products in aquaculture diets is generally limited by their low levels of digestible protein and/or energy. The seed cakes of soybeans, lupins, pea and Jatropha represent some of those plant products that contain considerable amount of proteins and efforts have been made to further enhance their protein levels by processing technologies (Glencross et al., 2005; Makkar and Becker, 2009; Makkar et al., 2009; Øverland et al., 2009). While protein isolate is commercially available from soybean, pea and canola derived sources, similar such products from other plant products are still in a dormant phase. Notable in the products evaluated thus far is an increasing protein content, usually at the expense of the lignin and carbohydrate content of the seed cake. Based on the reports from other studies, it could be reasoned that an increase in the protein content of these product would be usually concomitant with a decline in the levels of antinutritional factors (Makkar and Becker, 2009). Many researchers (Murai et al., 1987; Olli and Krogdahl, 1994; Kaushik et al., 1995; Bureau et al., 1998) evaluated nutritional value of SPI in salmonid fish and reported improved feed intake and growth compared to diets containing soybean meal. Clearly these observations are consistent with those observed in the present study for Jatropha protein isolate. Growth performance is higher in the present study compared to previous study wherein a Jatropha kernel meal was used as a protein source in common carp (Kumar et al., 2010c).

Phorbol esters and antinutrients content in protein isolate

Phorbol esters content in untreated protein isolate was 1.2 mg/g. However, these were not detected in DJPI. Trypsin inhibitor and lectins were also not detected in DJPI and SPI; whereas phyate levels in DJPI and SPI were 2.95% and 0.94%, and NSP levels 10.5% and 1.04% respectively (Table 2).

Proximate and amino acid composition of feed ingredient and experimental diets

Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 2 and 3. Diets contained about 38% crude protein and were isonitrogenous. Crude lipid and ash were in the range of 8.8–9.7% and 9.2–10.9% respectively. All experimental diets had almost similar amino acid composition (Table 3). In all the diets, contents of essential amino acids were essentially as per the requirement of the common carp (NRC 1993). Calculated digestible protein/digestible energy (DP/DE) ratios of the experimental diets were in the range from 19.1 to 20.7 g MJ^{-1} (Table 3). Optimum dietary DP/DE ratio studied in various commercially important protein resources ranged from 17 – 26 (g MJ^{-1}) (Page and Andrew, 1973; Garling and Wilson, 1976; Takeuchi et al., 1979; Anderson, 1996; Einen and Roem, 1997). A diet containing DP/DE in the range of 18 – 24 g MJ^{-1} was reported to promote optimum growth of common carp (Takeuchi et al., 1979). Our value falls within the range of the optimum for growth and feed utilization.

Table 2 Proximate composition, antinutrients content and amino acid contents of feed ingredients

	Fish meal	Jatropha protein isolate	Soy protein isolate	Wheat meal
Proximate composition (g kg $^{-1}$)				
Dry matter	940	945	957	941
Crude protein	655	808	922	145
Crude lipid	88	9.7	10	16.3
Crude ash	142	93	37.9	14
Gross energy (KJ/g)	21.1	19.3	22.0	18.7
Antinutrients				
Trypsin inhibitor (mg trypsin inhibited per g sample)	-	ND	ND	-
Lectin[a]	-	ND	ND	-
Phytate (g kg $^{-1}$ dry matter)	-	29.5	9	-
Non-starch polysaccharides (g kg $^{-1}$)	-	105	10.4	-
Essential amino acids composition (g kg $^{-1}$)				
Arginine	35.3	86.0	67.9	5.4
Histidine	17.7	24.0	24.4	3.4
Iso leucine	22.8	33.8	36.5	4.2
Leucine	41.6	55.8	68.1	9.1
Lysine	40.9	18.9	52.1	3.3
Phenylalanine	21.8	38.6	43.2	6.5
Methionine	16	11.8	12.1	2.0
Threonine	23	26.5	31.1	3.7
Tryptophan	4.9	8.9	10.4	1.4
Valine	29.3	58.6	37.4	5.1
Non-essential amino acids composition (g kg $^{-1}$)				
Alanine	43.3	32.8	40.9	4.6
Asparginine	60.5	76.5	122.8	7.2
Cystine	4.3	1.4	9.8	2.9
Serine	25.5	35.1	46.0	6.3
Glutamine	79.4	119.4	174.9	44.9
Glycine	59.8	36.4	37.2	5.6
Tyrosine	14.8	20.0	31.0	3.3
Proline	36.9	41.0	50.2	14.5

ND: Not detected

[a]Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Table 3 Proximate, amino acid composition (g kg⁻¹) and digestible protein to digestible energy ratio (DP/DE, g MJ⁻¹) of the experimental diets

| Treatment*	Control	J_{50}	J_{75}	S_{50}	S_{75}
Proximate (g kg⁻¹)					
Dry matter	952	951	955	961	962
Crude protein	384	382	381	380	385
Crude lipid	92	94	97	88	91
Crude ash	109	105	103	96	92
Gross energy (KJ/g)	19.1	19.8	20.4	19.4	20.1
Essential amino acids (g kg⁻¹)					
Arginine	24.53	27.75	31.99	22.57	24.14
Histidine	12.30	10.47	10.70	9.96	9.92
Iso leucine	15.85	13.97	14.56	13.62	14.00
Leucine	28.91	24.96	25.45	25.74	26.56
Lysine	28.43	20.44	19.96	20.29	19.83
Phenylalanine	15.15	15.67	16.85	15.53	16.61
Methionine	11.12	7.05	6.29	6.82	5.93
Threonine	15.99	12.37	12.21	12.52	12.42
Tryptophan	3.41	3.54	3.83	3.58	3.89
Valine	20.36	20.79	23.05	15.74	15.41
Non-essential amino acids (g kg⁻¹)					
Alanine	30.09	18.91	16.92	19.51	17.79
Asparginine	42.05	32.77	33.02	38.89	42.13
Cystine	3.34	2.58	2.19	3.99	4.31
Glycine	17.72	15.79	16.18	16.82	17.69
Glutamine	55.18	62.15	64.36	68.83	74.26
Proline	41.56	24.04	20.41	23.31	19.27
Serine	10.29	8.94	9.13	10.35	11.22
Tyrosine	25.65	23.27	22.87	23.87	23.72
DP/DE (g MJ⁻¹)	19.6	19.7	19.1	20.5	20.7

Amino acid compositions of the experimental diets were calculated from amino acid profile of individual feed ingredients.
*See footnotes to Table 1.

Fish behaviour, feed intake and growth

Sometimes the use of plant protein products in aquaculture diets is limited by the effects of the ingredients on the palatability of the diets to the fish (Burel et al., 1998). Based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was

normal. No left feed was observed in the aquaria. These findings are in contrast with those reported by others who observed a decline in feed intake of soy and lupin protein isolates fed at higher than 40% inclusion levels to common carp and rainbow trout (Escaffre et al., 1997; Glencross et al., 2004).
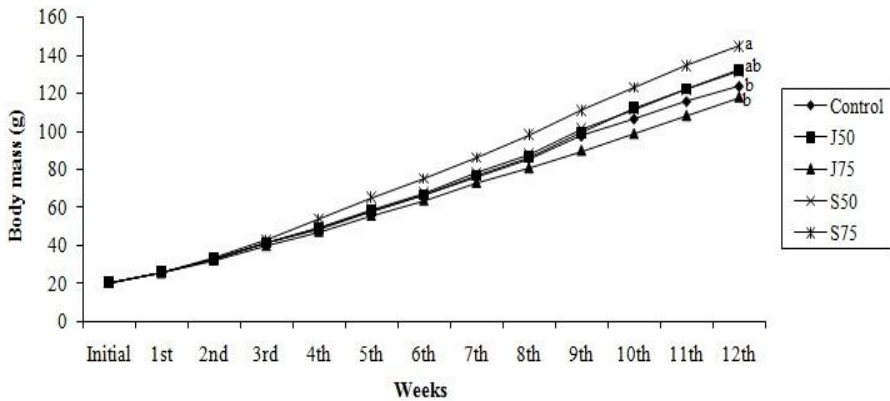
Figure 1 Body mass gain of common carp (*Cyprinus carpio* L.) fed experimental diets for 12 weeks

Weekly body mass gains of fish are given in Figure 1. These indicate that from third week onwards there was differential growth among the groups and lower body mass development was observed in J_{75} and control groups than other groups. This trend was maintained till the end of the experiment. Growth performance and nutrient utilization parameters are presented in Table 4. Highest BMG, SGR, MGR, PER, LPV were observed for S_{75} group, which were not statistically different from those for J_{50} and S_{50} groups and significantly higher (P < 0.05) than those for all other groups. Lowest PPV was observed in control, which was not statistically different from that for J_{75} group and significantly lower (P < 0.05) than those for all other groups. Lowest feed gain ratio was observed for S_{75} group, and this value significantly (P < 0.05) differed from those for all other groups.

Significantly higher (P < 0.05) LPV was observed in plant protein fed groups than control group. Since overall growth performance and protein or energy utilisation of this group was similar to the FM fed group, the current study demonstrates that a high replacement level (up to 75%) of FM by a single plant-protein source such as DJPI is possible in common carp. Escaffre et al. (1997) have reported reduced growth in carp fed a diet containing SPI at levels $\geq$ 60%. On the other hand, many researchers (Glencross et al., 2005;

Kaushik et al., 2004; Overland et al., 2009) have shown that soy, lupin and pea protein isolates can replace 50-75% of FM protein in trout and Atlantic salmon diets without impairing the growth performance and nutrient utilization. Despite the existence of some variability between fish species in the utilisation of plant products, results of most studies confirm that high dietary levels (>40% of total protein) of plant derived proteins depress growth and feed efficiency in carp (Hasan et al., 1997; Mazurkiewicz, 2009). This poor growth performance commonly found in fish fed plant protein-rich diets, in most cases, was related to reduction in the voluntary feed intake (consequently a lower intake of essential nutrients and digestible energy) (Gomes et al., 1995). However, in some studies conducted with rainbow trout, the total replacement of FM by soybean products (with or without supplemented L-methionine) was successfully achieved (Wilson, 1992; Kaushik et al., 1995).

Table 4 Growth performance and nutrient utilisation of common carp (*Cyprinus carpio* L.) fed the experimental diets for 12 weeks

Treat*	IW (g)	FW (g)	BMG	SGR	MGR	FGR	PER	PPV	LPV	EPV
Control	20.3 ± 0.12	$124^b \pm 9.0$	$510^b \pm 42$	$2.15^b \pm 0.08$	$13.2^b \pm 0.50$	$1.36^a \pm 0.06$	$1.91^b \pm 0.11$	$30.4^c \pm 2.84$	$35.3^c \pm 7.78$	$22.1^b \pm 1.06$
J$_{50}$	20.3 ± 0.11	$128^{ab} \pm 5.7$	$533^{ab} \pm 25$	$2.20^{ab} \pm 0.05$	$13.5^{ab} \pm 0.29$	$1.31^a \pm 0.03$	$2.01^{ab} \pm 0.05$	$34.2^b \pm 1.37$	$48.0^b \pm 8.15$	$23.8^{ab} \pm 5.95$
J$_{75}$	20.2 ± 0.08	$118^b \pm 13.5$	$481^b \pm 65$	$2.09^b \pm 0.14$	$12.8^b \pm 0.83$	$1.39^a \pm 0.10$	$1.86^b \pm 0.14$	$33.7^{bc} \pm 2.83$	$56.0^a \pm 10.50$	$24.1^{ab} \pm 2.19$
S$_{50}$	20.4 ± 0.14	$131^{ab} \pm 7.0$	$546^{ab} \pm 38$	$2.22^{ab} \pm 0.07$	$13.6^{ab} \pm 0.40$	$1.29^a \pm 0.05$	$2.02^{ab} \pm 0.05$	$36.4^{ab} \pm 1.14$	$53.7^{ab} \pm 4.21$	$27.1^a \pm 1.65$
S$_{75}$	20.4 ± 0.12	$145^a \pm 2.4$	$610^a \pm 9$	$2.33^a \pm 0.01$	$14.3^a \pm 0.10$	$1.19^b \pm 0.03$	$2.17^a \pm 0.07$	$38.7^a \pm 2.21$	$67.2^a \pm 8.27$	$28.9^{ab} \pm 1.91$
SEM	0.04	3.04	14.47	0.03	0.18	0.02	0.03	1.01	3.42	0.94

*See footnotes to Table 1. Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
IW- Initial weight, FW - Final weight, BMG (%) - Body mass gain, SGR (%) – Specific growth rate and FGR – Feed gain ratio; MGR (gkg$^{0.8}$ day^{-1}) - Metabolic growth rate, PER - Protein efficiency ratio, PPV (%)- Protein productive value, LPV (%) - Lipid production value and EPV (%) - Energy production value.

Chemical composition of whole body of fish

Whole body chemical composition of fish was significantly affected by dietary treatments (Table 5). Moisture content exhibited inverse relationship with lipid and protein contents. Similar trend has been reported in a study by Hasan et al. (1997), wherein FM protein was replaced by plant protein such as mustard, sesame, linseed, copra and groundnut oil cakes. Significantly higher crude lipid deposition was observed in all plant protein fed groups compared to control group. As plant protein increased in the common carp diet, lipid retention in the whole body also increased. Higher lipid content in fish of plant protein fed groups led to higher value of LPV and EPV in these groups (Table 4). Similarly, Hasan et al. (1997) and Mazurkiewicz (2009) observed that FM protein replaced by plant protein in common carp diet exhibited higher lipid deposition. Makkar et al. (2009) observed higher LPV in common carp fed detoxified Jatropha kernel meal and soybean meal based diets compared to FM based diet. There is evidence that replacement of FM by plant protein sources such as corn gluten meal and soy protein concentrates increases hepatic lipogenic enzyme activities in seabass that leads to higher whole body lipid (Dias, 1999; Kaushik et al., 2004). In the present study, higher value of HSI in plant protein fed groups suggests higher lipid deposition in liver. Hepatosomatic index values of about 1, as observed here, are common in common carp (Yılmaz and Genc, 2006; Makkar et al., 2009).

Crude protein of whole body was higher in plant protein fed groups than control group (Table 5), which concurs with the higher value of PPV in plant protein groups (Table 4). Efficient protein synthesis requires sufficient availability of all essential amino acids. Unbalanced amino acid concentrations in a diet or different solubility of individual amino acids results in increased protein degradation, and thereby increased protein turnover. Cheng et al. (2003) reported that the plant protein (soy protein) based diets decrease nitrogen retention in salmon and trout because these diets have less digestible energy and an amino acid profile that is sub optimal for muscle growth. Interestingly in our study crude protein content in whole body was higher in DJPI and SPI fed groups. In accordance with this, other researchers (Hasan et al., 1997; Mazurkiewicz, 2009) also found that the body protein content increased significantly when plant protein replaced FM in common carp diet. Almost similar amino acid contents in DJPI, SPI and FM (Table 3) along with supplementation of lysine in the diets

(J[50] and J[75] diets) might have resulted in the increased protein accretion in test groups (J[50], J[75], S[50] and S[75]) compared to control group. Jahan et al. (2003) have shown that proper combination of FM and plant proteins increases protein retention in carp (Jahan et al., 2003). These observations suggest that DJPI and SPI containing diets contained optimum digestible energy and a balanced amino acid profile for optimum growth of common carp.

Table 5 Chemical composition of whole body of common carp (*Cyprinus carpio* L.) of different experimental groups at the start and at the end of the experiment (g kg $^{-1}$ wet basis ± SD)

Treatment*	Moisture	Crude protein	Crude lipid	Ash	Gross energy (kJ/g)
Initial fish	783 ± 15.4	141 ± 11.3	42 ± 2.2	33 ± 1.3	43 ± 1.4
Control	764[a] ± 12.1	156[b] ± 6.8	53[b] ± 11.5	27[a] ± 3.1	5.4[b] ± 0.26
J[50]	747[b] ± 11.5	165[ab] ± 5.1	66[ab] ± 15.0	28[a] ± 3.5	5.9[a] ± 0.29
J[75]	737[b] ± 5.8	170[a] ± 3.1	71[a] ± 9.6	27[a] ± 3.6	6.4[a] ± 0.45
S[50]	747[b] ± 5.8	166[a] ± 4.1	67[ab] ± 5.4	27[a] ± 2.1	6.4[a] ± 0.21
S[75]	743[b] ± 7.1	170[a] ± 7.1	73 [a] ± 9.6	25[a] ± 2.6	6.6[a] ± 0.47
SEM	4.2	2.1	3.4	0.7	0.17

* See footnotes to Table 1
Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 6 Apparent digestibility coefficient of the dry matter, nutrients and energy of diets, and efficiency of digestible nutrients and energy of diets in common carp (*Cyprinus carpio* L.)

Treatment*	Dry matter digestibility	Protein digestibility	Lipid digestibility	Energy digestibility	Digestible protein retained (%)	Digestible lipid retained (%)	Digestible energy retained (%)
Control	82[b] ± 1.53	90[b] ± 1.03	94[a] ± 1.58	88[b] ± 1.28	33.6[b] ± 3.09	44.8[b] ± 11.97	25.1[a] ± 1.45
J[50]	86[a] ± 2.54	93[a] ± 1.57	95[a] ± 1.67	91[a] ± 1.64	36.7[a] ± 1.64	61.5[ab] ± 16.35	26.2[a] ± 6.74
J[75]	83[b] ± 3.21	89[b] ± 2.01	94[a] ± 2.53	89[b] ± 2.08	37.2[ab] ± 3.58	75.8[a] ± 15.78	27.1[a] ± 2.32
S[50]	83[b] ± 3.21	91[b] ± 1.94	94[a] ± 2.52	88[b] ± 2.08	39.6[a] ± 1.21	68.2[ab] ± 7.19	31.0[a] ± 2.17
S[75]	88[a] ± 2.08	94[a] ± 1.59	96[a] ± 1.15	92[a] ± 2.67	41.0[a] ± 2.54	83.7[a] ± 11.01	31.4[a] ± 1.90
SEM	0.82	0.53	0.43	0.58	0.89	4.54	1.03

* See footnotes to Table 1. Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Nutrient digestibility and digestible nutrients and energy retained

Highest ADCs of protein and energy were observed for the S_{75} group, which were not statistically different to those for J_{50} group and significantly higher ($P < 0.05$) than for all other groups; whereas, ADCs of dry matter and lipid did not differ significantly among the five groups (Table 6). Apparent digestibility coefficients of oil seed meal proteins and plant protein isolates are 80-95% and 90-98% respectively for tilapia, trout, Atlantic salmon and common carp (Jauncey and Ross, 1982; Glencross et al., 2005; Makkar et al., 2009). Common carp is reported to be able to digest the plant proteins well, generally slightly better than carnivorous fish species (NRC, 1983). The protein digestibility coefficient is a key factor in the evaluation of the quality of a diet and in particular in determination of its potential for the synthesis of new tissues. Detoxified Jatropha protein isolate and SPI, in combination with FM protein, showed excellent dry matter, crude protein, lipid and energy digestibilities in the present study (Table 6), indicating excellent utilization of feed ingredients. Makkar et al. (2009) reported that apparent protein digestibility values ranged between 80–92% for carp fed plant protein (detoxified Jatropha kernel meal and soybean meal) based diets. In the present study, when compared to FM proteins, Jatropha and soy protein isolates have higher apparent protein digestibility (at 50% replacement of FM protein), which could be attributed to the absence of trypsin inhibitor and lectin, presence of low levels of NSPs, and addition of phytase to mitigate the effects of phytate, if any. In another study Kaushik et al. (1995), have used a SPI (containing protease inhibitors <3 mg/g) as a FM replacer in trout diet and found ADCs of protein of SPI fed groups was similar to FM fed group. However, digestibility of protein from corn gluten meal was significantly lower than that from FM in carp and rainbow trout (Escaffre et al., 1997; Gomes et al., 1995). Energy digestibility of DJPI and SPI protein based diets was considerably lower than protein digestibility. Our results are in concurrence with those obtained in studies by Gouveia et al. (1993) and Makkar et al. (2009) in trout and common carp respectively wherein they observed considerably lower energy digestibility of plant protein based diets than protein digestibility.

Digestible protein, lipid and energy retained were in the range of 34-41%, 45-84% and 25-31% respectively (Table 6). Digestible energy retained of diets did not differ significantly among the five groups. Plant protein fed groups exhibited higher retention of digestible protein and lipid than control group,

indicating that common carp has utilized DJPI and SPI better than FM and retained more efficient than control group.

Digestive enzyme activities and intestinal somatic index

Dietary inclusion of DJPI and SPI did not alter the digestive enzymes (amylase, protease and lipase) activities (Table 7). Phytate and trypsin inhibitor inhibit activities of digestive enzymes such as trypsin, pepsin and alpha-amylase (Alarcon et al., 1999), and phytate forms complexes with minerals (Sugiura et al., 1999) and proteins (Moyano et al., 1999); thereby modifying digestion processes and thus impairing intestinal absorption of nutrients. Heat labile antinutrients (trypsin inhibitors and lectins) were not detected in the DJPI and SPI; whereas, heat stable enzyme inhibitor (phytate) was present in DJPI and SPI. Phytate content in DJPI and SPI were 2.9% and 0.95% respectively. For plant based feeds, we used 500 FTU phytase per kg feed, which might be sufficient to hydrolyse the phytate content in DJPI and SPI. No difference in activities of digestive enzymes could be due to the absence of trypsin inhibitors and lectins and addition of phytase in the plant protein based diets. Our results are in contrast with those of Escaffre et al. (1997), who observed that increasing levels of dietary soy protein concentrate induced a significant decline in intestinal trypsin activity in common carp. In our previous study, common carp fed with detoxified Jatropha kernel meal and soybean meal exhibiting lower protease, amylase and lipase activities (Makkar et al., 2009). In that study detoxified Jatropha kernel meal and soybean meal contained 9.3% and 2.5% phytate respectively, which is almost 4 to 5 times higher than in DJPI and SPI. High content of phytate could inhibit the digestive enzyme activities by forming complexes with minerals and proteins during digestion processes.

It is known that carnivorous and omnivorous fish require longer time to digest plant protein based diets (Buddington et al., 1997; Makkar et al., 2009). Direct relationship between the amount of dietary plant protein and ISI has been reported earlier in fish (Makkar et al., 2009). In carp, higher intestinal somatic index was observed in fish fed DJPI and SPI based diets compared with FM fed diet (Table 7). Similarly, intestinal somatic index was higher in the plant protein fed groups than in control group in the common carp and trout (Santigoga et al., 2008; Makkar et al., 2009). From a physiological view point, a longer intestinal somatic index would facilitate an increase in digestibility and retention time by enhancing contact time of the digestive enzymes and the feed components, resulting in increase in their

digestion and absorption. Omnivorous fish like common carp appear to possess compensation mechanism, such as an increase in intestinal index and as a result increase in digestive activity, to achieve a digestive balance and growth rates similar to those observed for FM fed group.

Table 7 Activities of digestive enzymes (U/mg protein), hepato somatic index (HSI) and intestinal somatic index (ISI) of fish and digestible protein to digestible energy (g digestible protein/MJ digestible energy) ratio of different experimental groups

Treatment*	Amylase	Protease	Lipase	HSI	ISI
Control	$20.1^a \pm 3.36$	$40.0^a \pm 2.82$	$8.5^a \pm 0.85$	$0.87^b \pm 0.15$	$1.48^b \pm 0.18$
J_{50}	$18.6^a \pm 5.81$	$37.1^a \pm 3.91$	$8.4^a \pm 0.46$	$0.99^a \pm 0.17$	$1.66^a \pm 0.24$
J_{75}	$18.4^a \pm 4.40$	$32.7^a \pm 3.04$	$7.8^a \pm 1.39$	$1.12^a \pm 0.20$	$1.63^a \pm 0.10$
S_{50}	$19.0^a \pm 4.59$	$37.2^a \pm 5.56$	$7.9^a \pm 0.93$	$1.04^a \pm 0.04$	$1.58^a \pm 0.11$
S_{75}	$18.1^a \pm 5.44$	$38.1^a \pm 3.39$	$7.7^a \pm 1.37$	$1.04^a \pm 0.05$	$1.62^a \pm 0.15$
SEM	1.15	1.02	0.25	0.04	0.06

* See footnotes to Table 1
Values are mean (n = 3) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Conclusions

Detoxified *Jatropha curcas* protein isolate (DJPI) fed groups exhibited good growth performance (almost seven times increase in fish body mass after 12 weeks). The DJPI showed a potential for use in aqua feed. It can be used as one of the promising fish meal replacers in the diets of common carp. It can replace up to 75% of fish meal protein without sacrificing fish yield.

Acknowledgements

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Chapter 13

Hemato-immunological and biochemical responses in common carp (*Cyprinus carpio* L) fed *Jatropha curcas* and soy protein isolates

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Abstract

Jatropha curcas L. is a biofuel plant and its seeds are rich in oil and protein. Jatropha seed cake (JSC) obtained after oil extraction is rich in protein. The principle of isoelectric precipitation was used to obtain Jatropha protein isolate (JPI) from JSC and it was detoxified (DJPI). Carp (n = 45, 20.3 ± 0.13g) were randomly distributed into five-groups with three-replicates and for 12-weeks fed iso-nitrogenous diets (crude-protein 38%): Control (fishmeal based protein); J_{50} and J_{75} (50% and 75% of fishmeal protein replaced by DJPI); S_{50} and S_{75} (50% and 75% of fishmeal-protein replaced by soy-protein-isolate). At the end of experiment highest growth-performance was observed for S_{75} group but that was not significantly different to J_{50} and S_{50} groups but was significantly higher than those for all other groups. Significantly higher ($P < 0.05$) red blood cells count, hematocrit content, albumin concentration in blood, plasma triglyceride level and lysozyme activity in serum were observed in plant protein fed groups compared to fishmeal fed group whereas mean cell volume, blood glucose level, plasma cholesterol and creatinine level in blood exhibited opposite trend. White blood cells count, hemoglobulin concentration, mean cell hemoglobin, mean cell hemoglobin concentration, albumin, total protein, total bilirubin, urea nitrogen, ions (sodium and potassium) and activities of alkaline phosphatase and alanine transaminase in blood did not differ significantly among the five groups. Hematological and biochemical parameters were in the normal range and suggesting no adverse effects of DJPI in carp. Conclusively, DJPI is a promising fishmeal replacer in common carp diet.

Keywords: *Jatropha curcas,* protein isolate, common carp, growth, blood chemistry, biochemical

Introduction

Modern intensive aquaculture tends towards higher stocking density and high feed ration, which often implies increased susceptibility to infections, nutritional diseases and various environmental stress reactions. Traditionally intensive aquaculture was mainly depending on fish meal (FM) as a protein in aqua feed because of its protein quality and palatability. Since the last decade the increasing demand, price and world supply fluctuations of FM has emphasized the need to look for alternative protein sources in aqua feeds. Thus, the research for alternatives to FM is an international research priority. Plants are widely available and reasonably priced; the use of plant protein sources in aqua feeds should be considered (SOFIA, 2007). Substantial effort is being invested in exploring the use of plant proteins as substitutes for FM and this presents several challenges. Firstly, the amino acid compositions of many plant proteins differ significantly from that of FM and feeding such diets may induce essential amino acid deficiencies that would restrict growth, protein utilisation unless supplemented and it could have also affects the fish health (Espe et al., 2006; Hansen et al., 2007).

Jatropha curcas L. is a biofuel plant and its seed rich in oil and protein. Jatropha seed cake obtained after oil extraction is an excellent source of protein. However, presence of shells containing approximately 45% lignin, antinutritional and major toxic components phorbol esters (PEs) restricts its use as fish feed. Recently we have prepared protein isolate from Jatropha seed cake which contains about 81% crude protein and it has higher essential amino acids (except lysine and methionine) than FM (Makkar et al., 2008; Makkar and Becker, 2009; Kumar et al., 2011a). A method for detoxification of Jatropha protein isolate has been developed in our laboratory. It is based on the removal of PEs using organic solvents and inactivation of trypsin inhibitors and lectin by heat treatment.

Biochemical and hematological tests have proved useful in the detection and diagnosis of metabolic disturbances in fish. In common carp, growth, biochemical and hematology are good indicators of the adverse effects of plant nutrients on fish health and welfare. It is worthy of note that no biochemical and hematological and histopathological findings have been reported in studies that have examined the effects of DJPI and soy protein isolate on common carp. Therefore the main aim of the present study is to find out the effects of DJPI and soy protein isolate (SPI) on biochemical and hematological features in young common carp (Cyprinus carpio L.).

Materials and methods

Preparation of the Jatropha protein isolates

Jatropha seed cake obtained using a mechanical screw press (German screw press type Komet D85-1G, Germany) was used for preparation of protein isolate. The chemical composition of the seed cake was: crude protein 23.6 %, oil 9.3 %, ash 5.8% and lignin 43%; all on dry matter basis). The principle of isoelectric precipitation was used to obtain protein isolate from the seed cake. To 500 g of defatted seed cake (in triplicate) was added 5000 ml of distilled water adjusted to pH 11 using 10 mol L^{-1} NaOH. The mixture was stirred for 1 h at room temperature (20 °C). Every 15 min the pH was checked and adjusted to 11 using 10 mol L^{-1} NaOH. The contents were centrifuged at 3000 × g for 20 min and the supernatant was collected. The supernatant was brought to pH 4 using 6 mol L^{-1} HCl, stirred for 10 min and kept at 4 °C for 1 h to precipitate proteins. The contents were centrifuge at 3000 × g for 20 min to obtain the protein isolate. The protein isolate was freeze-dried. Organic solvents were used to detoxify the protein isolate (patent application has been filed for the process of detoxification). After removal of PEs, the protein isolate was autoclaved (121 °C for 15 min) to inactivate heat labile antinutrients, trypsin inhibitor and lectin.

Diet formulation, experimental system and animals

Fish meal was purchased from Kurt Becker GmbH, Bremen, Germany. Prior to feed formulation, the proximate composition of DJPI, SPI, wheat meal, and FM was determined. Five isonitrogenous diets were formulated. Experimental diets contained crude protein 38%, crude lipid 9%, gross energy 19.5 KJ/g, vitamin premix 2%, mineral premix 2% and titanium oxide (TiO_2) 1%. Lysine monohydrochloride (lysine 80% in this salt) was supplemented 2.71% of DJPI inclusion in the diet. Each experimental feed, except the control, contained 500 FTU phytase (NATUPHOS 5000G, BASF, Ludwigshafen, Germany) per kg. The inclusion level of the DJPI and SPI were as follows: Control diet (Control) was prepared with FM and wheat meal, without DJPI and SPI; J_{50} and J_{75} (50% and 75% of FM protein replaced by DJPI), and S_{50} and S_{75} (50% and 75% of FM protein replaced by SPI). The final mixture of each diet was made into 2 mm diameter moist pellets and then freeze-dried (Table 1).

Table 1 Composition of the experimental diets (g kg^{-1} feed)

Ingredients	Experimental diets*				
	Control	J$_{50}$	J$_{75}$	S$_{50}$	S$_{75}$
Fish meal	484	242	121	242	121
[1]Wheat meal	436	435	435	435	435
Jatropha protein isolate	-	196	295	-	-
Soya protein isolate	-	-	-	172	258
Cellulose	-	23	33	47	70
Sunflower oil	40	64	76	64	76
[2]Vitamin premix	20	20	20	20	20
[3]Mineral premix	20	20	20	20	20
Total	1000	1000	1000	1000	1000
Lysine monohydrochloride (g)	-	5.4	8.0	-	-
Phytase (FTU/kg)	-	500	500	500	500
TiO$_2$	10	10	10	10	10

*Experimental diets

Control: Fish meal and wheat meal, without any Jatropha protein isolate and soy protein isolate

J$_{50}$: 50% of fish meal proteins replaced by Jatropha protein isolate

J$_{75}$: 75% of fish meal protein replaced by Jatropha protein isolate

S$_{50}$: 50% of fish meal protein replaced by soy protein isolate

S$_{75}$: 75% of fish meal protein replaced by soy protein isolate

[1]Whole wheat meal.

[2]Vitamin premix (g or IU kg-1 premix): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[3]Mineral premix (g kg-1): CaCO$_3$, 336; KH$_2$PO$_4$, 502; MgSO$_4$. 7H$_2$O, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; MnSO$_4$. H$_2$O, 3.12; ZnSO$_4$. 7H$_2$O, 4.67; CuSO$_4$. 5H$_2$O, 0.62; KI, 0.16; CoCl$_2$. 6H$_2$O, 0.08; ammonium molybdate, 0.06; NaSeO$_3$, 0.02.

Common carp (*Cyprinus carpio* L.) fingerlings obtained from the Institute of Fisheries Ecology and Aquaculture of the Federal Research Center for Fisheries at Ahensburg, Germany were transferred to the University of Hohenheim, Stuttgart, Germany and kept in two 500 l capacity tanks for acclimatisation. After acclimatisation, 45 fish were randomly distributed into five groups with three replicates; each replicate contained three fish (av. wt. 20.3 ± 0.13 g) in an aquarium (45 l capacity). All the aquaria were supplied with water from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 26.2 – 27.1°C, pH 7.0 – 7.5, dissolved

oxygen 6.9 – 7.4 mg l⁻¹, total NH_3 0.1– 0.2 mg l⁻¹, nitrite 0.07 – 0.1 mg l⁻¹ and nitrate 1–3 mg l⁻¹). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved and during the experimental period fish were fed at 16 g feed per kg metabolic body mass (kg$^{0.8}$) per day (equal to five times their maintenance requirement) and split into 5 equal rations per day (at 8:00, 10:30, 13:00, 15:30 and 18:00 h). The feeds were dispensed using an automatic feeder. Fish were weighed individually at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feeding level for the subsequent week. The fish were not fed on the weighing day.

The experiment lasted 12 weeks and fish were sampled thereafter. At the end of experiment, one fish per group was anaesthetized with tricaine methanesulfonate (MS222; 250 mg/L). Blood was drawn near caudal peduncle from one fish from each replicate and transferred into a heparinized tube for hematological study. Blood was drawn from another fish from each group and divided into two equal part, one part was centrifuged at 1500×g for 5 min at room temperature (24 ℃) to obtain plasma, which was then stored at −20 ℃ for determination of cholesterol and triglycerides. Another part of blood was kept outside at room temperature for few minutes to collect serum. Serum was stored at −20 ℃ for lysozyme determination. Muscle was isolated from one fish per replicate and stored at −20 ℃ for determination of cholesterol and muscle lipid peroxides.

The University of Hohenheim Animal Welfare Committee approved all the experimental procedures involving in keeping, feeding and sampling of common carp.

Proximate analysis and determination of phorbol esters, antinutrients and amino acid

The proximate composition of diet ingredients, diets and whole body of fish was determined using standard methods (AOAC, 1990). Phorbol esters (PEs) were determined according to Makkar et al. (2007) based on the method of Makkar et al. (1997). The results were expressed as equivalents to a standard phorbol-12-myristate 13-acetate (Sigma, Saint Louis, USA). Detection limit of PEs by HPLC was 3 μg/g protein isolates. Trypsin inhibitor activity was determined according to Smith et al. (1980) except that the enzyme was added last as suggested by Liu and Markakis (1989). Analysis of the lectin content was conducted by haemagglutination assay (Makkar et al., 1997).

Phytate content of samples was determined by a spectrophotometric procedure (Vaintraub and Lapteva, 1988). Non-starch polysaccharides (NSP) were determined according to Englyst et al. (1994). Amino acid composition of FM, DJPI, SPI and wheat meal was determined using an automated amino acid analyser after hydrolysing the samples with 6M HCl at 110 ^{0}C for 24 h (Bassler and Buchholz, 1993). The sulphur-containing amino acids were oxidised using performic acid before the acid hydrolysis. Tryptophan content was determined spectrophotometrically by the method of Pinter-Szakacs and Molnar-Perl (1990).

Determination of lipid peroxides, cholesterol and triglyceride

Lipid peroxides in fish muscle were determined using the procedure of Utley et al. (1967). The determinations of the plasma cholesterol and triglycerides were using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color intensity was determined photometrically and was directly proportional to the concentration of cholesterol and triglycerides.

Haematological parameters

Total erythrocyte count (RBC) and total leucocyte count (WBC)

RBC and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope.
The following formula was used to calculate the number of RBC per mm^3 of the blood sample:
Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid

Haemoglobin (Hb) and hematocrit (Hct) content

The Hb content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hct was determined on the basis of sedimentation of blood. Heparinised blood (50

μl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Haematocrit [in L/L] x 1000) ÷ (RBC count [in millions/μL]); mean corpuscular haemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/μL]) and mean cell haemoglobin concentration, MCHC [in g/dL] = Haemoglobin [in g/dL] ÷ Haematocrit [in L/L].

Lysozyme activity in serum

Lysozyme activity of serum was measured by EnzChek Lysozyme Assay Kit (E-22013) Leiden, The Netherlands. The assay measures lysozyme activity on *Micrococcus lysodeikticus* cell wall, which is labeled to such a degree that the fluorescence is quenched. Lysozyme action relieves this quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase was measured by using spectrofluorometer that detects fluorescein. Lysozyme hydrolyzes β-(1-4)-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of the microorganism.

Blood parameters analysis by Vet Scan

VetScan Chemistry Analyzer (Scil Animal care company GmbH, Technischer Service, Germany) was used for determinations of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium (Ca^{++}), creatinine, globulin, glucose, phosphorus, potassium (K^+), sodium (Na^+), total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Growth performance

Growth performance was assessed in terms of body mass gain (BMG) = [(Final body mass - initial body mass) / Initial body mass] X 100 and specific growth rate (SGR) = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test (P < 0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results and discussion

Substantial efforts have been made by many researchers (Glencross et al., 2005; Makkar et al., 2008; Makkar and Becker, 2009; Øverland et al., 2009; Kumar et al., 2011a; Nepal et al., 2010)   to enhance protein content by processing technologies from seed cakes of soybeans, lupins, pea and Jatropha. Protein isolate from soybean are available in the market whereas protein isolates from other plant products are still in an undeveloped stage. Usually increase in the protein content of these products concomitant with a decrease in the levels of antinutritional factors and carbohydrate content (Makkar and Becker, 2009; Makkar et al., 2008).

Toxic compound (phorbol esters), antinutrients, proximate and amino acid composition

Untreated Jatropha protein isolate contains toxic compound (PEs) about 1.2 mg/g. Phorbol esters was not detected in DJPI. Heat labile antinutrients (trypsin inhibitor and lectins) were also not detected in DJPI and SPI; whereas phyate levels in DJPI and SPI were 2.95% and 0.94%, and NSP levels 10.5% and 1.04% respectively (Table 2). Proximate composition and amino acid profiles of feed ingredients and diets are shown in Tables 2 and 3. Diets were isonitrogenous (38% crude protein), containing 8.8–9.7% crude lipid and 9.2–10.9% ash. Almost similar amino acid composition (except methionine content, which is slightly lower in plant protein based diets than control diet) was in all experimental diets (Table 3). According to NRC (1993), the contents of essential amino acids were present in all the diets as per the requirement for the common carp. Experimental diets containing digestible protein/digestible energy (DP/DE) ratios in the range from 19.1 – 20.7 g MJ^{-1} (Table 3). Many researchers (Page and Andrew, 1973; Garling and Wilson, 1976; Takeuchi et al., 1979; Anderson, 1996; Einen and Roem, 1997) have found that optimum dietary DP/DE ratio should be in the range 17 – 26 (g MJ^{-1}) for various commercially important protein sources. For optimum

growth of common carp the diet should contain DP/DE in the range of 18 – 24 g MJ[-1] (Takeuchi et al., 1979). In the present study DP/DE (19.1-20.7 MJ[-1]) comes under the optimum range for maximum growth of common carp.

Palatability and growth performance

The use of plant protein products in aqua feed is limited by the effects of the ingredients on the palatability of the diets (Burel et al., 1998). In the present study based on the visual observation during feeding time, palatability or acceptability of feed was good and the behaviour of fish was normal. There was no residual feed in the aquaria. Our observations are in contrast with Escaffre et al. (1997) and Glencross et al. (2004a) they observed that a decline in feed intake of soy and lupin protein isolates fed at higher than 40% inclusion levels to rainbow trout and common carp diets.

Table 2 Proximate composition, antinutrients content and amino acid contents of feed ingredients

	Fish meal	Jatropha protein isolate	Soy protein isolate	Wheat meal
Proximate composition (g kg [-1])				
Dry matter	940	945	957	941
Crude protein	65.5	808	922	145
Crude lipid	88	9.7	10	16.3
Crude ash	142	93	37.9	14
Gross energy (KJ/g)	21.1	19.3	22.0	18.7
Antinutrients				
Trypsin inhibitor (mg trypsin inhibited per g sample)	-	ND	ND	-
Lectin[a]	-	ND	ND	-
Phytate (% dry matter)	-	2.95	0.95	-
Non-starch polysaccharides (g kg [-1])	-	105	10.4	-
Essential amino acids content (g kg [-1])				
Arginine	35.3	86.0	67.9	5.4
Histidine	17.7	24.0	24.4	3.4
Iso leucine	22.8	33.8	36.5	4.2
Leucine	41.6	55.8	68.1	9.1

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Table 2 continued

Lysine	40.9	18.9	52.1	3.3
Phenylalanine	21.8	38.6	43.2	6.5
Methionine	16	11.8	12.1	2.0
Threonine	23	26.5	31.1	3.7
Tryptophan	4.9	8.9	10.4	1.4
Valine	29.3	58.6	37.4	5.1
Non-essential amino acids content (g kg^{-1})				
Alanine	43.3	32.8	40.9	4.6
Asparginine	60.5	76.5	123	7.2
Cystine	4.3	1.4	9.8	2.9
Serine	25.5	35.1	46.0	6.3
Glutamine	79.4	119	175	44.9
Glycine	59.8	36.4	37.2	5.6
Tyrosine	14.8	20.0	31.0	3.3
Proline	36.9	41.0	50.2	14.5

ND: Not detected

[a]: Minimum amount of material (mg mL^{-1} assay medium) that produced agglutination.

Growth performance parameters are presented in Table 4. Highest growth performance (final body mass, BMG and SGR) was observed for S_{75} group, which were not statistically different from those for J_{50} and S_{50} groups and significantly higher ($P < 0.05$) than those for all other groups. Since overall growth performance of J_{75} group was similar to the FM fed group, which demonstrates that a high replacement level (up to 75%) of FM by a single plant-protein source such as DJPI is possible in common carp. In another study, Escaffre et al. (1997) have observed that when carp fed a diet containing SPI at levels $\geq$ 60% lead to lower growth performance whereas Glencross et al. (2005), Kaushik et al. (2004) and Overland et al. (2009) observed that soy, lupin and pea protein isolates can replace 50-75% of FM protein in trout and Atlantic salmon diets without compromising on growth performance. Carp fed high level of plant protein (>40-75% of total protein) diet depress growth and nutrient utilization (Hasan et al., 1997; Mazurkiewicz, 2009; Kumar et al., 2011b) because plant protein-rich diets reduces feed intake that leads to lower intake of essential nutrients (Gomes et al., 1995). Wilson (1992), Kaushik et al. (1995) observed that the total replacement of FM by soybean products (with or without supplemented L-methionine) was successfully achieved in rainbow trout.

Table 3 Proximate, amino acid composition (g kg^{-1}) and digestible protein to digestible energy ratio (DP/DE, g MJ^{-1}) of the experimental diets

Treatment*	Control	J$_{50}$	J$_{75}$	S$_{50}$	S$_{75}$
Proximate (g kg^{-1})					
Dry matter	952	951	955	961	962
Crude protein	384	382	381	380	385
Crude lipid	92	94	97	88	91
Crude ash	109	105	103	96	92
Gross energy (KJ/g)	19.1	19.8	20.4	19.4	20.1
Essential amino acids (g kg^{-1})					
Arginine	24.5	27.7	32.0	22.6	24.1
Histidine	12.3	10.5	10.7	10.0	9.9
Iso leucine	15.8	14.0	14.6	13.6	14.0
Leucine	28.9	25.0	25.5	25.7	26.6
Lysine	28.4	20.4	20.0	20.3	19.8
Phenylalanine	15.1	15.7	16.8	15.5	16.6
Methionine	11.1	7.0	6.3	6.8	5.9
Threonine	16.0	12.4	12.2	12.5	12.4
Tryptophan	3.4	3.54	3.8	3.6	3.9
Valine	20.4	20.8	23.0	15.7	15.4
Non-essential amino acids (g kg^{-1})					
Alanine	30.1	18.9	16.9	19.51	17.8
Asparginine	42.0	32.8	33.0	38.89	42.1
Cystine	3.34	2.6	2.2	3.99	4.3
Glycine	17.7	15.8	16.2	16.82	17.7
Glutamine	55.2	62.1	64.4	68.83	74.3
Proline	41.6	24.0	20.4	23.31	19.3
Serine	10.3	8.9	9.1	10.35	11.2
Tyrosine	25.6	23.3	22.9	23.87	23.7
DP/DE (g MJ^{-1})	19.6	19.7	19.1	20.5	20.7

Amino acid compositions of the experimental diets were calculated from amino acid profile of individual feed ingredients.

*See footnotes to Table 1.

Table 4 Growth performance and nutrient utilisation of common carp (*Cyprinus carpio* L.) fed the experimental diets for 12 weeks (Source: Kumar et al., 2011a)

Treatment*	Initial body mass (g)	Final body mass (g)	Body mass gain (%)	Specific growth rate (%.day^{-1})
Control	20.3 ± 0.12	124[b] ± 9.0	510[b] ± 42	2.15[b] ± 0.08
J$_{50}$	20.3 ± 0.11	128[ab] ± 5.7	533[ab] ± 25	2.20[ab] ± 0.05
J$_{75}$	20.2 ± 0.08	118[b] ± 13.5	481[b] ± 65	2.09[b] ± 0.14
S$_{50}$	20.4 ± 0.14	131[ab] ± 7.0	546[ab] ± 38	2.22[ab] ± 0.07
S$_{75}$	20.4 ± 0.12	145[a] ± 2.4	610[a] ± 9	2.33[a] ± 0.01
SEM	0.04	3.04	14.47	0.03

* See footnotes to Table 1.
Values are mean (n = 3) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 5 Cholesterol and triglyceride (mg/dl) level in plasma and muscle cholesterol (mg/100 g), muscle lipid peroxide (nMol MDA (malondialdehyde) /100g tissue) and blood glucose (mg/dl) level common carp (*Cyprinus carpio*) fingerlings

Treatment*	Plasma cholesterol	Plasma triglycerides	Muscle cholesterol	Muscle lipid peroxide	Blood glucose
Control	140[a] ± 15	76[c] ± 4.1	1.52[a] ± 0.05	1.89 ± 0.78	95[a] ± 7.8
J$_{50}$	118[b] ± 25	88[b] ± 3.2	1.24[b] ± 0.03	2.01 ± 0.87	84[b] ± 6.4
J$_{75}$	92[c] ± 16	102[a] ± 7.6	1.06[c] ± 0.06	2.22 ± 0.69	75[c] ± 3.2
S$_{50}$	112[b] ± 14	84[b] ± 4.4	1.27[b] ± 0.04	2.01 ± 0.58	84[b] ± 11
S$_{75}$	81[c] ± 12	98[a] ± 9.5	1.01[c] ± 0.05	2.17 ± 0.77	69[c] ± 9.2
SEM	3.08	2.2	0.02	0.31	2.01

* See footnotes to Table 1.
Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Blood glucose; Cholesterol and triglycerides in plasma

Dietary inclusion of DJPI and SPI in common carp significantly reduced (P > 0.05) blood glucose level compared to FM fed group (Table 5). Glencross et al. (2004b) observed that plant protein (yellow lupin as a substitute to FM) did not have any effects on blood glucose level in rainbow trout. On the other hand, Kikuchi (1999) and Kumar et al. (2010) observed that dietary inclusion of detoxified Jatropha kernel meal, soybean meal and corn gluten in fish diet increased blood glucose level. The differences in above findings might be due to the presence of higher carbohydrate content in their plant

protein based diet (kernel meal) compared to our plant protein diet (protein isolate).

Cholesterol and triglyceride levels in plasma, and muscle cholesterol concentration were significantly lower ($P < 0.05$) in plant protein fed groups compared to FM fed group whereas triglyceride levels in plasma exhibited opposite trend. Muscle lipid peroxide value did not differ significantly ($P > 0.05$) among the five groups (Table 5). The decrease in plasma cholesterol concentrations in fish fed diets with plant proteins is in accordance with the results of other researchers (Yamamoto et al., 2007; Kumar et al., 2008; Kumar et al., 2010, 2011c). Usually, plant products are generally considered to have a hypocholesteromic effect in animals, because of presence of the relatively high levels of estrogeno-mimetic isoflavones (Setchell and Cassidy, 1999). Although cholesterol metabolism in terrestrial animals and fish could differ, the fish hypocholesterolemia in response to dietary plant protein supply could be due either to an increased excretion of bile salts, to an inhibition of cholesterol intestinal absorption, or just to the withdrawal of FM rather than to the direct effects of plant protein (Kaushik et al., 2004; Kumar et al., 2010).

Triglyceride level in plasma was higher in plant protein fed groups compared to FM fed groups (Table 5). Serum triglycerides act as a short-term indicator of feeding or nutritional status (Bucolo and David 1973). The increase in whole body fat content in plant protein fed group (DJPI and SPI fed groups) (data not presented, Kumar et al. 2011a) along with the increase in plasma triglyceride concentrations also suggest lipid mobilisation in these groups. Similar trend was observed by Kumar et al. (2010) wherein they found that inclusion of detoxified Jatropha kernel meal and soybean meal in the diet of common carp increased the plasma triglyceride level compared to FM fed group. In another study when yellowtail fed with soybean meal based diet that leads to decrease the plasma triglyceride level compared to FM fed group (Shimeno et al., 1993).

Hemato-immunology

The WBC counts, Hb content, MCH and MCHC did not differ significantly ($P > 0.05$) among the three groups (Table 6). Red blood cells counts, Hct content and MCV were higher in plant protein (DJPI and SPI) fed groups than FM fed group (Table 6). As the plant protein content increased, an increase in the RBC count and Hct content was observed. Plant ingredients may cause early release of immature erythrocytes (Hemre et al., 2005), that

leads to increase the RBC count and Hct content. Consequently, MCV value was changed at the same time. Mean cell volume indicates that cell size and higher MCV was observed in plant protein fed groups which suggest bigger cell size in plant protein fed groups compared to FM fed group. Similar trend has been observed in our previous study (Kumar et al., 2010) wherein detoxified Jatropha kernel meal replaced FM in common carp diet. Whereas opposite trend was observed in another study (Hemre et al., 2005) they found that significant reduction of MCV in Atlantic salmon when FM was replaced with soybean meal. In the present study MCHC did not differ significantly among the five groups whereas Hansen et al. (2007) reported that lower MCHC in the plant protein fed group might be due to release of immature erythrocytes with less Hb concentration.

The Hb and Hct assays are normally used as general indicators of fish health (NRC, 1981). Red blood cells count, WBC count, Hb and Hct content and MCV, MCH and MCHC were in the normal range reported by Radu et al. (2009) for common carp, Barham et al. (1980) for rainbow trout and Zhang et al. (2009) for Tibetan catfish and Sun et al. (1995) for tilapia.

Table 6 Effects of experimental diets on the haematological parameters (RBC (10^6 cells/mm³), WBC (10^3 cells/mm³), Hb (g/dl), Hct (%), MCV (fL), MCH (pg), MCHC (g/dl), albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood and lysozyme activity (IU/ml) in the serum of common carp (*Cyprinus carpio* L.) fingerlings

Group*	RBC	WBC	Hb	Hct	MCV	MCH	MCHC
Control	1.21[c] ± 0.05	1.29 ± 0.04	5.0 ± 0.00	31[c] ± 2.03	256[b] ± 16.3	41.3 ± 4.5	16.1 ± 1.32
J$_{50}$	1.38[b] ± 0.02	1.30 ± 0.04	5.9 ± 1.50	41[b] ± 2.31	297[a] ± 18.3	42.7 ± 11.6	14.3 ± 1.54
J$_{75}$	1.43[a] ± 0.02	1.30 ± 0.06	6.4 ± 1.85	45[a] ± 1.15	314[a] ± 12.8	44.8 ± 8.4	14.2 ± 1.74
S$_{50}$	1.34[b] ± 0.04	1.31 ± 0.02	5.6 ± 1.10	42[b] ± 2.04	313[a] ± 18.4	41.8 ± 8.1	13.4 ± 2.38
S$_{75}$	1.42[a] ± 0.03	1.30 ± 0.03	6.0 ± 1.20	44[a] ± 1.51	309[a] ± 19.2	42.2 ± 7.7	13.6 ± 2.25
SEM	0.02	0.02	0.29	0.81	6.2	2.11	0.58

*See footnotes to Table 1. Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05)
MCV: Mean cell volume (fL); MCH: Mean corpuscular hemoglobin (pg); MCHC: Mean corpuscular hemoglobin concentration (g/dl).

Albumin, globulin and total protein in blood and lysozyme activity in serum on common carp are presented in Table 7. Albumin and total protein in blood did not differ significantly among the five groups whereas globulin concentration in blood and lysozyme activity in serum were higher in plant protein fed groups compared to control group. The concentration of total protein in blood is used as a basic index for health and nutritional status in fish (Martinez, 1976). Among the blood proteins, albumin and globulin are the major proteins, which play a significant role in the immune

response. Albumin is used as an indicator of liver impairment (Silverman et al., 1986). Blood protein did not differ significantly (P > 0.05) among the five groups (Table 7), indicating that there are no nutritional deficiencies and no impaired protein metabolism in the liver. Albumin, globulin and total protein blood values for all groups were within the normal range of (Wedemeyer and Chatterton, 1970; Sandnes et al. 1988). Globulin is important for the immunological responses. Higher globulin concentration in blood was observed in plant protein fed groups (DJPI and SPI) compared to FM fed group. These results are consistent with the higher lysozyme activity observed in plant protein (DJPI and SPI) fed groups. This suggests an immunostimulating effect of DJPI and SPI inclusion in the diet of common carp. Lysozme is regarded as a participant of the first line of defense, with high activities in mucus, serum, gills and alimentary tract (Lie et al., 1989). Innate immunity due to lysozyme is caused by lysis of bacterial cell wall and this stimulates the phagocytosis of bacteria. The suppression of the non-specific immune capacity by high concentrations of dietary soybean proteins has been reported in rainbow trout (Burrells et al., 1999). However, other reports wherein soybean meal was fed to rainbow trout (Rumsey et al., 1994) and Atlantic salmon (Krogdahl et al., 2000) or alginate to Atlantic salmon (Gabrielsen and Austreng, 1998), increased values of different non-specific immune parameters have been reported, which have been interpreted as immunostimulating effects of plant protein sources. In the present study lysozyme activity was higher in plant protein fed groups, indicating immunostimulating effect of DJPI and SPI in common carp.

Enzymes activities and ions in blood

Alkaline phosphatase and ALT activities, TBIL, BUN and creatinine level in blood of common carp are shown in Table 8. Alkaline phosphatase and ALT activities, TBIL and BUN did not differ significantly among the five groups. Creatinine level was higher in FM fed group compared to plant protein fed groups. Alkaline phosphatase and ALT are released into blood during organ damage (Racicot et al., 1975). Alkaline phosphatase level rises during bile duct obstruction, and in intrahepatic infiltrative diseases of the liver and kideny (Goel et al., 1984). Alanine transaminase also called serum glutamic pyruvate transaminase is an enzyme present in hepatocytes (liver cells). When liver cell and kideny are damaged, it leaks into the blood. Alanine transaminase rises dramatically in acute liver and kidney damage (Racicot et al., 1975). Thus, detection of blood level of ALP and ALT gives information

on the damage of organs and in particular of liver cells. Activities of ALP and ALT were similar in all the groups, indicating normal organ function on feeding of DJPI and SPI. Hemre et al. (2005); Sanden et al. (2006) and Kumar et al. (2010, 2011c) also reported similar results on feeding plant protein (soybean meal and detoxified Jatropha kernel meal) containing diets to Atlantic salmon, trout and common carp. Alkaline phosphatase and ALT level in all groups were in the normal range reported by Zhang et al. (2009) which suggests that inclusion of DJPI in fish feed does not impair liver function.

Table 7 Lysozyme activity (IU/ml) in the serum, albumin (g/dl), globulin (g/dl) and total protein (g/dl) in the blood of common carp (*Cyprinus carpio* L.) fingerlings fed different experimental diets

Treatment*	Lysozyme activity	Albumin	Globulin	Total protein
Control	375[b] ± 22	1.97 ± 0.38	0.93[c] ± 0.06	2.90 ± 0.44
J$_{50}$	422[a] ± 21	2.17 ± 0.16	0.97[b] ± 0.12	3.13 ± 0.15
J$_{75}$	428[a] ± 34	1.77 ± 0.35	1.07[a] ± 0.15	2.83 ± 0.12
S$_{50}$	424[a] ± 25	1.97 ± 0.42	1.03[ab] ± 0.15	3.00 ± 0.26
S$_{75}$	435[a] ± 38	1.73 ± 0.16	1.23[a] ± 0.16	2.97 ± 0.17
SEM	19	0.08	0.04	0.06

*See footnotes to Table 1.
Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05)
IU- The amount of enzyme required producing a change in the absorbance at 450 nm of 0.001 units per minute at pH 6.24 and 25^{0}C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

Blood urea nitrogen levels are thought to be associated with liver or gill dysfunction (Stoskopf, 1993), as these are the sites of urea production and excretion, respectively. Blood urea nitrogen concentration did not differ significantly among the five groups, suggesting that DJPI and SPI fed groups were normal and healthy. Blood urea nitrogen concentrations were in the normal range (Wedemeyer and Chatterton, 1970; Witters, 1986; Wedemeyer, 1996). Bilirubin is the predominant bile pigment found in the circulation in fish derived from the breakdown of hemoglobin (Cornelius, 1992). An elevation in total bilirubin indicates increased erythrocyte destruction that indicates obstructive diseases of the bile system and hepatocellular disease (Duncan, 1995; Tietz, 1986). Total bilirubin concentration in blood was similar for all groups which indicate that liver and kidney function was normal. Creatinine is used as an indicator of kidney damage or malfunction

(Tietz, 1986). Creatinine is a degraded product of creatine, which is involved in muscle energy metabolism. Blood creatinine is normally quite stable. Its level in the blood becomes elevated if kidney function is impaired. Creatinine was highest in control group but it was within the normal range (Ceschia et al., 1978; Tietz, 1986). Creatinine is a metabolite of animal protein and its highest level in control is due to highest content of FM in this group (Table 8).

Table 8 Effects of experimental diets on alkaline phosphatase (ALP, U/l) and alanine transaminase (ALT, U/l), total bilirubin, TBIL (mg/dl), blood urea nitrogen, BUN (mg/dl) and creatinine (mg/dl) in blood of *Cyprinus carpio* L. fingerlings

Treatment*	ALP	ALT	TBIL	BUN	Creatinine
$C_{control}$	140 ± 6	88 ± 14	0.27 ± 0.06	2.87 ± 0.81	2.00[a] ± 1.58
J_{50}	160 ± 15	74 ± 17	0.23 ± 0.06	3.37 ± 0.58	0.67[b] ± 0.08
J_{75}	181 ± 61	87 ± 10	0.30 ± 0.00	3.17 ± 0.68	0.57[b] ± 0.06
S_{50}	191 ± 62	71 ± 15	0.23 ± 0.06	3.00 ± 0.00	0.63[b] ± 0.06
S_{75}	245 ± 32	76 ± 12	0.23 ± 0.06	2.67 ± 0.58	0.41[b] ± 0.38
SEM	19.52	3.51	0.02	0.16	0.29

*See footnotes to Table 1.
Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).
1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute).

Table 9 Effects of experimental diets on blood ions (calcium (mg/dl), phosphorus mg/dl, sodium (mmol/l) and potassium (mmol/l) of common carp (*Cyprinus carpio* L.) fingerlings

Treatment*	Calcium	Phosphorus	Sodium	Potassium
$C_{control}$	10.13[c] ± 0.32	12.83[b] ± 2.74	131 ± 1.73	1.50 ± 0.00
J_{50}	10.80[b] ± 0.26	14.60[a] ± 1.51	133 ± 1.58	1.50 ± 0.00
J_{75}	11.67[a] ± 0.61	15.13[a] ± 1.45	132 ± 1.15	1.57 ± 0.12
S_{50}	10.2[c] ± 0.31	10.7c ± 1.66	133 ± 2.31	1.50 ± 0.11
S_{75}	9.8[c] ± 0.22	8.9[d] ± 0.26	136 ± 2.31	1.55 ± 0.15
SEM	0.11	0.95	0.56	0.04

*See footnotes to Table 1. Values are mean (n = 3) ± standard deviation.
Mean values in the same column with different superscript differ significantly (P < 0.05).

Calcium and phosphorus ions concentration in blood were higher in DJPI fed groups compared to other groups whereas potassium and sodium ions in blood did not differ significantly among the five groups (Table 9).

Detoxified Jatropha protein isolate contains higher amount of phytate (2.95%) compared to SPI (0.95%) and these diets were supplemented with phytase that could lead to increased release of phosphorus and calcium from DJPI based feed compared to SPI based diets. All these levels of ions in blood were within the normal range (Zietoun et al., 1977; Handy et al., 1999).

Conclusions

Hematological and biochemical examination of fish is an integral part of evaluating their health status with regard to endogenous and exogenous factors of intensive aquaculture system. In the present study, all hematological and biochemical parameters were within the normal range as reported for healthy fish. Detoxified *Jatropha curcas* protein isolate (DJPI) fed groups exhibited good growth performance and good health of fish which indicate that DJPI can be used as a fish meal replacer in common carp diet.

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Isolation of phytate from *Jatropha curcas* kernel meal and effects of isolated phytate on growth, digestive physiology and metabolic changes in Nile tilapia (*Oreochromis niloticus* L.)

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Abstract

Jatropha curcas seeds are rich in oil and protein. The oil is used for biodiesel production. The defatted Jatropha kernel meal obtained after oil extraction is rich in protein (58−66%) and phytate (9−11%). The phytate rich fraction was isolated from defatted kernel meal using organic solvents (acetone and carbon tetracholride). It had 66% phytate and 22% crude protein. In Nile tilapia (*Oreochromis niloticus* L.) fingerlings, an eight week experiment was conducted to evaluate effects of the added phytate rich fraction and of a commercial phytase on the growth, feed utilization, blood chemistry, metabolic responses, and biochemical features of electrolyte in the blood. The fingerlings (n=50, 16.2 ± 0.64 g) were randomly distributed into five groups containing 10 replicates and fed iso-nitrogenous diets (crude protein 36%): control diet containing casein and gelatin as proteins; control diet containing 1.5% and 3% Jatropha phytate ($PWP_{1.5}$ and PWP_3 respectively); and control diet containing 1.5% and 3% Jatropha phytate supplemented with phytase (1500 FTU/ kg) ($PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ respectively). Significantly lower (P<0.05) growth and feed utilization in $PWP_{1.5}$ and PWP_3 groups than for control and both phytase containing groups were observed; whereas feed gain ratio exhibited opposite trend. Protein and lipid digestibilities of the diets, amylase and protease enzyme activities in the intestine were significantly higher (P<0.05) in $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ groups than for $PWP_{1.5}$ and PWP_3 groups. Lowest red blood cell counts, and hemoglobin and hematocrit concentrations were observed in PWP_3 group which were not statistically different to those for $PWP_{1.5}$ group, but were significantly (P<0.05) lower than those for all other groups. Highest albumin, globulin and total protein concentrations were observed in $PP_{3+Phytase}$ group and lowest in $PWP_{1.5}$ group; and values for the latter were statistically similar to those for control group. Calcium, phosphorus and glucose

concentrations in blood and cholesterol concentration in plasma were significantly lower ($P<0.05$) in the phytate enriched groups compared with control and phytase treated groups ($PP_{1.5+Phytase}$ and $PP_{3+Phytase}$). Higher ($P<0.05$) alkaline phosphatase activity was observed in phytase supplemented groups compared with that in non-supplemented groups which ($PP_{1.5+Phytase}$) was statistically similar to that in control group, whereas alanine transaminase activity in blood exhibited opposite trend. In conclusion, Jatropha phytate present in detoxified Jatropha kernel meal (DJKM) is an antinutrient and addition of phytase in the diet containing DJKM is recommended.

Introduction

Jatropha curcas grows in wide climatic conditions including marginal land, drought areas and nutrient deficient soil in the tropical and subtropical regions. It is a non edible oil seed plant, exhaustively researched and promoted as a feedstock for biodiesel production. The utilization of agriculturally unsuitable land for *J. curcas* cultivation is therefore attractive, as it would not displace food-producing crops. *Jatropha curcas* kernel contains about 60% oil that can be converted into biodiesel of high quality upon transesterification. The kernel meal is a rich source of nitrogen, which could be an economic protein supplement (contains 58−66 % crude protein) in aqua and livestock feeds, provided the antinutrients (phytate, lectin and trypsin inhibitors) and toxins (phorbol esters) present are removed (Makkar and Becker, 2009; Kumar et al., 2010a). The levels of essential amino acids (except lysine) are higher in *J. curcas* kernel meal than soybean meal (Makkar and Becker, 2009; Kumar et al., 2010a). Jatropha kernel meal contains high amount (9.1%) of phytate; much higher than soybean meal (2.5−3% phytate) (Makkar & Becker 2009; Kumar et al., 2010a). Phytate in natural form found in many plant species and purified phytate such as phytic acid or its salt sodium phytate are not digested by fish. The unique structure of phytic acid offers it the ability to strongly chelate with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts (Denstadli et al., 2006; Kumar et al., 2010a). It, therefore, adversely affects the absorption and digestion of minerals in animals. Besides, phytate has also

been reported to form complexes with proteins at both low and high pH values. Formation of complexes with lipids has also been reported. Formation of these complexes alters the protein structure, which result in decreased protein solubility, enzymatic activity and proteolytic digestibility; as well as abnormalities in intestinal epithelium thus reducing the availability of nutrients and energy, ultimately leading to growth depression in fish (Spinelli et al., 1983; Richardson et al., 1985; Hossain and Jauncey, 1993; Vielma et al., 2000).

In addition, addition of phytate in the diet has also been investigated as a strategy to control blood lipid and glucose levels in mice (Lee et al., 2007) and rats (Szkudelski, 2005). Lee et al. (2007) and Liu et al. (2010) reported that dietary phytate decreased hepatic triacylglycerol, and total cholesterol, and the digestibility of lipid and cholesterol in mice and chickens. On the other hand, Eengelman et al. (2005) reported that phytate had no significant effect on blood lipids in humans. However, little is known about the effect of phytate on blood chemistry and metabolic process in fish.

The complexity of natural phytates in plant ingredients, and their functional properties being dependent on their structure (Reddy and Pierson, 1987) have led us to isolate phytate from Jatropha kernel meal and evaluate their effects. This approach resulted in attribution of the observed effects exclusively to Jatropha phytate. The effects of addition of phytase were also investigated, with the aim to confirm that the effects obtained on addition of the isolated phytate are solely due to Jatropha phytate, because the addition of phytase should counteract the effects of isolated phytate if that is the case. The study using phytase was also conducted to evaluate whether the phytase units added in the diet are sufficient for the phytate levels investigated or not.

To our knowledge, this is first study wherein effects of an isolated phytate from a plant species and of commercial phytase on growth, nutrients utilization, blood chemistry, metabolic responses and biochemical features of electrolyte in the blood have been investigated in a fish species.

Materials and methods

Isolation of phytate from Jatropha curcas kernel meal

Jatropha seeds were deshelled manually to obtain kernels, which were ground to fine powder by using a coffee grinder (Severin, KM 3872, 90 W, Severin Elektogerate GmbH, Sundern, Germany). The defatting of Jatropha

kernel meal was carried out in a Soxhlet apparatus using petroleum benzene (b.p. 40–60 ^{0}C).

Defatted Jatropha kernel meal (DJKM) was mixed with 0.36 N HCl (1:2 w/v, meal: HCl) and kept for 2 h at room temperature. This mixture was then freeze dried. In preliminary studies the optimal concentration of HCl for the maximal recovery of phytate was 0.36 N. The phytate recovery increases with decrease in pH (Kwanyuen and Burton, 2005). The freeze dried HCl treated DJKM was used to isolate the phytate fractions using the method reported by Reddy and Pierson (1987) with some modifications. The freeze dried HCl treated DJKM was fractionated by differential centrifugation using organic solvents. Five phytate containing fractions were obtained using differential centrifugation using organic solvents (Fig. 1). In brief, freeze dried HCl treated DJKM (37.5 g) was suspended in 100 ml acetone and carbon tetrachloride solution (36: 64 v/v, ρ = 1.27) mixture at room temperature (21°C) and stirred using a magnetic stirrer for 10 min. The suspension was centrifuged at 2600 g for 15 min. The residue was collected (supernatant discarded). The residue (obtained at ρ = 1.27) was further fractionated by re-suspension and centrifugation using mixtures of acetone and carbon tetrachloride at various densities (ρ = 1.28, ρ = 1.36, ρ = 1.42 and ρ = 1.45) as shown in Fig. 1. All five density gradient supernatant fractions (Fractions I, II, III, IV, and V) were air dried. The protein and phytate contents in these five fractions indicated that the fractions III and IV had most of the phytate and protein (Table 1). These two supernatant fractions (III and IV) were combined and washed twice with acetone (1: 5 w/v ratio), air dried and referred to as combined density fraction (CDF). The CDF was further purified based on sedimentation. The CDF was solubilized in Tris base (0.1 M, pH 7; 1:10, w/v) and centrifuged at 1100 g for 20 min to sediment the high molecular weight compounds. The supernatant rich in phytate was further centrifuged for 24000 g for 30 min and the resulting residue was washed sequentially with water (1:5, w/v) and acetone (1:5, w/v) to obtain phytic acid-rich fraction.

Isolated phytate rich fraction contained 66% phytate, 22% crude protein and 1.38 mg/ g phorbol esters (Table 1). The phytate rich fraction was washed four times with methanol to remove phorbol esters. Phorbol esters free phytate rich fractions were incorporated in the experimental diets.

Diet formulation

There were five iso-nitrogenous (36% crude protein) and iso-energetic (20 KJ per g) diets were prepared, (a) The control diet was prepared using casein and gelatin as proteins, (b) control diet containing phytate and without phytase addition (1.5% ($PWP_{1.5}$) and 3% (PWP_3) Jatropha phytate respectively), and (c) control diet containing Jatropha phytate (1.5% ($PP_{1.5+phytase}$) and 3% ($PP_{3+phytase}$) Jatropha phytate) treated with phytase (1500 FTU phytase per kg). The final mixture of each diet was made into 2 mm diameter moist pellets using a Bosch, Type UM60ST 2-M pelletizer (Robert Bosch Hausgerät GmbH, 70839 Gerlingen, Germany) and then air dried for only 2 h to keep the high moisture content (12−13%) of feed (Table 2).

Table 1 Protein, phytate and yields of various density fractions from Jatropha kernel meal.

	Protein (%)	Phytate (%)	Phorbol esters (mg/g)	Yield (g)
Jatropha kernel meal	61	9.1	1.82	100
Fraction I	49	18.8	1.75	1.21
Fraction II	43	20.5	1.72	2.28
Fraction III	42	28.2	1.48	6.24
Fraction IV	38	23.4	1.43	4.98
Fraction V	24	3.9	1.88	74.42
Combined density fraction (Mixture of fractions III and IV)	39	25.9	1.45	9.2
Phytate enrich fractions	22	66	1.38	-
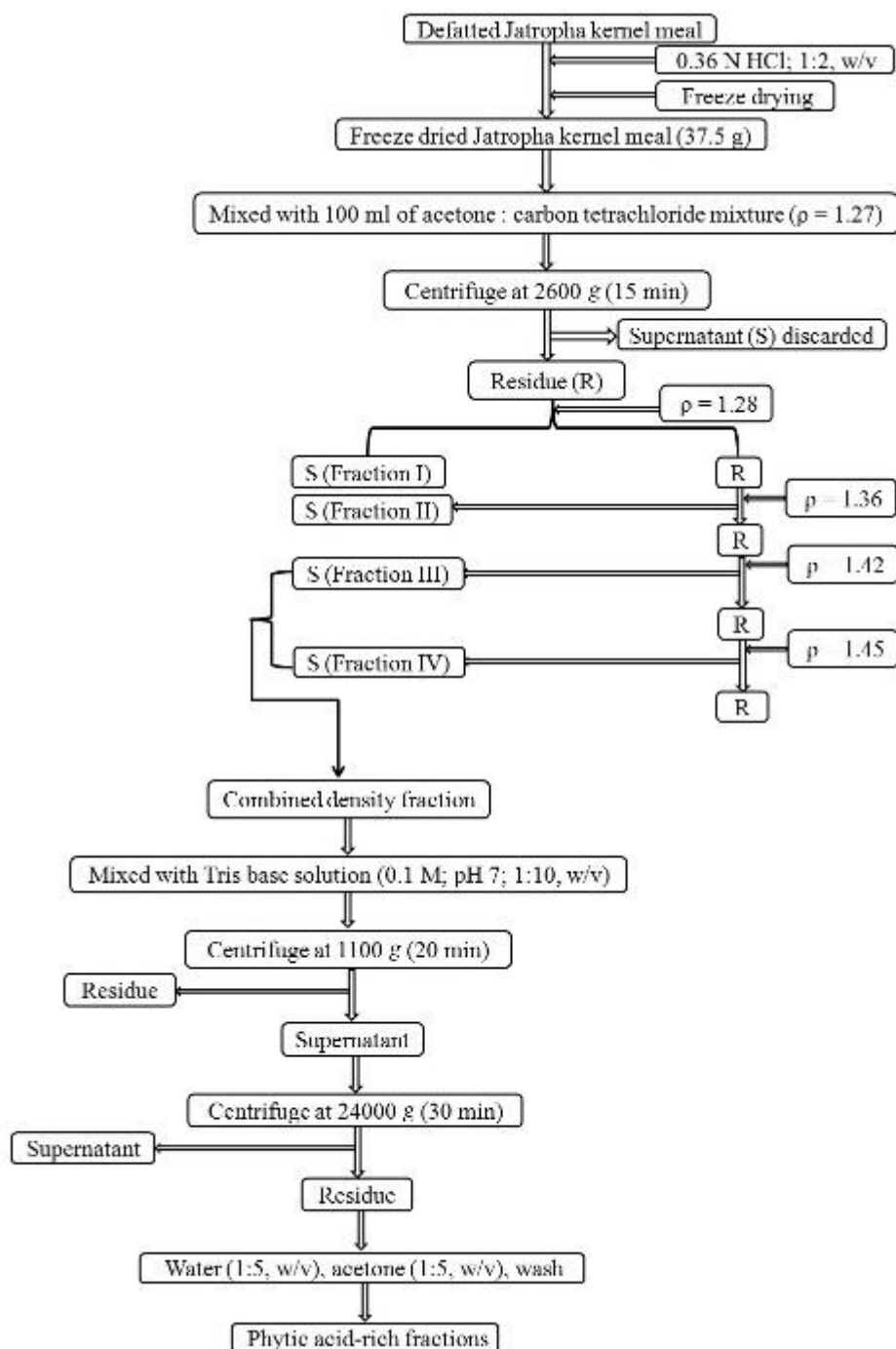
Fig. 1. Isolation of phytate rich fraction from Jatropha kernel meal.

Experimental design and general husbandry

All male Nile tilapia (*Oreochromis niloticus* L.) were selected from our fish laboratory (University of Hohenheim, Germany). In our initial trial, six fish per aquarium (45 L capacity) were kept so as to have six fish per replicate. However this resulted in high mortality due to aggressive/hierarchical behaviour of Nile tilapia. Therefore, after the acclimatization (for 12 days), 50 fish were randomly distributed into five groups with 10 replicates (av. wt. 16.2 ± 0.64 g). Two fish kept in an aquarium were separated from each other by a perforated plastic sheet. All the aquaria were supplied with water at 25−27 ℃ from a recirculatory system. The system was subjected to a photoperiod of 12 h light : 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 25−27 ℃, pH 7.0–7.5, dissolved oxygen 6.2–7.6 mg L^{-1}, total NH$_3$ 0.08–0.18 mg L^{-1}, nitrite 0.06–0.09 mg L^{-1} and nitrate 1–2 mg L^{-1}). Water flow was adjusted to keep the oxygen saturation above 80%. One day before start of the experiment, the fish were starved. One time maintenance requirement equals 3.2 g feed per kg metabolic body mass (kg$^{0.8}$). Therefore, 16 g feed per kg metabolic body mass (kg$^{0.8}$) was fed to satisfy 5 times maintenance requirements, and the feed was split into 3 equal rations per day (at 8:00, 12:00 and 16:00 h). As the moisture content in the experimental diets were high (12−13%), they were fed manually. All the fish were weighed individually at the beginning of the experiment and at weekly intervals during the experimental period to adjust the feed amount for the subsequent week. The fish were not fed on the weighing day. The faeces collection was qualitative, as the experimental diets contained an inert marker (TiO2). During last three weeks of the experiment, faeces were collected daily. After each feeding the aquaria were controlled for remaining feed; generally, there were no feed residues left. Every day prior to the faeces collection, aquaria were siphoned out to clean any residues. Subsequently, faeces excreted by the fish were collected in separate beakers for each aquarium by siphoning with a short small pipe. The collected mixture of water and faeces was centrifuged at 4000 × *g* for 10 min, the supernatant was discarded and the faeces were then stored at -20 °C until analysis. For the analysis, faeces from all the experimental periods from the same fish were pooled. During the experiment there was no mortality. At the start of the experiment, 12 fish of the same population were killed and preserved at −20 ℃ for the evaluation of initial body composition.

The experiment was terminated after 8−week and the fish were sacrificed. At the end of experiment, fish were anesthsized by tricaine methanesulfonate (MS222) at 250 ppm in water.

Blood was drawn near caudal peduncle from four fish from each group and transferred into a heparinized tube for hematological study. Same four fish per group were carefully dissected to isolate muscle and stored at −20 °C for determination of cholesterol and intestine was also isolated and stored in liquid nitrogen for digestive enzymes assay. Blood was drawn from another four fish from each group and divided into two equal part, one part was centrifuged at 1500 ×g for 5 min at room temperature (24 °C) to obtain plasma, which was then stored at −20 °C for determination of cholesterol and triglycerides. Another part of blood was kept outside at room temperature for few minutes to collect serum. Serum was stored at −20 °C for lysozyme determination. Same four fish and another two fish from each group were stored at −20 °C for chemical composition analysis. Prior to determination of the chemical composition, the fish were autoclaved at 121 °C for 20 min, thoroughly homogenised using an Ultra-Turrax T25 (Janke and Kunkel, GmbH & Co.KG, Heitersheim, Germany), frozen overnight and freeze-dried.

The University animal welfare committee (University of Hohenheim, Germany) approved all experimental procedures involving Nile tilapia.

Table 2 Formulation and proximate composition of the experimental diets (g kg^{-1} feed) for Nile tilapia (*Oreochromis niloticus* L.).

Ingredients	Experimental diets[*]				
	Control	PWP$_{1.5}$	PWP$_3$	PP$_{1.5+phytase}$	PP$_{3+phytase}$
[1]Casein	330	330	330	330	330
[2]Gelatin	91	91	91	91	91
[3]Wheat flour	439	416.3	393.6	416.3	393.6
[4]Fish oil and [5]Soy oil (1:2)	80	80	80	80	80
[6]Carboxymethyl cellulose	10	10	10	10	10
[7*]Vitamin premix	20	20	20	20	20
[8**]Mineral premix	20	20	20	20	20
[9]Titanium oxide	10	10	10	10	10
Phytate enrich fractions	0	22.7	45.4	22.7	45.4

Total	1000	1000	1000	1000	1000
[10]Phytase (FTU/kg)	0	0	0	1500	1500

Proximate composition of the experimental diets (g kg⁻¹ feed)					
Dry matter	872	882	879	881	879
Crude protein	356	361	367	362	368
Crude lipid	82	82	85	84	86
Crude ash	52	53	56	55	57
Gross energy (MJ/kg)	21.5	20.6	20.1	20.7	20.4

[*]Experimental diets:

Control: containing casein and gelatin as proteins

PWP$_{1.5}$: control diet containing 1.5% Jatropha phytate

PWP$_3$: control diet containing 3% Jatropha phytate

PP$_{1.5}$: control diet containing 1.5% Jatropha phytate treated with phytase (1500 FTU per kg)

PP$_3$: control diet containing 3% Jatropha phytate treated with phytase (1500 FTU per kg).

[1]Sigma, lot 56H0841, Sigma-Aldrich Chemie, GmbH, Steinheim, Germany

[2]Merck, lot K14180270, E-Merck, Darmstadt, Germany

[3]Cerestar, Deutchland GmbH, Zülpich, Germany

[4]Menhaden, batch # 102k0126, Sigma-Aldrich, Chemie GmbH Steinheim, Germany.

[5]Thomy, Nestle Deutchland AG, 41415 Neuss; Reg. Trademark of Societe des, Produits Nestle S.A..

[6]Sigma, lot 109H0196, Sigma-Aldrich Chemie, GmbH, Steinheim, Germany

[7]Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Vitamin premix (g or IU kg⁻¹): retinol palmitate, 500000IU; thiamine, 5; riboflavin, 5; niacin, 25; folic acid, 1; pyridoxine, 5; cyanocobalamine, 5; ascorbic acid, 10;cholecalciferol; 50000IU; α-tocopherol, 2.5; menadione, 2; inositol, 25; pantothenic acid, 10; choline chloride,100; biotin, 0.25.

[8]Altromin Spezialfutter GmbH & Co. KG, Lage, Germany. Mineral premix (g kg⁻¹): CaCO$_3$, 336; KH$_2$PO$_4$, 502; MgSO$_4$.7H$_2$O, 162; NaCl, 49.8; Fe(II) gluconate, 10.9; MnSO$_4$.H$_2$O, 3.12; ZnSO$_4$.7H$_2$O, 4.67; CuSO$_4$.5H$_2$O, 0.62; KI, 0.16; CoCl$_2$.6H$_2$O, 0.08; ammonium molybdate, 0.06; NaSeO$_3$, 0.02.

[9]Sigma, lot 50030, Sigma-Aldrich, Laborchemikalien GmbH, Seeize, Germany

[10]Natuphos 5000 G (EU) 3-phytase (EC 3.1.8) BASF Ludwigshafen, Germany.

Extraction and estimation of phorbol esters by HPLC and determination of phytate

The samples (kernel meal and phytate rich fractions) were extracted in methanol and phorbol esters were determined on a reverse phase C18 (LiChrospher 100, endcapped 5 lm) 250 x 4 mm I.D. column protected with a guard column containing the same material as the main column according to the procedure outlined by Makkar et al. (2007a), which was based on the method of Makkar et al. (1997). The results were expressed as equivalent to a standard, phorbol-12-myristate 13-acetate. Detection limit of phorbol esters was 3 µg/g samples.

Phytate content of the samples (kernel meal and phytate rich fractions) was determined by a spectrophotometric procedure described by Vaintraub and Lapteva (1988). Results are expressed as g/100 g phytic acid, using standard phytic acid (sodium salt; Sigma, St. Louis, MO, USA).

Chemical analysis

The proximate composition of diet ingredients, diets and whole body of fish was determined using the standard methods of the Association of Official Analytical Chemists (1990). Samples were analysed for dry matter, crude protein, lipid (ether soluble lipid) and ash. Gross energy of diet ingredients, diets and fish bodies was determined with bomb calorimeter (IKA C7000) (Janke and Kunkel, GmbH & Co.KG, Heitersheim, Germany) using benzoic acid as a standard.

Growth and nutrient utilization parameters

Growth performance and diet nutrient utilization were assessed in terms of body mass gain (BMG), specific growth rate (SGR), metabolic growth rate (MGR), feed gain ratio (FGR), protein efficiency ratio (PER), protein productive value (PPV), lipid productive value (LPV) and energy retention (ER). These were calculated as follows:
BMG (%) = [(Final body mass - initial body mass) / Initial body mass] X 100; SGR = [(ln final body mass in g) - ln initial body mass in g) / number of trial days] X 100; MGR = (Body mass gain in g) / [{(initial body mass in g / 1000)$^{0.8}$ + (final body mass in g / 1000)$^{0.8}$}/2] / number of trial days; FGR = dry feed fed (g)/body mass gain (g); PER = body mass gain (g)/crude protein fed (g); PPV (%) = [(final fish body protein in g - initial fish body protein in g) / total protein consumed in g] X 100; LPV (%) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid consumed in g] X 100; ER (%) = [(final fish body energy - initial fish body energy)/(gross energy intake)] X 100.

Digestibility measurement, hepatosomatic index (HSI), and gastro somatic index (GSI)

Titanium dioxide in the feed and faeces was determined according to the method described by Richter et al. (2003). The percentage of apparent dry matter digestibility of diets was calculated according to Maynard et al. (1981).

Apparent dry matter digestibility (%) = [1 – {(% TiO$_2$ in feed) / (% TiO$_2$ in faeces)}] X 100

Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets were calculated according to Maynard and Loosli (1969).

The nutrient and energy digestibility were obtained using the following formula:

Apparent nutrient digestibility (%) = [1 – {(% TiO$_2$ in feed) / (% TiO$_2$ in faeces) X (% Nutrient or energy in faeces) / (% Nutrient or energy in feed)}] X 100

Efficiency of digestible nutrients and gross energy = (Nutrient and energy retained in the whole body/Digestible nutrient and digestible energy) x 100

Digestible nutrients and energy = Total offered of nutrients and gross energy through feed X digestibility coefficient.

HSI = Liver mass (g) X 100 / body mass (g)

GSI = Intestine mass (g) X 100 / body mass (g)

Digestive enzymes assay, cholesterol and triglyceride estimation

The reducing sugars produced due to the action of glucoamylase and α-amylase on carbohydrate was estimated using dinitro-salicylic-acid (DNS) method (Rick and Stegbauer, 1974). Amylase activity was expressed as mmol of maltose released from starch per min at 37 ^{O}C. Protease activity was determined by the casein digestion method of Drapeau (1974), and one unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to Δ0.001A$_{280}$ per minute at 37 ^{O}C and pH 7.8. Lipase activity was assayed by the method of Cherry and Crandall (1932), and one unit of enzyme was the amount of enzyme that hydrolysis 1.0 microequivalent of fatty acid from a triglyceride in 24 h at pH 7.7 at 37 ^{O}C.

The determinations of the plasma cholesterol and triglycerides were carried out using enzymatic colorimetric kits: Hitado Diagnostic system, Nobiflow cholesterin (kit lot number 60041889) and Nobiflow triglyceride-GPO (kit lot number 60040710) respectively. Muscle cholesterol content was determined using a kit (lot no. 10139050035) (Boehringer Mannheim, Germany). The color intensity was determined spectrophotometrically and was directly proportional to the concentration of cholesterol and triglycerides in the plasma sample.

Haematological parameters

Total erythrocyte count (RBC) and total leucocyte count (WBC)

RBC and WBC were counted by Neubauer's counting chamber of haemocytometer. Care was taken to avoid trapping of air bubbles. The RBC lying inside the five small squares were counted under high power (40X) of light microscope.

The following formula was used to calculate the number of RBC per mm^3 of the blood sample:

Number of RBC/mm^3 = (N x dilution)/area counted x depth of fluid

Haemoglobin (Hb), hematocrit (Hct) content and lysozyme activity in serum

The Hb content of blood was analyzed by Reflotron Hemoglobin test (REF 10744964, Roche diagnostic GmbH, Manheim Germany). Hct was determined on the basis of sedimentation of blood. Heparinised blood (50 µl) was taken in the hematocrit capillary (Na-heparinised) and centrifuged in the Hematocrit 210 Hettich Centrifuge (Tuttlingen Germany) to obtain hematocrit value.

From analyses of Hct, Hb and RBC, the following parameters were calculated: mean cell volume, MCV (fL) = (Haematocrit [in L/L] x 1000) ÷ (RBC count [in millions/µL]); mean corpuscular haemoglobin, MCH (pg) = (Hemoglobin [g/dL] x 10 ÷ (RBC count [in millions/µL]) and mean cell haemoglobin concentration, MCHC [in g/dL] = Haemoglobin [in g/dL] ÷ Haematocrit [in L/L].

Lysozyme activity of serum was measured by EnzChek Lysozyme Assay Kit (E-22013) Leiden, The Netherlands. The assay measures lysozyme activity on *Micrococcus lysodeikticus* cell wall, which is labeled to such a degree that the fluorescence is quenched. Lysozyme action relieves this quenching, yielding a dramatic increase in fluorescence that is proportional to lysozyme activity. The fluorescence increase was measured by using spectrofluorometer that detects fluorescein. Lysozyme hydrolyzes β-(1-4)-glucosidic linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues present in the mucopolysaccharide cell wall of the microorganism.

Blood parameters analysis by Vet Scan

VetScan Chemistry Analyzer (Scil Animal care company GmbH, Technischer Service, Germany) was used for determinations of alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total calcium (Ca^{++}), creatinine, globulin, glucose, phosphorus, potassium (K^+), sodium

(Na^{+}), total bilirubin (TBIL), total protein, and urea nitrogen (BUN) in heparinized blood.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test (P < 0.05). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). Values are expressed as means ± standard deviation.

Results and discussion

The DJKM used in this study contained 9.1% phytate (Table 1), which is similar to those observed in earlier studies (Makkar et al., 1997; Makkar et al., 2007b; Kumar et al., 2010a). The DJKM was fractionated into five density fractions using differential centrifugation with a yield of 1.21%, 2.28%, 6.24%, 4.98% and 74.42% for fractions I, II, III, IV and V respectively (Table 1). The phytate content in fractions I, II, III, IV and V was 18.8%, 20.5%, 28.2%, 23.4%, and 3.9% respectively (Table 1). In addition, fractions III and IV contained more than 31% of total phytate and 41% of total crude protein of DJKM (Table 1). Fraction V contained mostly starch (data not shown), protein (24%) and phytate (3.9%) (Table 1). The combined density fraction (CDF) consisting of fraction III and IV, contained 25.9% phytate and 39% protein (Table 1). The phytate rich fraction obtained from CDF contained 66% phytate, 1.38 mg/g phorbol esters and 22% crude protein (Table 1). Phorbol esters were not present in the four-times methanol treated phytate rich fraction, used for adding into the diets.

Chemical composition of experimental diets and whole body of fish

The content of moisture, main nutrients and energy were fairly consistent among the diets (Table 2). Experimental diets were iso-nitrogenous (36% crude protein) and isoenergetic (20−21 KJ/g gross energy) (Table 2). Dietary inclusion of phytate and phytase markedly influenced the whole body composition of the fish (Table 3). The crude protein and lipid contents of whole body of fish were significantly lower (P < 0.05) in fish fed diets containing phytate (PWP$_{1.5}$ and PWP$_3$) than in control and phytase treated (PP$_{1.5+phytase}$ and PP$_{3+phytase}$) groups whereas moisture content in fish exhibited opposite trend. The protein and lipid contents in fish were found to be significantly lower (P < 0.05) in fish fed with diet containing 1.5%

phytate compared with those fed diet containing 3% phytate (Table 3). This consequently resulted in decrease in whole body energy content of the fish in those respective groups ($PWP_{1.5}$ and PWP_3) compared with $PP_{1.5+phytase}$ and $PP_{3+phytase}$ groups (Table 3). Our results are consistent with those of (Richardson et al., 1985; Usmani and Jafri, 2002). These authors reported that fish fed phytate supplemented diets had lower protein and lipid contents in whole body compared with those in control diet (without phytate). The phytate inhibits rise in hepatic total lipids, exhibiting hypolipidaemic effect and is attributed to the inhibition of hepatic enzymes involved in lipogenesis (Katayama, 1997; Kumar et al., 2010b). This mechanism might have appeared in the present study and we found that significantly lower (P < 0.05) value of HSI in $PWP_{1.5}$ and PWP_3 (phytate fed groups) suggests lower lipid deposition in liver compared with control and phytase treated groups ($PP_{1.5+phytase}$ and $PP_{3+phytase}$) (Table 3). The GSI did not differ significantly among the five groups (Table 3). The values for HSI and GSI of about 2 and 3 respectively observed here are common in Nile tilapia (Dongmeza et al., 2006). Our results also indicate that phytase supplementation in the diets increases the protein and lipid contents in the whole body of fish compared with non- supplemented groups. Our results are in contrast with those of Cheng and Hardy (2003), wherein phytase inclusion in the fish diet did not affect body composition; whereas, it was effective in releasing most minerals and trace mineral. The ash content was found to be significantly lower (P < 0.05) in fish fed PWP_3 (3% phytate without phytase) diet and highest ash content was observed for PP_3 (3% phytate with phytase) group (Table 3). Supplementation of phytase can hydrolyse phytate and increase the concentration of minerals that leads to higher ash content in the whole body of fish (Masumoto et al., 2001; Debnath et al., 2005; Liebert and Portz, 2005). Phytase supplementation in the diets increased ash content in whole body of fish which is confirmed by researchers (Sugiura et al., 2001; Cheng and Hardy, 2003; Li et al., 2004; Liebert and Portz, 2005); they have found that phytase supplementation increased the ash content via increasing apparent absorption of Ca, Mg, Cu, Fe, Sr and Zn from phytate enriched diets. Increment in ash in fish fed phytase-supplemented diets is an indication that the mineral bioavailability was significantly increased bydietary manipulation.

Table 3 Chemical composition of whole body (at the start and at the end of the experiment, g kg $^{-1}$ wet basis ± SD), hepato somatic index (HSI) and gastro somatic index (GSI) of Nile tilapia (*Oreochromis niloticus* L.) fingerlings of different experimental groups.

| Treatment*	Moisture	Crude protein	Crude lipid	Gross energy (kJ/g)	Ash	HSI	GSI
Initial fish	784 ± 3.5	127 ± 2.9	39.5 ± 1.4	39.6 ± 1.6	31.7 ± 0.7		
Control	762^b ± 10.3	148^b ± 8.4	52.8^{ab} ± 6.1	55.4^a ± 4.0	34.3^b ± 1.8	1.83^b ± 0.17	2.88 ± 0.20
PWP$_{1.5}$	766^b ± 16.4	142^{bc} ± 9.1	49.0^{bc} ± 4.1	48.8^{ab} ± 4.0	32.9^b ± 1.6	1.69^c ± 0.12	2.84 ± 0.49
PWP$_3$	792^a ± 8.11	132^c ± 6.1	42.0^c ± 2.6	43.2^b ± 3.4	28.6^c ± 2.2	1.72^c ± 0.11	2.87 ± 0.33
PP$_{1.5+phytase}$	759^b ± 12.5	152^{ab} ± 8.1	54.5^{ab} ± 5.1	51.9^a ± 3.7	33.3^b ± 1.8	1.93^{ab} ± 0.14	2.93 ± 0.24
PP$_{3+phytase}$	732^c ± 11.3	163^a ± 8.3	60.7^a ± 4.6	54.4^a ± 4.4	39.2^a ± 2.9	2.09^a ± 0.11	2.94 ± 0.23
SEM	2.81	2.10	1.51	0.7 2	1.13	0.04	0.06

* See footnotes to Table 2

Values are mean (n = 6) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 4 Growth performance and nutrient utilization of Nile tilapia (*Oreochromis niloticus* L.) fingerlings fed with the experimental diets for eight weeks

Treat*	Initial weight (g)	Final weight (g)	Weight gain (g)	BMG (%)	SGR ((% day^{-1})	MGR (gkg$^{0.8}$ day^{-1})	FGR	PER	PPV	ER	LPV
Control	16.2 ± 0.65	76.7^a ± 5.81	60.6^a ± 5.71	375^a ± 37	2.78^a ± 0.14	13.1^a ± 0.65	1.16^c ± 0.04	2.43^a ± 0.07	37.5^a ± 3.11	24.0^a ± 2.22	66.1^a ± 8.50
PWP$_{1.5}$	16.3 ± 0.62	64.8^{bc} ± 7.12	48.5^{bc} ± 7.21	300^{bc} ± 48	2.46^{bc} ± 0.21	11.6^{bc} ± 1.33	1.29^b ± 0.15	2.21^b ± 0.08	32.5^b ± 2.01	19.9^b ± 1.81	50.2^b ± 4.76
PWP$_3$	16.2 ± 0.71	57.9^c ± 8.25	41.6^c ± 8.49	257^c ± 57	2.25^c ± 0.29	10.6^c ± 0.98	1.43^a ± 0.07	1.96^c ± 0.17	26.4^c ± 3.32	15.7^c ± 2.35	35.7^c ± 4.65
PP$_{1.5+phytase}$	16.3 ± 0.64	73.4^a ± 5.21	57.1^a ± 5.67	352^{ab} ± 46	2.69^{ab} ± 0.18	12.7^{ab} ± 0.78	1.20^c ± 0.04	2.35^{ab} ± 0.06	37.4^a ± 2.36	22.6^{ab} ± 1.99	58.9^{ab} ± 7.23
PP$_{3+phytase}$	16.2 ± 0.61	72.2^{ab} ± 5.57	56.0^{ab} ± 5.61	345^{ab} ± 39	2.66^{ab} ± 0.16	12.5^{ab} ± 0.71	1.22^{bc} ± 0.04	2.29^{ab} ± 0.04	39.6^a ± 2.68	23.8^a ± 2.49	64.4^a ± 6.27
SEM	0.09	1.31	1.32	8.68	0.04	0.18	0.02	0.03	1.00	0.69	2.34

* See footnotes to Table 2. Values are mean (n = 10 for final weight, weight gain, BMG, SGR, MGR, FGR and PER; and n=6 for PPV, ER and LPV) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

BMG (%) - Body mass gain, SGR (% day^{-1}) – Specific growth rate and MGR (gkg$^{0.8}$ day^{-1}) – Metabolic growth rate. FGR – Feed gain ratio; PER - Protein efficiency ratio, PPV (%) - Protein productive value, ER (%) - Energy retention and LPV (%) - Lipid productive value.

Feed intake, growth and nutrient utilization

The data on growth and nutrient utilization are presented in Table 4. Body mass development of fish is presented in Figure 2. These data indicate that from second week onwards there was differential growth among the groups and lower body mass development was observed in phytate groups ($PWP_{1.5}$ and PWP_3) than other groups. This trend was maintained till the end of the experiment. Body mass development of $PP_{1.5+phytase}$ and $PP_{3+phytase}$ groups were similar till fifth week, thereafter higher body mass development was observed for $PP_{1.5+phytase}$ group than $PP_{3+phytase}$ group till end of the experiment.
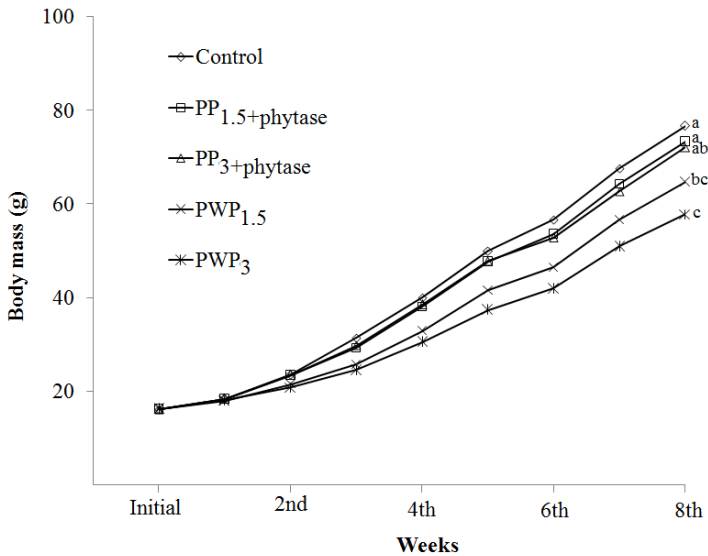
Fig. 2. Weekly body mass gain (g) of Nile tilapia (*Oreochromis niloticus* L.) fingerlings fed with experimental diets for eight weeks.

The growth performance and nutrient utilization parameters were significantly affected by dietary treatments (Table 4). The lower ($P < 0.05$) growth performance and nutrient utilizations were observed in $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups compared with control and phytate with phytase ($PP_{1.5+phytase}$ and $PP_{3+phytase}$) groups. Our results are in accordance with those of other researchers (Table 5). In contrast, McClain and Gatlin (1988) and Cheng and Guillaume (1984) have reported a beneficial effect of 0.72 and 1.08% phytate on growth and exoskeleton development in tilapia (*Oreochromis aureus*) and shrimp (*Penaeus japonicas*)

respectively. The authors did not give any explanation for these unexpected results. Gatlin and Phillips (1989) have reported that dietary phytate (1.5%) did not depress growth and feed utilization of channel catfish but significantly reduced bone zinc content. The phytate chelate with di- and trivalent mineral ions such as Ca^{2+} (Fredlund et al., 2006), Mg^{2+} and Zn^{2+} (Denstadli et al., 2006), Cu^{2+} and Fe^{3+} preventing their availability for fish growth (Duffus and Duffus, 1991). In presence of phytate and added calcium, absorption of other mineral was depressed due to the formation of insoluble complexes (Sandberg et al., 1993). For example, calcium-bound phytate shows affinity with Zn and forms co-precipitates. The mineral binding strength becomes progressively lower as the solubility increases when phosphate groups are removed from the inositol hexaphosphate.

Supplementation of phytate-containing diets with phytase neutralises the negative effects of phytate and increases growth and feed utilization in fish. Phytase supplementation may also stimulate appetite and therefore increase growth directly through increase in feed intake (Jackson et al., 1996; Li and Robinson, 1997) and increase in nutrient and energy digestibilities of the diets (Debnath et al., 2005; Gabaudan et al., 2006; Fox et al., 2006; Baruah et al., 2007). Positive impact of phytase on growth and nutrient utilization of fish has been observed in the present study. Similar results have been obtained by a number of authors: Jackson et al. (1996) in channel catfish, VanWeerd et al. (1999) in African catfish, Forster et al. (1999) in trout, Papatryphon and Soares (2001) in striped seabass, Vielma et al. (2002) in rainbow trout, Sajjadi and Carter (2004) in Atlantic salmon, Debnath et al. (2005) in pangus catfish; Portz and Liebert (2004) and Liebert and Portz (2005) in Nile tilapia, Nwanna et al. (2005) in common carp, Phromkunthong and Gabaudan (2006) in red tilapia and Baruah et al. (2007) in rohu. These reports confirmed that supplementing exogenous microbial phytase in feed ration enhance growth rate and performance which could be attributed to various factors, in individual or/and combined form, namely better bio-availability of P (Vielma et al., 2002; Baruah et al., 2007) and minerals (Vielma et al., 2004; Debnath et al. 2005), improved protein digestibility (Vielma et al., 2004; Liebert and Portz, 2005, Debnath et al., 2005; Baruah et al., 2007) and increased absorption of nutrients owing to well functioning of the pyloric cecal region of the intestine of fish (NRC, 1993). Our findings were in contrasts with those of other researchers, Oliva-Teles et al. (1998), Forster et al. (1999), Sajjadi and Carter (2004), Masumoto et al. (2001) and Yoo et al. (2005) for sea bass, rainbow trout, Atlantic salmon, Japanese flounder (*Paralichthys olivaceus*) and Korean rockfish (*Sebastes schlegeli*) respectively) who reported that there was no significant effect on growth

performance, protein digestibility and energy retention on phytase supplementation in the diet (phytate enriched) of fish.

The discrepancy in above findings may be associated with differences in their diet composition and also with different rearing conditions. These contrasting results also suggest that dietary substrate levels of phytate are an important determinant of the magnitude of phytase responses.

Apparent digestibility and efficiency of digestible nutrients and energy

Apparent digestibilities of protein and lipid and efficiency of digestible nutrients and energy were significantly influenced by dietary supplementation of phytate and phytase in Nile tilapia (Table 6). Significantly higher ($P < 0.05$) ADCs of protein and lipid were observed for the $PP_{3+phytase}$ and $PP_{1.5+phytase}$ (phytate with phytase) diets compared with $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups (Table 6). Energy digestibility did not differ significantly among the five groups (Table 6). The ADCs of protein and lipid were generally high (in the range of 81-94%), as expected from the use of highly digestible feed ingredients (casein and gelatin). The protein and lipid digestibilities ranged from 81% to 91% and 84% to 94% respectively; protein and lipid digestibility in fish fed diets with 15 g phytate kg^{-1} was significantly higher ($P < 0.05$) than the 30 g phytate kg^{-1} (Table 6). The digestibilities of protein and lipid decreased significantly as the inclusion of phytate increased. The reason could be the phytate depresses protein and amino acid digestibility and utilisation efficiency in fish and other higher animals. Interactions of phytate with proteins are dependent on pH (Cheryan, 1980) as the phytate molecule is polyanionic with phosphoric acid group moiety. In acidic environments such as Nile tilapia stomach (pH 1-2), half of the phosphate moieties are negatively charged. This favours binding of soluble proteins at amino-groups on lysine, imidazole groups on histidine and guanidyl groups on arginine. In alkaline environment such as Nile tilapia intestine, (pH 8.5 - 8.8), ternary complexes are favoured. Both complexes are resistant to proteolytic digestion (Riche and Garling, 2004).

Table 5 Dietary effects of purified phytate in different fish and shrimp species.

Nature of phyate included	Fish/shrimp species	Phytate content (%)	Effects	References
Calcium phytate and Sodium phytate	Rainbow trout	0.5	Inclusion of calcium and sodium phytate in diet leads to depression in growth and feed conversion efficiency	Spinelli et al. (1983)
Sodium phytate	Juvenile Chinook salmon	0.16, 0.65 and 2.58	2.58% group showed depressed growth, feed and protein conversion and thyroid function; while, other not. Other treatments exhibit similar performance as control.	Richardson et al. (1985)
Sodium phytate	Blue tilapia	1.5%	Weight gain was generally improved; however, feed efficiency was not affected.	McClain et al.(1988)
Sodium phytate	Channel catfish	1.1 and 2.2%	2.2% phytate significantly reduced weight gain and feed efficiency compared with that with $\leq$1.1% phytate in diet	Satoh et al. (1989)
Sodium phytate	Channel Catfish	0.5 and 1.5%	No adverse effect on weight gain and feed efficiency	Gatlin and Phillips (1989)
Sodium phytate	*Penaeus japonicas* and *Penaeus vannamei*	0.75, 0.94, 1.19 and 1.44%	No effect on growth rate or survival rate of *P. Japonicas* even at 1.44%. In contrast, growth rate of *P. Vannamei* was strongly depressed by the presence of even 0.75% phytate. Feed conversion was markedly increased in both species when	Civera and Guillaume (1989)

			sodium phytate added at $\geq$ 0.75%.	
Sodium phytate	Common carp	0.5 and 1.0%	Depression in growth, feed utilization and protein digestibility; effect was exacerbated by simultaneous increases in dietary calcium and magnesium levels. Intestinal epithelium showed abnormalities.	Hossain and Jauncey (1993)
Sodium phytate	Mrigal	0.5, 1.0, 1.5, 2.0 and 2.5%	Growth performance and nutrient utilization were significantly reduced at > 1% levels.	Usmani and Jafri (2002)
Sodium phytate	Atlantic salmon	1%	No significant effect on feed intake or weight gain; significantly reduced protein digestibility although there was no reduction in trypsin activity.	Sajjadi and Carter (2004)
Sodium phytate	Atlantic salmon	0.1, 0.21, 0.47, 1.0 and 2.07 %	Reduction in feed intake, growth, feed conversion efficiency, nutrient digestibilities and nutrient retentions at 2.07% level only.	Denstadli et al. (2006)
Sodium phytate	Japanese Flounder	0.51, 1.04, 1.35, and 2.06%	Reduction in growth performances and nutrient utilization at ≥1.35% level.	Laining et al., (2010)
Jatropha phytate	Nile tilapia	1.5 and 3.0%	Growth performance, nutrient utilization and digestive enzymes activities were significantly reduced at $\geq$ 1.5% levels.	Kumar et al., unpublished data

When phytate reacts with minerals and other nutrients, the formed complexes are insoluble in the intestine and it is highly unlikely that they provide absorbable essential elements. Thus, they lead to lower digestibility of protein in $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups compared with control diet and phytase treated ($PP_{1.5+phytase}$ and $PP_{3+phytase}$) groups; whereas supplementation of phytase increased the protein digestibility of those diets. Formation of sparingly digestible phytate–protein complexes was found to be the main reason for growth depression in Nile tilapia. In the present study, casein and gelatin were used as protein sources. The capacity of phytate to complex with protein sources varies according to the protein. Phytate probably complexed with casein (Spinelli et al., 1983) in the present study and the digestibility values showed that protein digestibility was significantly reduced for the diet containing phytate (without phytase) compared with other groups (phytase supplemented). In the present study inclusion of 1.5-3% phytate reduced protein digestibility in Nile tilapia and was in agreement with previous studies (Spinelli et al., 1983; Sajjadi and Carter, 2004).

Phytate is a strong chelator and forms complexes with lipid and derivatives along with other nutrients (Vohra and Satyanarayan, 2003). The complex of Ca/Mg–phytate and lipids is referred as 'lipophytins' and is the major constraint for energy utilisation derived from lipid source (Leeson, 1993). However there is a paucity of evidence supporting the existence of lipid–phytate complexes in fish. In the present study significantly lower ($P < 0.05$) lipid digestibility was observed for $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups compared with control diet. Our results concur with those of Denstadli et al. (2006); these authors observed that 20.7 g phytate per kg diet exhibited lower lipid digestibility compared with control diet (devoid of phytate) in Atlantic salmon. Usmani and Jafri (2002) observed that significantly lower fat content in carcass of *Cirrhinus mrigala* when fed with high dietary phytate as compared with dephytated diet which could be because of lower lipid digestibility of the phytate diet compared with control diet.

In the present study, the negative effect of phytate on protein and lipid digestibility was counteracted by the addition of 1500 FTU per kg phytase. The effects of phytase on digestibility may depend on a variety of dietary factors such as source and concentration of phytate in the diet, source and concentration of protein in the diet, digestibility of protein source, mineral levels, calcium and P levels and phytase inclusion rate (Sugiura et al., 2001).

Digestible protein, lipid and energy retained were in the range of 33-46%, 43-71% and 19-28% respectively (Table 6). Significantly lower ($P < 0.05$) retention of digestible protein, lipid and energy were observed for phytate fed groups ($PWP_{1.5}$ and PWP_3) compared with phytate with phytase groups ($PP_{1.5+phytase}$ and $PP_{3+phytase}$). This trend indicates that phytate inhibited the deposition of nutrients and energy in body of Nile tilapia whereas phytase supplementation in these diets counteracts those adverse effects.

Digestive enzyme activities

Amylase and protease acitivities in intestine were significantly influenced by dietary treatments (Table 6). Significantly lower ($P < 0.05$) activities of amylase and protease were observed for $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups compared with $PP_{1.5+phytase}$ and $PP_{3+phytase}$ (phytate with phytase) groups (Table 6). Lipase activity did not differ significantly among the five groups (Table 6). Other workers (Liener, 1994; Robaina et al., 1995; Alarcon et al., 1999; Sajjadi and Carter, 2004; Kumar et al., 2010a) have also shown decrease in pepsin, trypsin and amylase activities by addition of phytate in the diets of fish. Denstadli et al. (2006) observed that on addition of 2.07% phytate in Atlantic salmon diet, trypsin activity in the intestinal tract decreased significantly. Sajjadi and Carter (2004) found that 0.8% phytate had no effect on trypsin activity in pyloric caeca in Atlantic salmon. Phytate also forms complexes with minerals (Sugiura et al., 1999) and proteins (Moyano et al., 1999), thereby modifying digestion processes and impairing intestinal absorption. *In vitro* studies have also shown that phytate-protein complexes are less likely to be attacked by proteolytic enzymes (Ravindran et al., 1995) and even digestive enzymes like pepsin, amylopsin and amylase are inhibited by phytate. Complexed protein substantially alters the pepsin digestion (Vaintraub and Bulmaga, 1991), presumably due to alterations in structure and solubility that render protein less susceptible to pepsin activity following aggregation with phytate. Phytate can nonselectively bind to proteins and it has been shown to inhibit activities of enzymes including pepsin, trypsin and amylase (Liener, 1994) as well as to decrease protein digestibility (Table 6).

Blood chemistry

The red blood cells count, hemoglobin and hematocrit were affected by dietary treatments (Table 7). The white blood cells count, MCV, MCH and MCHC did not differ significantly ($P > 0.05$) among the five groups (Table

7). Higher (P < 0.05) RBC, hemoglobin and hematocrit were observed for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ (phytate with phytase) groups than for $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups. Similarly, both hemoglobin and hematocrit contents were also higher for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ which in turn did not change significantly the MCV, MCH and MCHC values. The hematological variables such as hemoglobin and hematocrit are considered as indicators of red blood cell formation (Sun et al., 1995). In the present study we found that the phytate decreased the hemoglobin and hematocrit concentration in $PWP_{1.5}$ and PWP_3 groups (phytate without phytase) and these parameters were improved on addition of phytase. Similarly Hallberg et al. (1989) found that the hemoglobin and hematocrit decreased in the presence of dietary sodium phytate. The lower hemeoglobin and hematocrit levels in Nile tilapia fed phytate containing diets without phytase could be attributed to the binding of phytic acid molecule to minerals (iron) and/or amine group of amino acids causing their low availabilities in the body and increased erythrocyte fragility (Sun et al., 1995).

Blood protein and lysozyme activity in serum

The dietary phytate and phytase significantly influenced the levels of albumin, globulin and total protein in blood (Table 7). The concentration of total protein in blood is used as a basic index for health and nutritional status in fish (Martinez, 1976). Two major groups of proteins in the blood are albumin and globulin and these proteins play a significant role in the immune response. Globulin is important for the immunological responses. The higher albumin, globulin and total protein in blood were observed (P < 0.05) for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ (phytate with phytase) groups compared with those for $PWP_{1.5}$ and PWP_3 (phytate without phytase) groups (Table 7). These results are consistent with the higher lysozyme activity observed for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ groups compared with $PWP_{1.5}$ and PWP_3 groups. As phytate content increased in the experimental diets ($PWP_{1.5}$ and PWP_3), the levels of albumin, globulin and total protein in blood also increased (P<0.05; Table 7). Lysozyme plays an important role in non-specific immune response and regarded as first line of defense with higher activity in mucus, gills, ova, alimentary tract and serum of fish. Innate immunity due to lysozyme is caused by lysis of bacterial cell wall and this stimulates the phagocytosis of bacteria. In the present study higher (P < 0.05) lysoyzme activity in serum was observed for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ groups compared with $PWP_{1.5}$ and PWP_3 groups indicate that hydrolysed phytate

such as free inositol phosphate acts as immunostimulating effect in fish (Cavazza, 2003).

Albumin, the most abundant blood protein, is essential for maintaining the osmotic pressure for proper distribution of body fluids between intravascular compartments and body tissues, and it also acts as a blood carrier by non-specifically binding hormones and a transport protein for hemin and fatty acids (Rosenoer et al., 1977; Schell and Blumberg, 1977). The decrease of blood albumin is resulted from sub-health or sub-nutritional status of the host (Zunszain et al., 2003). In this study, phytate significantly decreased the blood albumin and phytase supplementation elevated it (Table 7), indicating the possible adverse effect of phytate on the nutrient transport and health status of fish (Liu et al., 2010). Phytase supplementation improved intestinal nutrition and health status of fish in the present study.

Cholesterol and triglyceride levels in plasma and blood glucose level

Both dietary phytate and phytase significantly influenced the plasma cholesterol and triglyceride levels, and muscle cholesterol level (Table 8). Phytate decreased plasma triglyceride levels ($P < 0.05$), while phytase addition increased it. Similarly, dietary phytate significantly decreased the levels of plasma cholesterol and muscle cholesterol levels compared with control, indicating that phytate may interfere with lipid metabolism. Dietary phytate in mice and chicken has also been shown to decrease the concentrations of cholesterol in the serum and liver; and the digestibility of lipid and cholesterol (Lee et al., 2007; Liu et al., 2010). The mechanism of cholesterol lowering effects by phytate remains to be clarified but it was assumed that in part it is related to an increase in faecal bile acid and lipid excretion (Lee et al., 2007) and a reduction in hepatic cholesterol synthesis. Dietary phytate also reduces the plasma triglyceride level compared with control and phytase treated groups. Similar trends have been observed in another study with rats and chickens (Szkudelski, 2005; Liu et al., 2010). These authors found that dietary phytate resulted in a substantial reduction in the concentration of triglyceride level in plasma. *In vitro* studies on mice have also demonstrated that dietary phytate supplementation results in significant lowering of serum cholesterol and triglyceride levels (Jariwalla et al., 1990).

Table 6 Apparent digestibility of the nutrients and energy of diets, efficiency of digestible nutrients and energy of diets; and activities of digestive enzymes (U/mg protein) in Nile tilapia (*Oreochromis niloticus* L.) fingerlings.

Treatment*	Protein digestibility	Lipid digestibility	Energy digestibility	Digestible protein retained (%)	Digestible energy retained (%)	Digestible lipid retained (%)	Amylase	Protease	Lipase
Control	90.0[a] ± 3.61	94.2[a] ± 3.96	85.8 ± 3.42	41.7[ab] ± 3.46	28.0[a] ± 2.59	70.1[a] ± 9.02	25.0 ± 4.30	45.2[a] ± 8.01	9.88 ± 2.51
PWP$_{1.5}$	85.0[ab] ± 3.54	88.0[ab] ± 2.24	83.2 ± 4.87	38.2[bc] ± 2.36	24.0[ab] ± 2.18	57.1[b] ± 5.41	21.4 ± 2.51	35.8[ab] ± 6.87	8.6 ± 1.82
PWP$_3$	80.6[b] ± 5.13	83.8[b] ± 3.49	81.8 ± 5.54	32.8[c] ± 4.12	19.2[b] ± 2.87	42.6[c] ± 5.54	17.6 ± 2.30	29.6[b] ± 7.27	7.52 ± 2.25
PP$_{1.5+phytase}$	86.2[ab] ± 1.48	93.4[a] ± 3.78	86.8 ± 2.28	42.3[ab] ± 3.67	26.0[a] ± 2.30	63.0[ab] ± 7.74	25.6 ± 5.18	44.2[ab] ± 9.02	9.92 ± 2.20
PP$_{3+phytase}$	88.4[a] ± 3.21	91.0[a] ± 3.87	85.8 ± 3.96	46.0[a] ± 3.11	27.8[a] ± 2.90	70.7[a] ± 6.89	25.6 ± 5.94	43.8[ab] ± 8.98	9.88 ± 2.96
SEM	0.93	1.01	0.85	0.99	0.74	2.26	1.13	2.15	0.53

* See footnotes to Table 2. Values are mean (n = 6) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 7 Effects of experimental diets on the haematological parameters (RBC (10^6 cells/mm^3), WBC (10^3 cells/mm^3), Hb (g/dl), Hct (%), MCV (fL), MCH (pg), MCHC (g/dl), albumin (A, g/dl), globulin (G, g/dl), albumin/globulin ratio and total protein (g/dl) in the blood; and lysozyme activity (LA, IU/ml) in the serum of Nile tilapia (*Oreochromis niloticus* L.) fingerlings.

Treat*	RBC	WBC	Hb	Hct	MCV	MCH	MCHC	Albumin	Globulin	A/G ratio	Total protein	LA
Control	1.42[a] ± 0.03	1.29 ± 0.02	6.93[a] ± 0.48	51.3[a] ± 7.50	361 ± 28	48.8 ± 5.84	12.32 ± 2.14	1.28[c] ± 0.10	2.57[b] ± 0.13	0.50[bc] ± 0.07	3.85[c] ± 0.10	397[b] ± 24
PWP$_{1.5}$	1.23[cd] ± 0.04	1.31 ±0.03	6.03[b] ± 0.95	41.3[b] ± 2.87	355 ± 33	49.1 ± 8.39	14.74 ± 3.01	1.10[c] ± 0.08	2.07[c] ± 0.05	0.53[b] ± 0.03	3.17[d] ± 0.13	348[c] ± 41
PWP$_3$	1.17[d] ± 0.02	1.29 ±0.02	5.63[b] ± 0.51	38.3[b] ± 1.89	337 ± 46	48.1 ± 6.67	14.69 ± 2.80	1.23[c] ± 0.10	2.52[b] ± 0.10	0.49[c] ± 0.04	3.75[c] ± 0.12	379[bc] ± 18
PP$_{1.5+phytase}$	1.31[b] ± 0.04	1.30 ± 0.01	7.05[a] ± 0.70	49.0[a] ± 5.16	374 ± 49	51.8 ± 5.97	14.39 ± 2.48	1.50[b] ± 0.08	2.65[ab] ± 0.24	0.57[ab] ± 0.04	4.15[b] ± 0.30	404[b] ± 22
PP$_{3+phytase}$	1.29[bc] ± 0.03	1.30 ± 0.02	7.40[a] ± 0.65	46.8[a] ± 4.06	364 ± 37	52.4 ± 6.73	15.21 ± 3.49	1.80[a] ± 0.08	2.85[a] ± 0.06	0.63[a] ± 0.02	4.65[a] ± 0.13	457[a] ± 29
SEM	0.02	0.01	0.13	0.81	13.18	1.32	0.57	0.03	0.08	0.02	0.04	10.4

*See footnotes to Table 2. Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05)
MCV: Mean cell volume (fL); MCH: Mean corpuscular hemoglobin (pg); MCHC: Mean corpuscular hemoglobin concentration (g/dl)
IU- The amount of enzyme required to produce a change in the absorbance at 450 nm of 0.001 units per minute at pH 6.24 and 25^{0}C, using a suspension of *Micrococcus lysodeikticus* as the substrate.

Blood glucose level was significantly lower (P < 0.05) for the $PWP_{1.5}$ and PWP_3 groups compared with $PP_{1.5+phytase}$ and $PP_{3+Phytase}$ and control groups (Table 8). Our results concur with those of Thompson et al. (1987) who reported that intrinsic dietary phytate as well as the addition of 1% sodium phytate to the diet significantly reduced (P < 0.05) blood glucose levels in humans. Lee et al. (2006) also found reduced blood glucose levels in mice depending on the dietary phytate contents (0–1.0%). In another study, Denstadli et al. (2006) observed that graded levels (1–20.7 g phytate kg^{-1}) of phytate in Atlantic salmon diet did not influence the blood glucose level. *In vitro* studies on humans verified that more dependence on phytate-enriched diets results in low blood glucose response (Yoon et al., 1983). Moreover, phytate can be a key element in modulating insulin secretion; diminished production of insulin. The real mechanism of action is not fully understood but it seems that phytate regulates insulin secretion via its effect on calcium channel activity because it specifically inhibits serine threonine protein phosphatase activity. This, in turn, opens intracellular calcium channels, driving insulin release (Barker and Berggren, 1999).

Alkaline phosphatase (ALP) and alanine transaminase (ALT) activities

In clinical diagnosis, the liver transaminases such as ALP and ALT indicate status of the liver function (Velick and Vavra, 1962; Niizuma et al., 1988). Large amount of ALP and ALT is released into blood, mostly during liver cell damage, and their detection could monitor liver cell damage. In fish, the normal range of fish blood transaminase cannot be established because of the limited reports related to effects of phytate on these enzyme activities, so it is difficult to judge whether the transaminase changes caused by the dietary factors interfered with liver function of fish in this study. The blood ALP and ALT enzyme activities in the present study are presented in the Table 8. As phytate concentration increased in the experimental diets, the ALP activity increased, whereas ALT activity exhibited opposite trend. Our results are in contrast with Denstadli et al. (2006) wherein they observed that ALP activity decreases in the stomach and intestine when phytate content increases (≥ 4.7 g Na-phytate per kg) in the feed in Atlantic salmon. However, phytase supplemented diets revealed significantly higher (P < 0.05) ALP activity compared with non phytase supplemented diets (Table 8). Highest ALP activity was observed for $PP_{3+Phytase}$ group (highest inclusion of phytate with phytase) compared with other groups (Table 8). Significantly lower (P < 0.05) ALT activity was observed for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ groups (phytate with phytase) compared with $PWP_{1.5}$ and PWP_3 groups

(phytate without phytase). Similarly Liu et al. (2010) found in chicken that higher phytate content in the diets decreases the ALT activity in blood. However, the lower transaminase activity in the blood observed in high phytate basal diets without phytase might suggest that phytate depresses transamination and consequently affects the nutrient metabolism of the fish. In this study, phytate significantly decreased the ALP level in blood compared with phytase treated groups, indicating that dietary phytate and phytase affect the P metabolism as reflected on the blood P level (Table 8). We observed significantly higher (P < 0.05) P level in the phytate + phytase treated groups (PP$_{1.5+Phytase}$ and PP$_{3+Phytase}$) compared with non phytase supplemented groups (PWP$_{1.5}$ and PWP$_3$) (Table 8).

Electrolytes and metabolites in the blood

The influence of phytate and phytase on the blood electrolytes were of interest in this study. Electrolytes in the body are important to maintain body fluid function because they affect the amount of body water, blood acidity, muscle action and other important processes (Manery, 1954). Calcium is a versatile intracellular messenger that is used throughout the life cycle of an organism to control diverse biological processes (Berridge et al., 2000). Sodium and K help keep the proper balance of body fluids and maintain the body's acid–base balance (Kirkendall et al., 1976). In this study, the blood concentrations of most electrolytes (P and Ca) were significantly influenced by dietary phytate and phytase, indicating that dietary factors exert a substantial influence on the electrolyte metabolism.

The concentrations of Ca and P in blood were significantly decreased by dietary phytate (P < 0.05) compared with PP$_{1.5+Phytase}$ and PP$_{3+Phytase}$ groups (Table 9). Sodium and K concentration in the blood were not influenced by phytate and phytase supplementation in the diets (Table 9). Phytase supplementation significantly increased (P < 0.05) the blood electrolytes (P and Ca) compared with PWP$_{1.5}$ and PWP$_3$ groups. Increase in phytate content in the diet decreased P and Ca concentrationsl in plasma. These results are consistent with those of Laining et al. (2010). They observed that more than 13 g IP6 per kg diet significantly decreases the plasma P and Ca level in Japanese Flounder, *Paralichthys olivaceus*.

Table 8 Cholesterol and triglyceride (mg/dl) levels in plasma and muscle cholesterol (mg/100 g), blood glucose (mg/dl) levels; alkaline phosphatase (ALP, U/l) and alanine transaminase (ALT, U/l), activities in blood of Nile tilapia (*Oreochromis niloticus* L.) fingerlings.

Treatment*	Plasma cholesterol	Plasma triglycerides	Muscle cholesterol	Blood glucose	ALP	ALT
Control	156[a] ± 5.12	176[a] ± 10.1	132[a] ± 3.77	50.7[a] ± 4.03	40[b] ± 3.42	51[c] ± 1.63
PWP$_{1.5}$	127[c] ± 4.43	138[bc] ± 10.1	88[c] ±5.48	42.5[b] ± 2.65	30[d] ± 1.83	62[a] ± 6.45
PWP$_3$	109[d] ± 2.36	121[c] ± 5.74	69[d] ± 3.50	39.2[b] ± 2.75	34[c] ± 3.59	49[d] ± 5.62
PP$_{1.5+phytase}$	153[a] ± 2.65	153[b] ± 4.41	123[a] ± 6.73	50.5[a] ± 2.89	39[b] ± 3.32	54[b] ± 2.94
PP$_{3+phytase}$	139[b] ± 7.50	152[b] ± 10.13	106[b] ± 3.11	50.5[a] ± 4.36	56[a] ± 5.12	45[d] ± 4.35
SEM	3.68	4.00	4.75	1.31	2.31	1.88

*See footnotes to Table 2. Values are mean (n = 4) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

1 U = 16.66 nKat/l; nKat = Amount of glandular kallikrein which cleaves 0.005 mmol of substrate per minute).

Table 9 Effects of experimental diets on blood ions [calcium (mg/dl), phosphorus mg/dl, sodium (mmol/l) and potassium (mmol/l)]; and total bilirubin (mg/dl), urea nitrogen (mg/dl) and creatinine (mg/dl) concentrations in blood of Nile tilapia (*Oreochromis niloticus* L.) fingerlings.

Treatment*	Calcium	Phosphorus	Sodium	Potassium	Total bilirubin	Urea nitrogen	Creatinine
Control	12.7[c] ± 0.24	22.4[d] ± 0.81	154 ± 19	1.63 ± 0.32	0.25 ± 0.06	2.25 ± 0.50	0.20 ± 0.08
PWP$_{1.5}$	12.8[c] ± 0.86	23.6[d] ± 0.51	158 ± 21	1.73 ± 0.33	0.24 ± 0.08	2.25 ± 0.96	0.21 ± 0.11
PWP$_3$	13.5[c] ± 0.64	25.4[c] ± 0.56	156 ± 29	1.65 ± 0.13	0.22 ± 0.10	2.00 ± 0.82	0.21 ± 0.05
PP$_{1.5+phytase}$	16.2[b] ± 0.47	27.5[b] ± 0.84	160 ± 17	1.95 ± 0.47	0.20 ± 0.00	2.00 ± 0.82	0.20 ± 0.00
PP$_{3+phytase}$	17.7[a] ± 0.59	30.9[a] ± 0.91	160 ± 14	1.88 ± 0.17	0.20 ± 0.00	1.75 ± 0.50	0.20 ± 0.08
SEM	0.48	0.71	4.19	0.07	0.01	0.15	0.01

*See footnotes to Table 2.

Values are mean (n = 4) ± standard deviation.

Mean values in the same column with different superscript differ significantly (P < 0.05).

Phytase supplementation enhances digestibility and availability of minerals which are bound to phytate. In the present study Ca and P level were higher for $PP_{1.5+Phytase}$ and $PP_{3+Phytase}$ groups compared with $PWP_{1.5}$ and PWP_3 groups (Table 9). Our results are in agreement with those of other studies (Sugiura et al., 2001; Vielma et al., 2004; Sajjadi and Carter 2004; Liebert and Portz, 2005) that reported a positive effect of phytase supplementation on mineral (P, Ca, Cu, Mg, Fe, Sr and Zn) availability in fish. Supplementation of phytase hydrolyses phytate and significantly increases the concentration of Ca, P, Mg, and Mn in plasma of fish (Jackson et al., 1996; VanWeerd et al., 1999; Papatryphon and Soares, 2001; Furuya et al., 2001; Yan and Reigh, 2002; Liebert and Portz, 2005; Cao et al., 2008). Likewise, the inclusion of exogenous phytase in tilapia diet could act as replacer for supplemented P and Ca in the diet. Apart from fish, inclusion of microbial dietary phytase in feed has been shown to bring a desirable effect on the utilisation of bound P and Ca in chicken, pig and broiler (Selle and Ravindran, 2008; Liu et al., 2010).

Blood urea nitrogen levels are thought to be associated with liver or gill dysfunction (Stoskopf, 1993), as these are the sites of urea production and excretion, respectively. In our study, urea nitrogen, bilirubin and creatinine concentration in the blood did not differ significantly among the five groups (Table 9). There were no dietary effects on blood urea nitrogen concentration and these values were in the normal range (Wedemeyer and Chatterton, 1970; Witters, 1986; Wedemeyer, 1996), suggesting that fish liver and gill are in normal condition in the phytate and phytase supplemented groups. Bilirubin is the predominant bile pigment found circulating in fish, which is derived from the breakdown of hemoglobin (Cornelius, 1992). According to Tietz (1986) the concentration of total bilirubin is used as an indicator of liver functionality. An elevation in total bilirubin indicates increased erythrocyte destruction that could be due to obstructive diseases of the bile system and hepatocellular disease (Duncan, 1995; Tietz, 1986). Total bilirubin concentration in blood was similar for all groups indicating that liver and kidney function was normal. Creatinine, a degraded product of creatine is used as an indicator of kidney damage or malfunction (Tietz, 1986). Blood creatinine is normally quite stable and its level in the blood becomes elevated if kidney function is impaired. The creatinine concentration in the blood was within the normal range (Ceschia et al., 1978; Tietz, 1986).

Conclusions

The defatted Jatropha kernel meal (DJKM) contains high amount of phytate. The supplementation of partially purified Jatropha phytate in the fish diet produced negative effects on growth performance (body mass gain, specific growth rate and metabolic growth rate), nutrient utilization (protein efficiency ratio, protein productive value, feed gain ratio and energy retention) and digestive physiology (nutrient digestibility and digestive enzymes) in Nile Tilapia. Inclusion of Jatropha-phytate in the fish diet also adversely influenced the biochemical changes such as metabolic enzymes and electrolytes/metabolites. The prominent changes include decreased red blood cell count and hematocrit content, decreased cholesterol and triglyceride concentrations in plasma and decreased blood glucose levels, when compared with control. The adverse changes observed may be due to the interaction of phytate with minerals and enzymes in the gastro intestinal tract, resulting in poor bioavailability of minerals. Phytate concentration used in the present study (1.5 and 3.0%) corresponds to 16.5% and 33.0% of DJKM in the fish diet. The DJKM at levels > 16.5% in the diet would be detrimental to tilapia. Addition of phytase (1500 FTU/kg) to the phytate containing diets would mitigate the adverse effects of at least up to 3% Jatropha phytate (or 33% DJKM) in the diet. Addition of phytase in diets containing DJKM is recommended to maximally utilize it in Nile tilapia.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

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Chapter 15

General discussion, conclusions and future perspectives

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15. General discussion

Aquaculture continues to grow at a higher pace than farming of terrestrial animals. In addition, the aquaculture industry is shifting away from the traditional extensive approach to more intensive practices. This shift will further increase the need for formulated feeds. Since fish stocks, including those used to make meals and oils, are near or at their limits of production, other sources will have to fill the upcoming gap in supply. For fish and shrimp feeds, the most pressing need is to find alternative protein sources. As fish meal is a limited primary source and plants are widely available and reasonably priced, the use of plant protein sources in aquafeeds should be considered. Soybean meal is one of the most nutritious of all plant protein sources and currently, the most commonly used protein source in aqua feed. However, production of soybeans often means that there is less land on which crops used for human food can be raised and hence there is a need to identify other protein rich plant resources that could be used in fish diets. Detoxified *Jatropha curcas* kernel meal (DJKM), heated *Jatropha platyphylla* kernel meal (H-JPKM) and detoxified Jatropha protein isolate (DJPI), are attractive from a nutritional standpoint and are thus candidates for replacing fishmeal. Detoxified Jatropha kernel meal, H-JPKM and DJPI in aquaculture are relatively new feed ingredients compared to soybean meal.

15.1 Effects of Jatropha derived feed ingredients (DJKM, H-JPKM and DJPI) on growth and health of fish and shrimp

15.1.1 Palatability of feed and retention of nutrients

The use of plant protein products in aquafeed is limited by the effects of the ingredients on the palatability of the diets to the fish (Burel et al., 1998). Based on visual observation during feeding time acceptability of the feeds based on DJKM, H-JPKM and DJPI was good and the behaviour of fish and shrimp was normal. No feed was left in the aquaria. These findings are in contrast with other researchers (Escaffre et al., 1997; Glencross et al., 2004) who observed a decline in feed intake when protein isolates of soy and lupin were included at levels >40% in common carp and rainbow trout diets. Detoxified Jatropha kernel meal inclusions (>50% replacement of fish meal protein) resulted in reduced protein utilisation, measured as protein efficiency ratio and protein productive value (Chapters 2 and 4); whereas H-JPKM and DJPI inclusion (62.5% and 75% replacement of fish meal protein

respectively) had no effect on the protein utilisation (Chapters 10, 11 and 12). These results revealed that higher (>50% replacement of fish meal protein) inclusion of H-JPKM and DJPI in fish diet meet the dietary demands for protein and energy.

Reduced plasma cholesterol was observed in common carp, rainbow trout and Nile tilapia when Jatropha based proteins (DJKM, H-JPKM and DJPI) were incorporated in their diets (Chapters 3, 6, 8 and 13). Similar results were observed by other researchers (Kaushik et al., 1995; Goto et al., 2001; Kaushik et al., 2004; Hansen, 2009) in some fish species given plant based diets. Reduced plasma cholesterol in fish as dietary fish meal level decreased, and explained that this was a consequence of the reduced amount of available cholesterol in the diet (Kaushik et al., 1995). In our study, dietary cholesterol levels in fish receiving plant protein based diets were lower than in those receiving the fish meal reference diet. Further, fibre and antinutrtional factors (ANF) reduce absorption of total fat, including cholesterol, when these factors are increased in the diet (Krogdahl et al., 2005; Hansen, 2009). Faecal excretion of steroids (bile acids) is the major pathway for elimination of cholesterol from the body (Hansen, 2009). De Schrijver (1990) showed that the faecal excretion of steroids by rats increased as their plasma cholesterol was decreased by feeding them plant protein. Further, Liaset et al. (2009) revealed that reductions in plasma cholesterols in rats could have resulted from changes in energy metabolism, caused by differences in the amino acid profile of a plant based diet.

15.1.2 Nutrient digestibility and digestive enzymes

Detoxified Jatropha kernel meal and DJPI in combination with fish meal protein were found to have excellent protein, lipid and energy digestibilities (Chapters 2, 6, 8 and 12). Generally, oil seed meal proteins have digestibilities of 80-95% in fish (Jauncey and Ross, 1982). The protein digestibility is a key factor in the evaluation of a diet for fish and its potential for the synthesis of new tissues. The digestibility of DJKM protein is relatively high in fish (~ 89-92%) (Chapter 2) suggesting that DJKM is an excellent protein source for the fish. However, DJKM is known to contain low levels of fibre and ANFs, and these factors even at low levels could still have caused the reduction in digestibility. Use of DJKM (>50% replacement of fish meal protein) resulted in reduced protein and lipid digestibility in common carp and trout (Chapters 2, 6 and 8), which is in consistent with the results of Regost et al. (1999) and Førde-Skjærvik et al. (2006) in whose

experiments plant protein (soybean meal and corn gluten meal) inclusion in salmon and turbot diets resulted in lower nutrient digestibility. Inclusion of DJKM (>50% fish meal protein replacement) in common carp and rainbow trout diets decreased lipid digestibility. The DJKM based diets had a higher content of non-starch polysaccharides (NSP) than the fish meal based diet (Chapters 2, 6 and 8). The lower lipid digestibility of the fish fed the DJKM based diets may be associated with the increase in the NSP content, which reduces fat absorption by disturbing micelle formation in the gastro intestinal tract and also increases the viscosity of the gut contents. It could also be that NSP entrap bile salts, thereby reduce their effectiveness in solubilising lipids (Krogdahl et al. 2003; Gatlin et al. 2007; Hansen, 2009; Øverland et al. 2009).

The digestibilties of nutrients and energy in detoxified Jatropha protein isolate based diets (75% replacement of fish meal protein) were similar to those of the control diet, suggesting that DJPI is a good quality protein source for fish feed.

Antinutrients (heat labile) such as trypsin inhibitors and lectins were absent in the DJKM and DJPI, whereas a heat stable antinutrient (phytate) was present. Phytate is known to inhibit digestive enzymes such as pepsin, trypsin and α-amylase (Robaina et al. 1995; Alarcon et al. 1999). It also forms complexes with minerals (Teskeredzic et al., 1995; Sugiura et al., 1999) and proteins (Moyano et al. 1999), thereby modifying digestion processes and impairing intestinal absorption. In our study digestive enzyme activity decreased on inclusion of DJKM in the common carp and rainbow trout diets at levels > 50% fishmeal protein replacement (Chapters 2 and 6). Decrease in digestive enzyme (amylase, protease and lipase) activity in common carp and trout intestine might be caused by the presence of phytate in the DJKM based diets. For plant based feeds, phytase has been added to the feed at a level of 500 FTU per kg (Forster et al., 1999; Cheng et al., 2004). We used 500 FTU phytase per kg feed, which might not be sufficient because of the high phytate content in DJKM. For any new feed resource such as Jatropha kernel meal separate systematic studies on optimization of phytase level in the diet would need to be undertaken.

An increase in the level of DJKM in the diets of common carp and rainbow trout led to a decrease in protein availabilty probably caused by the presence of unutilised phytate. This resulted in lower protease activity. Similar results were observed many researchers (Sandholm et al., 1976; Krogdahl et al., 1994; Santigosa et al., 2008), wherein they observed that the intestinal trypsin activity (protein digesting enzyme) decreased as plant

ingredients addition increased in fish feed. They concluded that trypsin is highly sensitive to plant antinutrients. Krogdahl et al. (2003) fed diets containing various levels of soybean meal. They found that increasing levels of soybean meal were associated with a dose dependent decrease in digestive enzyme activity, reduced nutrient digestibility and morphological changes in the distal intestinal mucosa leading to digestive and absorptive dysfunctions. In the present study we found that lower activity of digestive enzymes in groups fed DJKM was associated with lower nutrient digestibility.

15.1.3 Growth

The overall growth rates were good in fish and shrimp (Chapters 2 - 10). The dietary effects on growth were dependent on the plant ingredients and their inclusion levels. Based on the results (Chapters 2-10), 50% of the fish meal protein can be replaced by DJKM in common carp, rainbow trout and white leg shrimp diets without compromising growth and nutrient utilization. However, greater than 50% replacement of fish meal protein by DJKM leads to significantly lower growth, which could be attributed to factors such as:

- Lower digestibilities of protein and energy in the diets, resulting in lower protein and energy availability from the DJKM (plant protein structures in general are much more compact than fish meal protein, so digestive enzymes act slowly on DJKM proteins)
- The presence of antinutrients such as phytate and NSP. Detoxified Jatropha kernel meal contains large amounts of these compounds that could adversely affect feed utilisation.
- The digestibility of synthetic lysine, which was added as a supplement to the diets, may be less than that of the natural amino acid present in the feed ingredients.
- The level of sulfur containing amino acids (methionine and cystine) in DJKM based diets was slightly lower than that in the control diet and did not reach the optimum requirement in common carp and rainbow trout diets.

Another constraint related to the digestion of diets based on plant sources (soybean meal and DJKM and H-JPKM) is their relatively high carbohydrate content, which is generally not well digested by fish (Singh and Nose, 1967; Refstie et al., 2000). Many researchers (Kaushik et al. 1995; Sanz et al. 1994; Hasan et al., 1997; Refstie et al. 2000; Morris et al. 2005;

Aksnes et al., 2006; Refstie et al., 2006a,b) reported that the high addition of plant ingredients (sunflower, lupin, peas, rapeseed and soybean meal; mustard, sesame and groundnut oil cakes) in fish feed resulted negative effects on growth performance and nutrient utilization.

Feeding DJPI to common carp has proved promising (Chapter 12). Common carp fed diets containing a relatively low level of DJPI (50% replacement of fish meal protein) grew better than those fed a fish meal based control diet (Chapter 12). However, high level (75% replacement of fish meal protein) of DJPI in common carp diet exhibited similar growth performance with control diet (Chapter 12). Since overall growth performance and protein or energy utilisation of this group were similar to that of the fish meal fed group, the current study demonstrates that a high replacement level (up to 75% replacement of fish meal protein) of fish meal by a single plant-protein source such as DJPI is possible in diets for common carp. Escaffre et al. (1997) have reported reduced growth in carp fed a diet containing soy protein isolate at levels $\geq$ 60%. On the other hand, many researchers (Glencross et al., 2005; Kaushik et al., 2004; Overland et al., 2009) have shown that soy, lupin and pea protein isolates can replace 50-75% of fish meal protein in trout and Atlantic salmon diets without impairing growth performance and nutrient utilization. Despite the existence of some variability between fish species in the utilisation of plant products, results of most studies show that high dietary levels (more than 40% of total protein) of plant derived proteins depresses growth and feed efficiency in carp (Hasan et al., 1997; Mazurkiewicz, 2009). This poor growth performance commonly found in fish fed plant protein-rich diets, was in most cases, related to reduction in the voluntary feed intake (consequently a lower intake of essential nutrients and digestible energy) (Gomes et al., 1995). However, in some studies with rainbow trout, the total replacement of fish meal by soybean products (with or without supplemented L-methionine) was successfully achieved (Wilson, 1992; Kaushik et al., 1995).

15.1.4 Energy metabolism

Imbalanced dietary amino acid profiles influence energy metabolism in all animals, including fish. In common carp, an increase in the whole body lipid content of fish fed diets containing DJKM and DJPI proteins was observed (Chapters 2 and 12). Similar results were reported by several authors when an increase in plant protein in fish diets led to high lipid deposition (Adelizi et al., 1998; Kaushik et al., 2004). When fish fed plant protein (100%)

compared with reference diet (100% fish meal) in rainbow trout thereafter an up-regulation of several proteins involved in energy metabolism in liver (Vilhelmsson et al., 2004). On the other hand, in Chapter 6, an increase in lysine level did affect lipid storage in rainbow trout; reducing lipid productive value and hepatosomatic index lysine intake increased. These results concurs with many researchers findings in fish and mammals (Tanphaichitr et al., 1976; Rodehutscord et al., 1997; Witte et al., 2000; Marcouli et al., 2006; Espe et al., 2007; Hansen, 2009), which could be related to the role of lysine as a precursor in the biosynthesis of carnitine (Tanphaichitr et al., 1971; Harpaz, 2005; Hansen, 2009). Carnitine is involved in the transport of fatty acids through the outer mitochondria membrane (Hansen, 2009). All amino acids in fish diet should be in correct proportions, excess amino acids can not be stored in the fish body and are therefore transdeaminated, and used either directly for energy or stored as lipid (El-Mowafi et al., 2010; Hansen, 2009). The increased liver size can therefore be explained by amino acids being used for *de novo* fat synthesis or burnt for energy thus saving fat for storage (Hansen, 2009). An increase in blood urea nitrogen in fish fed DJKM compared to that of fish fed fish meal was observed in common carp (Chapter 3). Urea nitrogen is the major end product of amino acid deamination in fish, contributing to 60 to 90% of the nitrogen excreted (Cowey and Walton, 1988). Therefore increased blood urea nitrogen concentration in DJKM fed groups compared with the control group (Chapter 3) can be an indicator of increased deamination of amino acids derived from the proteins present in DJKM based diets.

15.1.5 Energy budget and metabolic efficiency

Major components of the energy budget (routine metabolic rate, heat released and metabolisable energy) were not affected by feeding DJKM or H-JPKM in the diets of common carp and Nile tilapia respectively when compared with fish meal fed groups (Chapters 4 and 10). Similar results have been reported by Suárez et al. (2009). They found no significant differences in the energy budgets of white shrimp (*Litopenaeus vannamei*) juveniles fed diets containing either soybean-canola meal (at 80% substitution level) or fish meal.

The routine metabolic rate is attributed to a multitude of factors associated with feed utilization and it is generally accepted that its magnitude is mainly due to deamination of ingested amino acids (Cho and Kaushik, 1990). However, while some authors observed a change in the

routine metabolic rate with different types of protein sources (Jobling and Davies, 1980; Helland et al., 2006; Hatlen et al., 2007), surprisingly we were not able to find any effect of DJKM or H-JPKM protein when compared with fish meal and soybean meal fed groups. The metabolizable energy observed in these studies (Chapter 4 and 10) were higher than those that have been observed in studies of other fish species in which estimates are usually between 60% and 70% (Carter and Brafield, 1991; Schwarz and Kirchgessner, 1995; Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999; Booth and Allan, 2003; Lupatsch et al., 2003; Lupatsch and Kissil, 2005). Cui and Liu (1990) constructed average energy budgets for six teleost species fed *ad libitum* and found that heat loss was always the largest component, 50 - 69% of consumed energy. Whereas the energy used for growth was much smaller, 21 – 35%. In the present study, for all the experimental diets, the energy retained for growth was between 28 and 37%, whereas energy expenditure was between 39 and 45%. The metabolizable energy of the diet ranged from 72 to 78%. These findings (Chapters 4 and 10) suggest that dietary protein sources DJKM and H-JPKM can be efficiently utilized for growth by common carp and Nile tilapia respectively, and that the efficiency is high as that for soybean meal and fish meal.

15.1.6 Growth hormone (GH) and insulin-like growth factor-1 (IGF-1) encoding genes

The GH-IGF-I axis provides an integrated signal for growth and nutrient partitioning (Beckman and Dickhoff, 1998; Mingarro et al., 2002). In the present study (Chapter 5) there was an over-all negative correlation between expression of GH gene and growth for all groups.

Highest growth was observed for the DJKM fed group (50% fish meal protein replaced by DJKM), which was similar to that of the control group and significantly higher than of the other group (75% replacement of fish meal protein by DJKM). However, GH gene expression in liver and brain was lowest in the DJKM fed group (50% fish meal protein replaced by DJKM), which was similar to that for the control group and significantly lower than for the other group (75% replacement of fish meal protein by DJKM). We can conclude that GH gene expression was inversely related to growth (Chapter 5). This conclusion agrees well with data from salmonids and other fish species that have shown elevated GH concentrations during extended periods of fasting or feed restriction (Wagner and McKeown, 1986; Sumpter et al., 1991; Pérez-Sánchez et al., 1995; Pierce et al., 2005). Our

results concur with those of Aksnes et al. (2006). They fed rainbow trout and gilthead sea bream on a diet in which ≥75% of the FM protein was replaced by a mixture of plant protein sources (soybean meal, soy protein concentrate, corn gluten meal, wheat gluten, extruded peas, rapeseed meal) balanced with essential amino acids and found that GH gene expression was increased in the liver. They concluded that growth performance was inversely related to GH gene expression in the liver. We observed a similar trend in our study.

Much evidence suggests that IGF-1 plays a role in the growth of fish (Duan, 1997,1998; Moriyama et al., 2000). Inclusion of DJKM in the diet of common carp did not influence the concentration of IGF-1 levels in plasma but there was a numerically similar trend in the expression of the IGF-1 gene in the liver and brain (Chapter 5) and IGF-1 gene expression in these organs was positively correlated with growth (Chapter 5). Our results are in agreement with Gomez-Requeni et al. (2004), Dyer et al. (2004), Aksnes et al. (2006) and Li et al. (2006), who observed that IGF-1 gene expression in liver was positively corelated with growth rates in trout, sea bream, channel cat fish, barramundi and Atlantic salmon. Results of the present study and the study of Dyer et al. (2004) suggest that mRNA expression and circulating plasma concentrations of IGF-1 are useful tools for the prediction of growth rates in fish.

IGF-1 levels in the blood plasma did not differ significantly among the treatment groups and correlated positively with growth. Our results concur with those of Gómez-Requeni et al. (2004, 2005) who showed that dietary protein sources may affect plasma GH concentrations, but not IGF-1 levels in rainbow trout and gilthead sea bream. IGF-1 is involved in the GH negative feedback loop (Perez-Sanchez et al. 1992; Weil et al. 1999), and consistent changes in plasma GH concentrations occur in response to shifts in ration size and dietary protein/energy ratio (Perez-Sa´nchez et al. 1995; Martı´-Palanca et al. 1996; Company et al. 1999). Gomez-Requeni et al. (2003) demonstrated that changes in the content of essential and nonessential amino acids in the diet are able to induce some state of liver GH resistance in conjunction with reduced growth rates. In comparison to fish fed the fish meal diet, no changes in the J_{50} group (50% replacement of fish meal protein by DJKM meal) were found, but in the J_{75} group (75% replacement of fish meal protein) increased GH expression level correlated positively with the decrease in IGF-I gene expression (Chapter 5).

15.1.7 Impact of plant proteins (DJKM, H-JPKM and DJPI) on fish health

In modern aquaculture, haematological, biochemical and histological measurements in commercially important fish are an integral part of evaluating their health status with regard to endogenous and exogenous factors.

15.1.7.1 Clinical markers

The activity of alkaline phosphatase (ALP) and alanine transaminase (ALT) in blood is used as an indicator of liver cell condition. Goel et al. (1984) and Racicot et al. (1975) reported that the level of ALP and ALT rises in blood during acute liver damage. Similar level of ALP and ALT observed for the control and the Jatropha based protein (DJKM, H-JPKM and DJPI) fed groups, indicated that there was no organ damage or dysfunction. Alkaline phosphatase and ALT level in all groups were in the normal range reported by Zhang et al. (2009) for healthy Tibetan catfish. Our result suggests that inclusion of DJKM, H-JPKM and DJPI in fish feed does not impair liver function (Chapter 3, 6, 8, 9, 11 and 13).

An elevated level of blood urea nitrogen is related to the liver or gill dysfunction because these are the sites where urea production and excretion take place (Stoskopf, 1993). Higher blood urea nitrogen was observed in the DJKM fed groups compared with the group fed fish meal; however the values for all groups were in the normal range of (1.9-9.6 mg/dl) (Wedemeyer and Chatterton, 1970; Giorgetti and Ceschia, 1977). This suggested that the livers and gills of the fish were in a normal condition in the DJKM fed groups. According to Tietz (1986) the concentration of total bilirubin (TBIL) is also used as an indicator of liver function. In the present study, TBIL concentrations in the blood of fish fed DJKM, H-JPKM and DJPI were similar to those of fish in the control group and these values were in the normal range, suggesting that the DJKM based diets did not have any harmful effect on liver function. Creatinine level in blood increases if kidney function is impaired (Tietz, 1986). Higher creatinine was observed in the control group, but the values in all groups were within the normal range (0.2-2.17 mg/dl) (Ceschia et al., 1978). Higher creatinine is caused by the raised levels of fishmeal in the control diet.

Haematology [haematocrit, haemoglobin and Red blood cells (RBC)] values were within normal ranges for fish (Ghittino, 1983; Lie et al., 1990) and in agreement with previously published data (Rosenlund et al., 2004). One of the few unusual effects observed was a significant reduction in blood

cell size, (measured as mean cell volume, MCV) as the content of plant proteins increased (paper 3 and 8). As this observation appeared to coincide with increased spleen size, it was suggested that some of the plant ingredients may have caused early release of immature erythrocytes. It may be noted that the spleen was larger in plant protein fed groups than in those groups offered the reference diet offered (Chapter 3).

Total protein in blood is used as a basic index for health and nutritional status in fish (Martinez, 1976). Moreover, albumin and globulin concentrations and lysozyme activity play a significant role in the immune response. According to Lie et al. (1989) lysozyme is regarded as the first line of defense, with high activity in mucus, serum, gills and the alimentary tract. Feeding soybean meal has been shown to increase the level of different non-specific immune parameters in rainbow trout (Rumsey et al., 1994) and Atlantic salmon (Krogdahl et al., 2000). Higher albumin, globulin and total protein concentrations and lysozyme activity in the blood was observed for DJKM and DJPI fed groups when compared with the control group, indicating an immuno stimulating effect of plant protein on the fish. Albumin, globulin and total protein concentration in blood were within the normal range for all groups (Wedemeyer and Chatterton, 1970; Sandnes et al., 1988).

15.1.7.2 Gut morphology

In common carp fed DJKM at 75% replacement of fish meal protein mild lesions were occasionally found in intestinal mucosal tissue samples. These lesions were not specific for the DJKM since similar lesions were found in the intestinal tissues from fish fed diets containing soybean meal. In other parts of the intestinal loops no lesions were detected. The intestinal mucosa was well developed and no morphological alteration was found (Chapter 3). In another study on rainbow trout we did not observe any signs of pathological lesions after feeding DJKM (Chapter 6, *section 2*).

Detoxified Jatropha kernel meal has a higher digestibility than unheated detoxified Jatropha kernel meal due to significantly reduced levels of trypsin inhibitors and indigestible oligosaccharides. The effects of phytate were also lessened by treating the feed with phytase. Diets containing DJKM are therefore unlikely to precipitate any histopathological changes in the intestine of common carp or rainbow trout. This assertion is supported by the results of Refstie et al. (2006a,b) which showed that an inclusion of 24%

bioprocessed soybean meal did not cause any alterations in intestinal morphology.

In the present study, all blood related parameters are within the normal range reported for healthy fish (Barham et al., 1980; Sandnes et al., 1988; Zhang et al., 2009). These results indicate that the Jatropha based protein (DJKM, H-JPKM and DJPI) can be used safely as a standard ingredient in aqua feed.

15.2 Effects of Jatropha-phytate on growth and blood chemistry in Nile tilapia

Phytate in its natural form is found in the seeds of many plant species. Purified phytate in the form of phytic acid or its salt sodium phytate is not digested by fish. Natural phytates in plant ingredients are complex and their functional properties are dependent on their structure so we isolated phytate from Jatropha kernel meal in order to evaluate its effects on Nile tilapia. The effects of adding the enzyme phytase to neutralise the negative effects of Jatropha-phytate were also investigated.

15.2.1 Growth and nutrient utilization

The poorer growth performance and nutrient utilization observed in phytate fed groups compared with phytate with phytase ($PP_{1.5+phytase}$ and $PP_{3+phytase}$) groups and the control (Chapter 14) are in accordance with those of other researchers (Spinelli et al., 1983; Richardson et al., 1985; Usmani and Jafri, 2002; Sajjadi and Carter, 2004; Laining et al., 2010). Phytate chelates di- and trivalent mineral ions such as Ca^{2+} (Fredlund et al., 2006), Mg^{2+} and Zn^{2+} (Denstadli et al., 2006), Cu^{2+} and Fe^{3+} so they become less available for fish growth (Duffus and Duffus, 1991). In the presence of phytate and added calcium, absorption of other minerals was depressed due to the formation of insoluble complexes (Sandberg et al., 1993).

Supplementation of phytate-containing diets with phytase neutralised the negative effects of phytate and improved growth and feed utilization in fish (Chapter 14). Significantly higher digestibilities of protein and lipid were observed in fish fed the phytate with phytase diets compared with those of groups fed phytate without phytase (Chapter 14). The digestibilities of protein and lipid were generally high (in the range of 81-94%), as expected because highly digestible feed ingredients such as casein and gelatin were used. The protein and lipid digestibilities ranged from 81%

to 91% and 84% to 94% respectively; protein and lipid digestibility in fish fed diets with 15 g phytate kg^{-1} was significantly higher than in fish fed diets with 30 g phytate kg^{-1} (Chapter 14). The digestibilities of protein and lipid decreased significantly as the inclusion of phytate increased (Chapter 14). Interactions of phytate with proteins are dependent on pH (Cheryan, 1980) because the phytate molecule is polyanionic with a phosphoric acid group moiety. In acidic environments such as the Nile tilapia stomach (pH 1-2), half of the phosphate moieties are negatively charged. This favours binding of soluble proteins at amino-groups on lysine, imidazole groups on histidine and guanidyl groups on arginine. In an alkaline environment such as the Nile tilapia intestine, (pH 8.5 - 8.8), ternary complexes are favoured. Both complexes are resistant to proteolytic digestion (Riche and Garling, 2004). When phytate reacts with minerals and other nutrients, the complexes formed in the intestine are insoluble and it is highly unlikely that they provide absorbable essential elements. All these factors appear to lower protein and lipid digestibilities. Supplementation with phytase overcomes the adverse effects of phytate and leads to increased digestibility of phytate containing diets. Formation of sparingly digestible phytate–protein complexes was found to be the main reason for depression of growth in Nile tilapia.

In the present study, the negative effect of phytate on protein and lipid digestibility was counteracted by the addition of 1500 FTU per kg phytase (Chapter 14). The effects of phytase on digestibility may depend on a variety of dietary factors such as source and concentration of phytate in the diet, source and concentration of protein in the diet, digestibility of protein source, mineral levels, calcium and P levels and level of phytase inclusion (Sugiura et al., 2001).

15.2.2 Blood chemistry

In the present study we found that phytate decreased the RBC count, hemoglobin concentration and hematocrit levels of the fish in the phytate containing groups and that these parameters were improved on addition of phytase (Chapter 14). Hallberg et al. (1989) also found that the hemoglobin and hematocrit decreased in the presence of dietary sodium phytate. The lower hemeoglobin and hematocrit levels in Nile tilapia fed phytate containing diets without phytase could be attributed to the binding of phytic acid molecules to minerals (iron) and/or the amine groups of amino acids. This would reduce the availability of these molecules for biosynthesis of

erythrocytes causing these cells to become more fragile (Sun et al., 1995). As phytate content was increased in the experimental diets from 1.5 to 3 %, the levels of albumin, globulin and total protein in blood also increased (Chapter 14). In the present study higher lysoyzme activity in serum was observed in the phytase treated groups compared with $PWP_{1.5}$ and PWP_3 (phytate without phyatse) groups which indicates that hydrolysed phytates such as free inositol phosphate have an immunostimulating effect in fish (Cavazza, 2003).

Phytate apparently decreased blood glucose level, while phytase addition increased it, indicating that phytate can be a key element in modulating insulin secretion by diminishing the production of insulin. The real mechanism of action is not fully understood but it seems that phytate regulates insulin secretion via its effect on calcium channel activity because it specifically inhibits serine/threonine protein phosphatase activity (Barker and Berggren, 1999; Kumar et al., 2010). Phytate decreased cholesterol and triglyceride levels in plasma, while phytase addition increased it, indicating that phytate may interfere with lipid metabolism. Dietary phytate in mice and chickens has also been shown to decrease the concentrations of cholesterol in the serum and liver; and the digestibility of lipid and cholesterol (Lee et al., 2007; Liu et al., 2010). The mechanism by which phytate lowers cholesterol remains to be clarified but it is assumed that it is partly related to an increase in faecal bile acid and lipid excretion (Lee et al., 2007) and a reduction in hepatic cholesterol synthesis.

In this study, the electrolytes (P and Ca) concentrations in blood were significantly influenced by dietary phytate and phytase, indicating that dietary factors exert a substantial influence on the electrolyte metabolism. The concentrations of Ca and P in blood were significantly decreased by dietary phytate compared with groups containing phyatse (Chapter 14). Sodium and K concentration in the blood were not influenced by phytate and phytase supplementation in the diets (Chapter 14). Phytase supplementation significantly increased the blood electrolytes (P and Ca) contrast to the phytate without phytase fed groups. Increase in phytate content in the diet decreased P and Ca concentrations in blood. These results are consistent with those of Laining et al. (2010). They observed that more than 13 g IP6 per kg diet significantly decreases the plasma P and Ca level in Japanese Flounder, *Paralichthys olivaceus*. Much evidence (Sugiura et al., 2001; Vielma et al., 2004; Sajjadi and Carter 2004; Liebert and Portz, 2005) suggest that positive effects of phytase supplementation on minerals (P, Ca, Cu, Mg, Fe, Sr and Zn) availability in fish. Supplementation with phytase

hydrolyses phytate and significantly increases the concentration of Ca, P, Mg, and Mn in the plasma of fish (Liebert and Portz, 2005; Cao et al., 2008). Similar trend was also observed in the current study, supplementation of phytase in diets containing phytate exhibited higher concentration of Ca and P in blood of fish compared non-supplemented diets.

15.3 Guidelines for using detoxified kernel meal and detoxified protein isolate from *Jatropha curcas* as a protein source in fish feed

Based on our studies the use of detoxified DJKM and DJPI in fish and shrimp aquaculture is likely to increase substantially in the future. The quality of DJKM and DJPI should be maintained. Recommended qualities of DJKM and DJPI are listed below in Table 11.

Table 11: Recommended quality measurements for detoxified Jatropha kernel meal and detoxified Jatropha protein isolate

	Detoxified Jatropha kernel meal	Detoxified Jatropha protein isolate
Protein (%), minimum	60-66	81-88
Fat (%), minimum	0.9-1.2	0.8 -1.0
Fibre (%), maximum	8-9	1
Ash (%), less than	9 – 11%	2-3%
Gross energy (KJ/g)	18.5	21.4
Moisture (%), maximum	6-8	4-6
Non-starch polysachharides (%), maximum	16	10
Lysine, higher than	2.3%	2.5%
Available lysine	Near 100%	Near 100%
Pepsin plus trypsin digestibility (% of total nitrogen)	> 92.0	> 97.0
Protein dispersibility index	15 – 40%	-
Trypsin inhibitor (mg trypsin inhibited per g sample)	Not detected	Not detectable
Lectin activity[a]	Not detected	Not detectable
Texture	Homogenous, free flowing, no lumps, not dusty	Homogenous, free flowing, no lumps, not dusty

Taste	Bland	Bland
Contaminants	Free of PEs	Free of PEs
	Free of urea	Free of urea
	Free of ammonia	Free of ammonia
	Free of mycotoxins and mold	Free of mycotoxins and mold

[a:]Based on haem-agglutination: PEs, phorbol esters (sensitivity: < 3 µg/g material)

15.3.1 Feeding guidelines for fish and shrimp

Detoxified Jatropha kernel meal (DJKM), H-JPKM and DJPI could replace 50%, 62.5% and 75% fish meal protein respectively in fish diets, without sacrificing growth and nutrient utilization; and without affecting physiological and haematological parameters. Interestingly, in shrimp when 50% fish meal protein replaced by DJKM exhibited higher growth and nutrient utilization parameters compared to control group. Jatropha based protein sources (DJKM, H-JPKM and DJPI) can be fed to most classes of fish and shrimps, and feeding guidelines need to be followed if optimum feed efficiency and fish and shrimp performance are to be achieved. The guidelines described below would increase the efficiency of feeding DJKM, H-JPKM and DJPI to fish and shrimp.

- Take into account that DJKM and H-JPKM both contain about 65% crude protein which is similar to the level in fish meal and can therefore substitute for fish meal on an equal weight basis.
- Detoxified Jatropha protein isolate contains about 81-88% crude protein which is much higher than fish meal (contains 65–70% crude protein), so addition of some filler (e.g. cellulose) in the diets is required.
- The palatability of DJKM, H-JPKM and DJPI based diets is similar to fish meal based diet.
- The DJKM, H-JPKM and DJPI are deficient in lysine therefore lysine monohydrochloride should be supplemented at the level of 1.5% of the DJKM, H-JPKM and DJPI (w/w) inclusion in the diet to equalize the lysine level.
- The DJKM and H-JPKM contain approximately 9-10% phytate which is almost 3 times more than that in soybean meal. Phytate is reported to lower the bioavailability of minerals and to reduce protein digestibility. These problems can be overcome by addition of

exogenous phytase to hydrolyse phytate. Add 1500 FTU phytase per kg of diet.

- Detoxified Jatropha kernel meal, H-JPKM and DJPI based diets should be fed to fish at 5 times maintenance requirements. One time maintenance requirement equals 3.2 g feed per kg metabolic body mass ($kg^{0.8}$) per day. Shrimp (juveniles, >10 g) should be fed 3-4% of the total body weight per day.

15.4 Potential benefits of DJKM and DJPI addition in fish and shrimp diets

- Detoxified Jatropha kernel meal and DJPI are especially valuable in feeding situations that require high protein levels (e.g. fish and shrimp diets). They can replace substantial amounts (up to 75%) of fishmeal in the diets of fish and shrimp.
- Detoxified Jatropha kernel meal and DJPI are an excellent source of nutrients; 60 to 66 % and 81-87% crude protein respectively. The levels of essential amino acids (except lysine) are higher in DJKM and DJPI than in soybean meal and soy protein isolate respectively and the palatability of DJKM and DJPI based diets is similar to fish meal based diets, indicating that DJKM and DJPI are good quality protein sources.
- The use of DJKM and DJPI will lower the need for soy meal and other plant protein sources (cotton seed meal, pea meal, copra meal, sun flower cake etc.) in fish and shrimp diets.
- These Jatropha derived products can be obtained from Jatropha planted in degraded lands, thus decreasing food-feed competition.

15.5 Potential problems of using DJKM and DJPI in fish and shrimp diets

- Inadequately heated Jatropha kernel meal that contains significant amounts of trypsin inhibitor and lectin will reduce the performance of fish and shrimp. Similarly, inadequately detoxified material will contain phorbol esters. Phorbol esters must be below the detectable limit (<3μg/g meal).
- Overheating Jatropha kernel meal will increase the portion of 'bound protein', which cannot be digested by fish or shrimp. A lab measurement of ADIN (acid detergent insoluble nitrogen) could be used as an indicator of the extent of bound protein.

- Incorporating a high level of DJKM into a diet requires rebalancing the ingredients in order to maintain a proper ratio of energy to other nutrients, especially protein.
- Fish and shrimp fed high levels of plant protein such as DJKM or DJPI will deposit more fat in their fish muscles, which could be more unsaturated and hence more susceptible to oxidation.

15.6 Environmental considerations

Detoxified Jatropha kernel meal and DJPI contain almost three times the phytate content of soybean meal and soy protein isolate respectively. Detoxified Jatropha kernel meal based feeds have higher phosphorus concentrations than traditional feeds. Feeding large quantities of these feeds increases the amount of phosphorus excreted by the fish and shrimp. In any given fish farming enterprise, this excess phosphorus would increase the acreage needed for spreading waste in order to comply with waste management regulations, which could potentially limit future expansion. To minimize these potential problems, it is suggested that supplemental phosphorus should not be included in the diet when DJKM based feeds containing phytase are fed. These diets would provide adequate phosphorus and meet the requirements as per the National Research Council (NRC) recommendations without further supplementation. Numerous research trials have demonstrated that feeding excess phosphorus does not improve the reproduction efficiency or health of fish or shrimps. When phosphorus is fed in excess of NRC recommendations, additional calcium may be required to maintain normal calcium-phosphorus ratios in the diet. Producers and their nutritionists may need to consider limiting the amount of mineral premix in DJKM based feeds to maintain phosphorus balance and to comply with nutrient management regulations.

15.7 Risk and additional responsibilities

The producer assumes complete responsibility for balancing rations to support a desired level of fish and shrimp growth and health when DJKM is one of the ingredients. Also, the producer assumes the responsibility for quality control including screening for any residual toxic components such as phorbol esters. Even though phorbol esters are removed from Jatropha kernel meal and protein isolate by the detoxification method, their presence in fish muscle must be monitored, at least in the initial phase of using

detoxified meal and detoxified protein isolate until it is absolutely certain that phorbol esters are not present in the muscle tissues.

15.8 Conclusions

As aquaculture continues to develop, there will be an increasing need to use alternative plant proteins in aquaculture diets so that aqua eco-systems will be sustainable. Jatropha (DJKM, H-JPKM and DJPI) can be used as protein rich sources in the diets of fish and shrimp.

There is a high potential for the safe use of DJKM, H-JPKM and DJPI in diets for fish and shrimp without compromising performance, provided that these ingredients are free of toxic factors. The detoxification process developed in Hohenheim is simple and robust and produces products that are safe and of good quality. Their addition to fish and shrimp diets gave excellent performance responses without any ill effects on animal health or safety.

Effects on growth and nutrient utilization:

- Detoxified Jatropha kernel meal, H-JPKM and DJPI could replace 50%, 62.5% and 75% fish meal protein respectively without compromising growth performance and nutrient utilization in fish. In addition, DJKM could also replace 50% fish meal protein with no adverse effects on growth and nutrient utilization in shrimp. If the replacement levels are exceeded, the producer must examine the nutrient profile of the feeds carefully to ensure that desired production levels can be achieved and fish and shrimp health maintained.
- High inclusion (>50% fish meal protein replacement) of DJKM decreased the efficiency of conversion of feed to body mass. This could be explained partly by the increased mean feed intake which was possibly a reaction to the reduced protein retention, measured as protein efficiency ratio and protein productive value. No such effects were seen with the use of DJPI in common carp diets.
- Increased DJKM inclusion in diets caused a significant lowering of protein, lipid and energy digestibilities. No such effects were seen when DJPI was used in common carp diets.

Effects on energy budget:

- Feeding DJKM and H-JPKM to common carp and Nile tilapia respectively did not change the major components of the energy budget (routine metabolic rate, heat released and metabolisable energy) compared to fish meal and soybean meal fed groups. These results revealed that dietary protein sources DJKM and H-JPKM can be efficiently utilized for growth by common carp and Nile tilapia respectively, as well as soybean meal and fish meal.

Effects on expression of growth hormone and insulin-like growth factor-1 encoding genes

- As the level of DJKM inclusion increased in the common carp diet, growth rate decreased. The expression of Insulin-like growth factor-1 (IGF-1) in liver also decreased with increase of DJKM in the diet and that of the growth hormone in liver decreased.

Effects on clinical health parameters and gut health:

- No mortality and unaffected haematological values suggested the fish were all in normal health. Alkaline phosphatase and ALT activities; urea nitrogen, bilirubin and creatinine concentration in blood were in the normal ranges which showed that there was no liver or kidney dysfunction.
- The measured plasma nutrient levels gave no indications of stress, but increasing the level of plant protein in the diet decreased plasma cholesterol. This may be related to high NSP content or reduced dietary intake of cholesterol. Decrease in muscle cholesterol level is also expected which could be considered good for human health.
- Histopathological evaluation of organs showed no damage to the stomach, intestine or liver of common carp or rainbow trout.

Effects of Jatropha-phytate in Nile tilapia

The defatted Jatropha kernel meal obtained after oil extraction is rich in protein (58−66%) and phytate (9 −11%). The phytate rich fraction was isolated from defatted kernel meal using organic solvents (acetone and carbon tetrachloride). It had 66% phytate and 22% crude protein and its inclusion in fish diets showed the following:

- Negative effects on growth performance, nutrient utilization and digestive physiology (nutrient digestibility and digestive enzymes).
- Adverse influences on biochemical entities such as metabolic enzymes (alkaline phosphatase and alanine transaminase) and electrolytes/metabolites. Salient changes include decreased red blood cell count and hematocrit content, decreased cholesterol and triglyceride concentrations in plasma and decreased blood glucose levels. The adverse effects observed may be due to the interaction of phytate with minerals and enzymes in the gastro intestinal tract, resulting in poor bioavailability of minerals and lower nutrient digestibility.

The level of phytate used in the present study (1.5 and 3.0%) corresponds to 16.5% and 33.0% of DJKM in the fish diet. The DJKM at levels > 16.5% in the diet would exhibit adverse effects in Nile tilapia. Addition of phytase to the phytate containing diets would mitigate the adverse effects of at least up to 3% Jatropha phytate (or 33% DJKM) in the diet. Addition of phytase (1500 FTU/kg) in diets containing DJKM is recommended to maximize their utilization by Nile tilapia.

15.9 Future perspectives

15.9.1 How to improve the growth of fish using Jatropha meal and protein isolate?

The studies reported in this thesis have shown that excellent opportunities exist for replacing fish meal with plant protein sources in the diets of common carp, Nile tilapia, rainbow trout and white leg shrimp. Supplements derived from DJKM, H-JPKM and DJPI were regarded as some of the best choices of plant proteins. Their use resulted in no reduction in the growth of fish or shrimp when replacing 50%, 62.5% or 75% of the fish meal protein in the original diets. Supplementing diets with crystalline lysine did not improve the growth of fish which reveals that there is more to plant proteins than protein content. Also the balance between the different amino acids was found to be important for growth performance. When we replace fish meal, we not only replace proteins and amino acids but also many other compounds. Therefore in the future it will be essential to balance all essential nutrients in any proposed new diet or supplement. This approach has already proved promising in trials with fish and shrimp. Further studies

on the utilization of DJKM and DJPI in other fish and shrimp species during long term feeding trials at farm level are required.

15.9.2 Is the use of *Jatropha curcas* kernel meal and isolate in aqua feed sustainable?

Jatropha curcas has spread beyond its original distribution because of its hardiness, its ease of propagation, its drought resistance, high oil content, low seed cost, short gestation period, and rapid growth. It has adapted to a wide range of agro-climatic conditions. It grows typically as a bush or shrub and many uses have been found for the various parts of the plant (King et al., 2009). The Jatropha kernel meal and protein isolate are excellent sources of protein. They contain 58 to 66 % and 81-85% crude protein respectively. The levels of essential amino acids (except lysine) in these resources are higher than in soybean meal or soy protein. The International Jatropha Organization has predicted that in 2017 there will be around 32.72 million hectares of land cultivated worldwide with Jatropha will produce 160 mt of seeds and that 95% of this will be in Asia (mostly in China and India) (Siang, 2009). The total projected annual Jatropha oil production in Asian countries will be ~ 47 mt by 2017 (Siang, 2009). With its present genetic makeup the Jatropha plant can yield up to 5 tons of seed per year from one hectare of plantation which in turn yields approximately one ton of protein rich kernel meal (Makkar and Becker, 1997). This means that there is a possibility of producing enough Jatropha kernel meal and protein isolate to meet the growing demands of the aquaculture industry.

Soybean meal is currently the most commonly used plant protein source in fish feeds (Yue and Zhou, 2009). The price of soybean meal has increased sharply (Azaza et al., 2009). The price of soybean meal increased by about 20% from September 2010 (Soybean meal monthly price, http://www.indexmundi.com/commodities/?commodity=soybean-meal). Based on the current supply and demand forecasts for the coming years, prices can be expected to remain high. The high cost of protein sources, their restricted availability and the unpredictability of their markets, increase the need for alternative sources of protein in fish feed. We expect that the market price of DJKM would be at least equivalent to soybean meal. Detoxified Jatropha kernel meal contains about 20% higher crude protein than soybean meal (65% crude protein in DJKM and 45% crude protein in soybean meal), and the value of this extra 20% of protein would be sufficient to meet the cost of detoxifying of Jatropha kernel meal.

The results reported in this thesis enlarge the portfolio of Jatropha based ingredients (DJKM, H-JPKM and DJPI) that can be used as good quality of protein source in aquafeed, and opens a new market opportunity for the use of a new feed resource in the aquafeed industry.

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