UNIVERSITÄT HOHENHEIM

Isolation, characterization and potential agro-pharmaceutical applications of phorbol esters from *Jatropha curcas* oil

Dissertation

"Doktor der Agrarwissenschaften"
(Dr. Sc. Agr. / Ph.D. in Agricultural Sciences)

Specialization: Agriculture and Environmental Biochemistry/Toxicology

Rakshit Devappa Kodekalra

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University of Hohenheim, Stuttgart, Germany

Department of Aquaculture and Animal Nutrition (480b)

Prof. Dr. Klaus Becker

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Examination committee:

Supervisor and Reviewer: Prof. Dr. Klaus Becker

Co-Supervisor and Reviewer: Prof. Dr. Harinder P.S. Makkar

Additional Examiner: Prof. Dr. Martin Mittelbach

Vice-Dean and Head of the Committee: Prof. Dr. Harald Grethe

Dedicated

To

My

Family, Friends and Mentors

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Declaration

I hereby declare that this work, submitted to the Faculty of Agricultural Sciences, University of Hohenheim, Stuttgart, for the award of Dr. Sc. Agr. is the result of original work carried out by myself under the supervision of Prof. Dr. Klaus Becker and co-supervision of Prof. Harinder P.S. Makkar. 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, 9th February, 2012 Rakshit Devappa Kodekalra

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List of Abbreviations

AV	Anisidine value
BHA	Butylated hydroxy anisole
BHT	Butylhydroxytoluene
CA	Chromic acid
cAMP	3'-5'-cyclic adenosine monophosphate
CV	Crystal violet
DAG	Di acyl glycerol
DCM	Dichloromethane
DHPB	12-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0]hexane-(13-O)-2'-[carboxylate]-(16-O)-3'-[8'-butenoic-10']ate
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration (50%)
ED_{50}	Effective dose (50%)
FAC	Fatty acid composition
FCE	Food conversion efficiency
HAART	Highly Active Anti-Retroviral Therapy
HCE	Human corneal epithelium
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IL-1α	Inter leukin-1α
IP_3	Inositol triphosphate
LC_{50}	Lethal concentration (50%)
LD_{50}	Lethal dose (50%)
MCA	3-methylcholanthrene
MIC	Minimum inhibitory concentration
NMR	Nuclear magnetic resonance
PE	Phorbol ester

PEEF	Phorbol ester enriched fraction
PEs	Phorbol esters
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PV	Peroxide value
RCR	Relative consumption rate
RGR	Relative growth rate
RHE	Reconstructed human epidermis
RT	Room temperature
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
TBHQ	Tertiary butylhydroquinone
THF	Tetra hydro furan
TIA	Trypsin inhibitor activity
TLC	Thin layer chromatography
TPA	12-*O*-tetradecanoylphorbol-13-acetate
VAZO-67	2, 2′-Azobis-(2-methyl-butyronitril)

Summary

Biodiesel is generally prepared from renewable biological sources such as vegetable oils by transesterification. *Jatropha curcas* seed oil is a promising feedstock for biodiesel production. During biodiesel production from Jatropha oil, many co-products such as glycerol, fatty acid distillate and seed cake, among others, are obtained. The efficient use of these co-products would enhance the economic viability of the Jatropha based biofuel industry. However, the possible presence of phorbol esters (PEs) in these co-products restricts their efficient utilization. During biodiesel production, Jatropha oil is subjected to many treatments (stripping, degumming and esterification) wherein PEs present in the oil undergo partial or complete destruction depending on the treatment conditions. One of aims of this study was to develop and integrate methodologies for using the PEs as a value added product instead of simply allowing them to be destroyed during biodiesel production. Potential uses of the phorbol ester enriched fraction (PEEF), obtained from Jatropha oil in agro-pharmaceutical applications were also investigated. The reason for choosing this group of compounds (PEs) was that they are highly bioactive both *in vitro* and *in vivo*, but they are currently considered to be merely toxic, unwanted biomaterial in the Jatropha biodiesel production chain. The recent increase in the cultivation of Jatropha means that there are potentially huge quantities of PEs that could be used for many purposes.

This study revealed that a large proportion (85.7%) of PEs was localized in the endosperm portion of the Jatropha seed. Interestingly, the kernel coat contained PEs in high concentration. The endosperm portion of the kernel also contained antinutritional factors such as phytate (96.5%) and trypsin inhibitor (95.3%). The presence of high levels of antinutritional/toxic components in the kernel was presumed to be one of the factors that protect Jatropha seeds against predatory organisms during post harvest storage.

Based on the presence or absence of PEs, a qualitative method was developed to differentiate between toxic/nontoxic Jatropha genotypes. In this method the methanol extract of seeds is passed through a solid phase extraction (SPE) column and the absorption (280 nm) of the resulting eluate is measured. After screening Jatropha seeds collected from different parts of

the world for toxic and non-toxic genotypes using the pre-established HPLC method for PEs, a cut off value of the absorbance was set up to differentiate toxic and nontoxic genotypes. Raw kernels whose SPE eluates had an absorbance ≥0.056 were considered as toxic and ≤0.032 as nontoxic. Corresponding absorbance for the SPE eluates of defatted kernel meal were ≥0.059 (toxic) and ≤0.043 (nontoxic). However, confirmation of the presence of PEs especially in Jatropha products for food applications should be carried out using the pre-established and validated HPLC method. The developed qualitative method could find its applications for screening the toxicity of products and co-products obtained from the Jatropha biodiesel industry.

Conditions were optimized for the extraction of PEs as a phorbol ester enriched fraction (PEEF) from Jatropha oil using methanol as a solvent and a magnetic stirrer/Ultra-turrax as extraction tools. The extent of PE reduction in Jatropha oil was >99.4% using methanol as the solvent. The PEEF obtained (48.4 mg PEs/g) was 14 fold higher in PEs than in the original oil and this fraction was highly bioactive as determined by the most sensitive snail bioassay (LC_{100}, 1 ppm) (see below). As the removal of PEs from oil took 60 min, which might be considered a long time in an industrial process, further conditions were optimized to extract maximum PEs in the shortest possible time with minimum solvent. The tools used for PE extraction (Ultra-turrax and magnetic stirrer) were effective with a treatment time of 2 and 5 min, resulting in 80 and 78% extraction of PEs, respectively. The biodiesel prepared from both the residual oils met European (EN 14214:2008) and American biodiesel standard (ASTM D6751-09) specifications. It was evident from the study that PEs could be easily extracted by either of the two methods with a high yield and the residual oil could be processed to produce high quality biodiesel. Also the residual oil with a lower PE content is expected neither to harm the environment nor the workers who had to handle it.

The extracted PEEF was evaluated for its agricultural potential as a bio control agent. The PEEF had a high biological activity in aquatic bioassays using snails (*Physa fontinalis*), brine shrimp (*Artemeia salina*) and daphnia (*Daphnia magna*), when compared with microorganisms. The EC_{50} (48 h) of the PEEF was 0.33, 26.48 and 0.95 ppm PEs for snail, brine shrimp and daphnia respectively. High MIC (minimum inhibitory concentration) values (≥215 ppm)

and EC_{50} values (≥58 ppm) were obtained for both the bacterial and fungal species. Among the bioassays tested, the snail bioassay was the most sensitive, producing LC_{100} at 1 µg of PEs/ml. The snail bioassay could be used to monitor the presence of PEs in various Jatropha derived products, contaminated soil and other matrices in the ecosystem that might be involved in the production or use of Jatropha and its products. The study also demonstrated that the PEs exhibit moluscicidal, antifungal and antibacterial activities.

The shelf life of the PEEF was investigated. The PEEF was more susceptible to degradation when stored at room temperature (50% degradation after 132 days) than when stored at 4 °C or -80 °C (9% and 4% degradation respectively). Similarly, the PEEF lost biological activity (the snail bioassay) more rapidly at room temperature becoming ineffective after 260 days; while at 4 °C and -80 °C, only 27.5% and 32.5% bio activity was retained after 870 days. The degradation of PEs was due to auto-oxidation. Changes in fatty acid composition increase in peroxide value and decrease in free radical scavenging activity of the PEEF reflected the auto-oxidation. Inclusion of antioxidants as additives (butylated hydroxyanisole (BHA), baynox and α-tocopherol) protected the PEs against degradation. The study demonstrated that the PEEF was susceptible to oxidation and addition of antioxidant stabilised the PEs during storage.

In soil, PEs present in both the PEEF (2.6 mg/g soil mixture) (silica was used to adsorb PEs) and Jatropha seed cake (0.37 mg/g soil mixture) were completely degraded as the temperature and moisture content of the soil increased. PEs from silica-bound PEEF were completely degraded after 19, 12, 12 days (at 13% moisture) and after 17, 9, 9 days (at 23% moisture) at room temperature (22−23°C), 32 °C and 42 °C respectively. Similarly, at these temperatures, PEs from seed cake were degraded after 21, 17 and 17 days (at 13% moisture) and after 23, 17, and 15 days (at 23% moisture). The toxicity of PE-amended soil extracts when tested using the snail bioassay decreased with the decrease in PE concentration. The study demonstrated that PEs present in the PEEF or Jatropha seed cake are completely biodegradable in soil and the degraded products are innocuous.

Summary / Zusammenfassung

In preliminary studies, the PEEF exhibited potent insecticidal activity against *Spodoptera frugiperda*, which is a common pest in corn fields damaging maize crop across the tropical/subtropical countries such as Mexico and Brazil. The PEEF produced contact toxicity with an LC_{50} of 0.83 mg/ml (w/v). The PEEF at higher concentration (0.25 mg/ml, w/v) also reduced food consumption, relative growth rate and food conversion efficiency (FCE) by 33%, 42% and 38% respectively. The study demonstrated that the PEEF has a potential to be used as a bio-control agent. Further in-depth field experiments on the effects of the PEEF on *S. frugiperda* will pave the way for its use under field conditions.

The pharmaceutical potential of Jatropha PEs was also investigated. The PEs from Jatropha oil was purified. At least six purified PEs (designated as factors C_1 to C_6) were present in Jatropha oil. The identities of the purified PEs (factors C_1 and C_2) were confirmed by NMR. Whereas, factor C_3 and factors (C_4 + C_5) were both obtained as mixtures. However, comparison of peak areas for phorbol 12-myristate 13-acetate (PMA) and Jatropha factor C_1 in the HPLC method showed a difference in sensitivity of absorption at 280 nm of 41.3 fold. All the individual purified Jatropha PEs (factors C_1, C_2, $C_{3mixture}$ and (C_4+C_5)) and PEs-rich extract (factors C_1 to (C_4 + C_5)) were biologically active when tested in the snail and brine shrimp bioassays. In addition, all the Jatropha PEs produced platelet aggregation *in vitro* with an effective order of (based on ED_{50} (μM)): Jatropha factor C_2 < factor $C_{3mixture}$ < factor C_1 < factor (C_4+C_5). The PEs-rich extract (contains factor C_1 to C_6) was toxic to mice upon intra gastric administration, with an LD_{50} of 27.34 mg/kg body mass as PMA equivalent or 0.66 mg/kg body mass as factor C_1 equivalent. The prominent histopathological symptoms were observed in lung and kidney.

The Jatropha purified PEs-rich extract, purified PEs (factor C_1, factor C_2, factor $C_{3mixture}$ and factors (C_4+C_5)) and toxic Jatropha oil produced severe cellular alterations/disintegration of the epithelium and also increased the inflammatory response (interleukin-1α and prostaglandin E_2 release) when applied topically to reconstituted human epithelium (RHE) and human corneal epithelium (HCE). In RHE, the nontoxic oil (equivalent to the volume used for toxic oil) produced a lower cellular and inflammatory response than the toxic oil and the response increased with an increase in concentration of the PEs. In HCE, nontoxic oil

(equivalent to the volume used for toxic oil) produced marked cellular alterations. The study demonstrated that the presence of PEs in Jatropha oil increased the toxicity, both towards RHE and HCE. In addition, all the purified Jatropha PEs gave positive responses in the tumour promotion assay and negative responses in the tumour initiation assay *in vitro* (the assay was based on foci formation in Bhas 42 cells). In the tumour promotion assay, the order of transformed foci/well formation was: PEs-rich extract > factor (C_4+C_5) > factor $C_{3mixture}$ > factor C_1 > factor C_2. The tumour promotion activity was mediated by the hyper activation of protein kinase C (PKC). The aforementioned studies demonstrated that Jatropha PEs are toxic when administered orally or when applied topically to the skin or eye tissues. The data obtained should help in establishing safety measures for people working with Jatropha PEs.

The potential of Jatropha PEs as a feedstock intermediate for the synthesis of Prostratin, a promising adjuvant in anti HIV therapy, was evaluated. The studies demonstrated that the Jatropha PEs could be synthesized sequentially by converting them first to crotophorbolone and then to prostratin. As analyzed by Nano-LC-ESI-MS/MSR, the prostratin synthesized from Jatropha PEs had similar mass and peak retention time to the reference prostratin (Sigma, St. Louis), The study showed that prostratin could be synthesized from Jatropha PEs. However, further optimization studies are required to ascertain the synthesis reactions and yield of prostratin synthesized from Jatropha PEs.

Some of the preliminary requirements for any successful bio-control agent are that it should have a high bioactivity on the target organism, a long shelf-life and a high biodegradability in soil. In addition, the bioactive phytochemical should be available in large quantities; it should be easily extractable and continuously available. The PEEF potentially satisfies these aforesaid requirements. The abundance and novelty of PEs present in Jatropha species could form a new ‘stock’ for the agro-pharmaceutical industries. Considering the projected oil yield of 26 million tons/annum by 2015 (GEXSI, 2008), huge amount of raw materials will be available for both biodiesel and pharmaceutical industries. PEs in the form of the PEEF could be used either as insect controlling agents in agricultural applications or as a ‘stock’ biomaterial for synthesizing prostratin in pharmaceutical applications.

Zusammenfassung

Biodiesel wird herkömmlich durch die Transesterifikation erneuerbarer biologischen Quellen, wie beispielsweise pflanzliche Öle, hergestellt. Das aus Samen von *Jatropha Curcas* gewonnene Öl ist eine vielversprechende alternative für die Biodieselproduktion. In der Herstellung des Biodiesels aus Jatrophaöl fallen viele brauchbare Nebenprodukte, unter anderem Glycerin, Fettsäuredestillate und Pressskuchen, an. Der sinnvolle Einsatz dieser Nebenprodukte könnte die Wirtschaftlichkeit der auf Jatropha Biodiesel basierenden Branche steigern. Allerdings verhindert das potentielle Vorkommen von Phorbolestern (PEs) in diesen Nebenprodukten deren effektiven Einsatz.

In der Biodieselproduktion wird das Jatrophaöl verschiedensten Behandlungen (Ausgasen flüchtiger Stoffe, Degummierung und Veresterung) ausgesetzt, wobei die im Öl vorkommenden PEs teilweise bis vollständig zerstört werden. Dies variiert je nach den vorherrschenden Bedingungen während der Behandlung. Eins der Ziele dieser Untersuchung war es, Methoden zu entwickeln, Verwendungen für die PEs zu finden und diese in die Produktionskette zu integrieren, anstelle zuzulassen dass sie ungenutzt bleiben bzw. sogar als Abfallstoffe übrig bleiben. Potentielle Einsatzmöglichkeiten der Phorbolesterangereicherten Fraktion (PEAF), gewonnen aus Jatrophaöl von agro-pharmazeutischen Produkte, wurden ebenfalls untersucht. Das große Interesse an den PEs rührt daher, dass sie sowohl *in vitro* als auch *in vivo* als hoch bioreaktiv einzustufen sind. Jedoch werden sie momentan nur als toxisches und unerwünschtes Nebenprodukt der Biodieselproduktion gesehen. Der starke Anstieg der Jatropha Kultivierung und deren Anbau lässt auf eine potentiell große verfügbare Menge an PEs schließen, welche vielseitig eingesetzt werden könnten.

Diese Untersuchung zeigte, dass ein großer Anteil (85,7 %) der PEs in dem Endosperm der Jatropha Samen vorkommen. Interessant ist außerdem, dass die Samenhaut auch eine beachtliche Menge an PEs enthält. Das Endosperm des Kerns beinhaltet zusätzlich große Anteile verschiedenster antinutritiver Substanzen wie Phytat (96,5 %) und Trypsin Inhibitoren (95,3 %). Es wird angenommen, dass das Vorkommen dieser antinutritiven, bzw.

toxischen, Substanzen einer der Eigenschaften der Jatropha Kerne ist, welche sie gegen Fraßfeinde während der Lagerung schützt.

Eine qualitative Methode um zwischen toxischen und nicht toxischen Jatropha Genotypen zu unterscheiden, wurde entwickelt basierend auf dem Vorkommen bzw. dem Fehlen von PEs. Hierbei durchläuft ein Methanolextrakt der Kerne eine Festphasenextraktionssäule (SPE). Die Absorption des Eluats wird dann bei 280 nm bestimmt. Zunächst wurden Jatrophakerne aus verschiedensten Teilen der Welt auf deren Toxizität anhand der bestehenden HPLC Methode für PEs geprüft. Dann wurde ein Grenzwert auf Basis derer Absorptionen definiert um nicht toxische von toxischen Arten zu unterscheiden. Unbehandelte Kerne deren SPE Eluate eine Absorption von ≥ 0,056 aufwiesen wurden als toxisch eingestuft, wohingegen Werte ≤0,032 als nicht toxisch angesehen wurden. Entsprechend wurden Absorptionen von ≥0,059 (toxisch) und ≤ 0,043 (nicht toxisch) für entfettetes Kernmehl festgelegt. Trotzallem sollte das Vorkommen von PEs in Jatropha Produkten, insbesondere in Lebensmittelprodukten, durch die bereits etablierte und validierte HLC Methode festgestellt werden. Die im Rahmen dieser Untersuchung entwickelte qualitative Methode könnte der toxikologischen Überprüfung von Produkten sowie Nebenprodukten der Jatropha Biodiesel Produktion dienen.

Die Bedingungen der Extraktion von PEs als PEAF aus Jatrophaöl mit Hilfe von Methanol als Lösungsmittel, einem Magnetrührer und einem Ultraturrax wurden optimiert. PEs des Jatrophaöls konnten um bis zu >99.4 % reduziert werden, wenn Methanol als Lösungsmittel eingesetzt wurde. Die PEAF beinhaltete (48,4 mg PEs/g) 14 mal mehr als die unbehandelten Öle. Außerdem war diese Fraktion äußerst bioreaktiv, wie Anhand eines empfindlichen Schnecken Biotests (LC_{100} - 1 ppm) festgestellt wurde (siehe unten). Das Entfernen der PEs aus dem Öl dauerte 60 min, eine für die industrielle Aufreinigung sehr lange Dauer. Daher wurden die Bedingungen, unter welchen man den höchsten Anteil an PEs in der geringsten Zeit extrahieren kann, optimiert. Die zur Extraktion verwendeten Geräte (Magnetrührer und Ultraturrax) funktionierten bei 2 und 5 min am effektivsten und konnten hier entsprechend 80 und 78 % der PEs entfernen. Der aus beiden resultierenden Ölen hergestellte Biodiesel entsprach sowohl den europäischen (EN 14214:2008) als auch den amerikanischen Biodiesel Standards (ASTM D6751-09). So konnte gezeigt werden, dass PEs

einfach und effizient Anhand beider Methoden zu extrahieren waren und das behandelte Öl ohne weiteres zu qualitativ hochwertigem Biodiesel weiterverarbeitet werden kann. Zudem stellt das durch die Extraktion aufgereinigte Öl keinerlei Gefahr für die Umwelt und den Menschen dar.

Die PEAF wurde auf den Einsatz als potentielles Spritzmittel biologischen Ursprungs getestet. Sie zeigten eine hohe biologische Aktivität in aquatischen Biotests bei Wasserschnecken (*Physa fontinalis*), Salzkrebse (*Artemia salina*) and Wasserflöhen (*Daphnia magna*), anders als bei Mikroorganismen. Die EC_{50} (48 h) der PEAF lagen bei 0,33 (Wasserschnecken), 26,5 (Salzkrebse) und 0,95 (Wasserflöhe) ppm PEs. Für Bakterien und Pilze wurden hohe minimale inhibitorische konzentrationen (MIC) (≥215 ppm) und EC_{50} (≥58 ppm) Werte ermittelt. Unter allen durchgeführten Biotests reagierten die Wasserschnecken am empfindlichsten mit einem LC_{100} Wert von 1 µg PEs/ml. Dieser Test könnte dem Nachweis der PEs in verschiedensten Jatropha Produkten, verunreinigten Böden und weiteren Matrizes verschiedenster Ökosysteme, welche dem Anbau von Jatropha und/oder der Verarbeitung deren Produkte dienen. Hier konnte nachgewiesen werden, dass die PEs molluskizide, bakterizide und fungizide Wirkungen aufweisen.

Die Lagerungsbeständigkeit der PEAF wurde auch untersucht. Sie wurden über 132 Tage bei verschiedenen Temperaturen gelagert und wiesen bei vorherrschender Raumtemperatur mit 50 % die höchste Abbaurate auf. Bei 4 °C und -80 °C konnte jedoch nur ein Abbau von 9 % bzw. 4 % nachgewiesen werden. Dementsprechend sank auch die biologische Aktivität (anhand des Wasserschneckentest überprüft) der bei Raumtemperatur gelagerten am schnellsten. Ihnen konnte bereits nach 260 Tagen kein Effekt mehr nachgewiesen werden, wohingegen die anderen nach 870 Tagen nur 27,5 % bzw. 32,5 % ihrer Aktivität zhoen. Dieser Effekt trat aufgrund der Autooxidation ein. Veränderungen der Fettsäurenmuster, ein Anstieg der Peroxid Werte und ein Abfall der freien Radikale in der PEAF spiegeln dies wider. Die Zugabe von Antioxidantien (Butylhydroxianisol (BHA), baynox und α-Tocopherol) konnte die PEs gegen diesen Abbau schützen. Hiermit wurde gezeigt, dass die PEAF eine begrenzte Lagerungsfähigkeit besitzt, welche jedoch mit Hilfe von Antioxidantien verbessert werden kann.

In Böden wurden sowohl die PEs in der PEAF (2,6 mg/g Boden) (Silikate wurden verwendet um die PEs zu absorbieren) als auch die PEs im Jatropha Presskuchen (0,37 mg/g Boden) wurden mit steigenden Temperaturen und Feuchtigkeitsgehalten im Boden komplett abgebaut. Die Silikatgebundenen PEs der PEAF wurden nach 19, 12 und 12 Tagen (bei 13 % Feuchtigkeit) und nach 17, 9 und 9 Tagen (bei 23 % Feuchtigkeit) entsprechend bei Raumtemperatur (22−23 °C), 32 °C und 42 °C komplett abgebaut. Bei denselben Temperaturen wurden die PEs im Presskuchen nach 21, 17 und 17 Tagen (bei 13% Feuchtigkeit) und 23,17 und 15 Tagen (bei 23% Feuchtigkeit) komplett abgebaut. Die Toxizität der mit PE belasteten Bodengemische (anhand des Wasserschneckentests bestimmt) wurde mit sinkenden PE Konzentrationen geringer. Die Untersuchung konnte aufzeigen, dass sowohl in Böden als auch in der PEAF vorkommende PEs komplett biologisch abbaubar und deren Abbauprodukte ungefährlich sind.

In Vorversuchen zeigte die PEAF eine insektizide Wirkung gegen *Spodoptera frugiperda*, ein häufig auftretender Schädling von Maispflanzen der tropischen/subtropischen Länder wie Mexiko und Brasilien. Die PEAF fungierte als Kontaktgift mit einem LC_{50} Wert von 0,83 mg/ml (w/v) Bei hohen Konzentrationen (0,25 mg/ml (w/v) wurden die Futteraufnahme, die relative Wachstumsrate und die Futterverwertung um 33%, 42% und 38% gesenkt. Hiermit konnte gezeigt werden, dass die PEAF potentiell als Insektizid eingesetzt werden könnte. Feldversuche wären an dieser Stelle notwendig um herauszufinden welchen Effekt sie unter Bedingungen im Freiland auf *S. frugiperda* hat.

Das pharmazeutische Potential der PEs wurde ebenfalls untersucht. Zunächst wurden die PEs aus Jatrophaöl aufgereinigt. Es konnten mindestens sechs verschiedene PEs (als C_1 bis C_6 ,bezeichnet) im Jatrophaöl nachgewiesen werden. Die Identität von C_1 und C_2 konnte durch Kernspinresonanzspektroskopie bestätigt werden. Der Vergleich der Flächen unter den durch die HPLC gewonnenen Kurven für "Phorbol 12-myristate 13-acetate (PMA)" und Jatropha Faktor C_1 zeigte eine 41,3-fach höhere Absorption bei 280 nm. Jedem der aufgereinigten PE Faktoren (C_1, C_2, $C_{3mixture}$ und (C_4+C_5)) und einem PE Gemisch konnte eine biologische Aktivität in den Wasserschnecken- und Salzkrebstests nachgewiesen

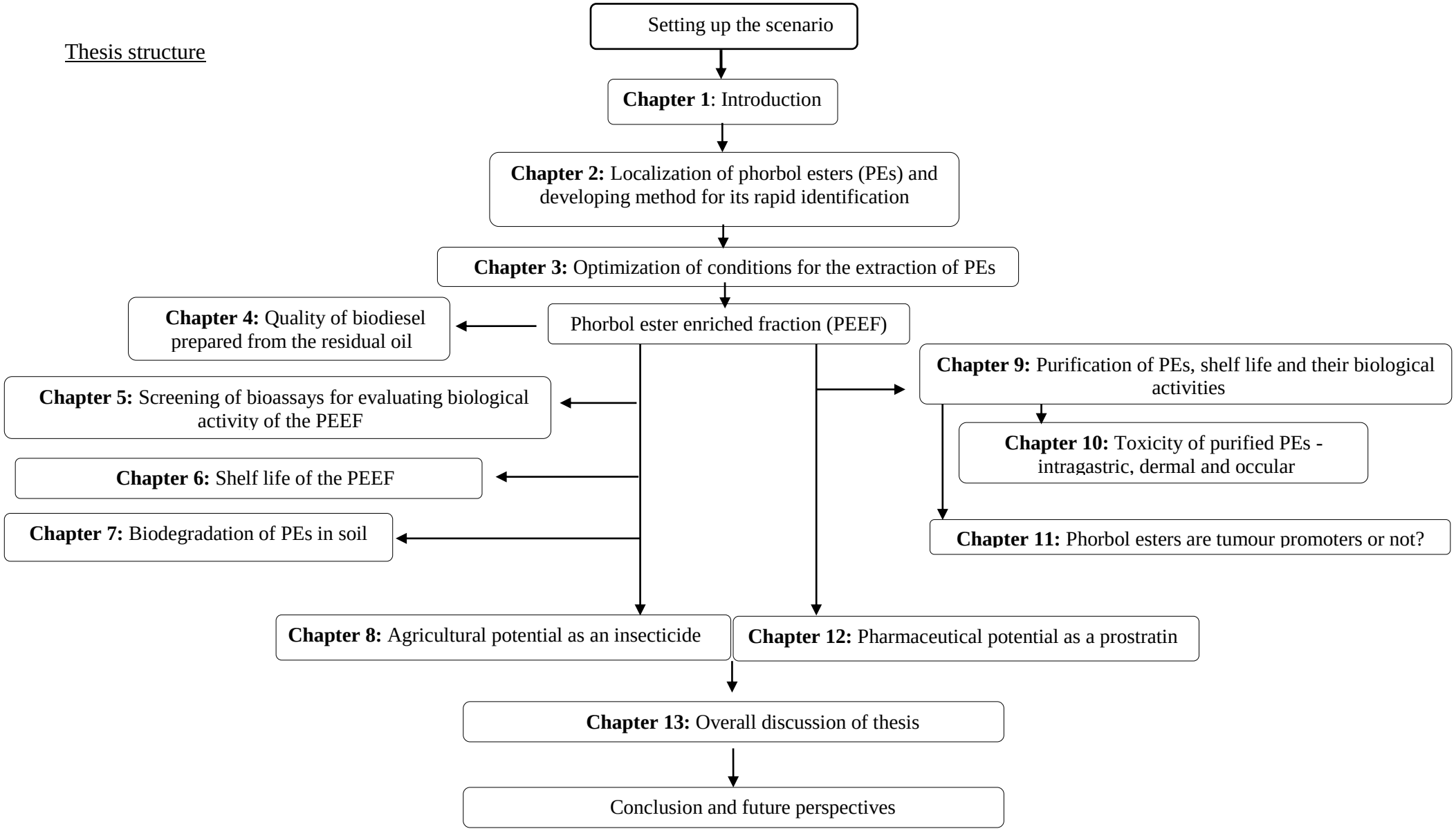
werden. Zusätzlich haben alle Jatropha PEs eine Blutplättchenaggregation verursacht. Diese Aggregation *in vitro* war am stärksten (basierend auf ED_{50} (µM)) für Jatropha Faktor C_2 < Faktor $C_{3mixture}$ > Faktor C_1 > Faktor (C_4+C_5). Das PE Gemisch zeigte bei intragastrischer Anwendung toxische Wirkungen mit einem LD_{50} Wert von 27,3 mg/kg Körpermasse als PMA Äquivalente oder 0,66 mg/kg Körpermasse als Faktor C_1 Äquivalente. Die auffälligsten histopathologischen Symptome konnten in der Lunge und den Nieren beobachtete werden. Die aufgereinigten Jatropha PEs, das PE Gemisch und das toxische Jatrophaöl riefen starke Veränderungen der Zellstrukturen, bis hin zum Auflösen der Gewebestruktur, der Epithelzellen hervor. Ferner erhöhte es die inflammatorische Reaktion (Interleukin-1α und Prostaglandin E_2 Ausschüttung) wenn es topisch an nachgebildetem menschlicher Haut (RHE) und nachgebildetem Hornhautgewebe (HCE) angewandt wurde. Im Falle des RHEs rief das nicht toxische Jatrophaöl eine geringere Zell-und inflammatorische Antwort als das toxische hervor, wenn sie in gleichen Volumina eingesetzt wurden. Die Reaktionen auf das toxische Öl wurden mit steigender PE Konzentration stetig stärker. Wurden wieder die gleichen Volumina für sowohl das toxische als auch das nicht toxische Öl eingesetzt, traten im Falle des nicht toxischen Öls im HCE Zellstrukturveränderungen auf. Diese Untersuchung zeigte, dass das Vorkommen von PEs in Jatrophaöl dessen Toxizität gegenüber RHE und HCE steigert. Dazu kommt, dass alle aufgereinigten Jatropha PEs positive Reaktionen auf das Tumorwachstum und negative Reaktionen auf die Tumorbildung *in vitro* zeigten (der Test basierte auf der Bildung von Entzündungsherden in Bhas 42 Zellen). In dem Tumorwachstumstest war die Reihenfolge von Entzündungsbildenden Substanzen wie folgt: PE-Gemisch > Faktor (C_4+C_5) > Faktor $C_{3mixture}$ > Factor C_1 > Factor C_2. Die Wachstumsförderung wurde durch die Hyperaktivierung der ProteinKinase C (PKC) reguliert. Die hier dargestellten Ergebnisse zeigen, dass Jatropha PEs toxische Wirkungen haben, sofern sie oral aufgenommen oder auf die Haut oder das aufgetragen werden. Die hieraus gewonnen Daten könnten in der Etablierung von Sicherheitshinweisen für den ungefährlichen Umgang mit Jatropha PEs dienen.

Jatropha PEs wurden auch auf deren potentielle Tauglichkeit als Futterzusatzstoff zur Förderung der Prostratrinsynthese, ein vielversprechendes Adjuvanz in der Therapie gegen HIV, untersucht. Die Untersuchungen zeigten, dass Jatropha PEs schrittweise synthetisiert

werden können. Zunächst werden sie zu "Crotophorbolone" umgewandelt und dann zu Prostratin. Wie die Nano-LC-ESI-MS/MSR Analyse ergab, besaß das aus Jatropha PEs hergestellte Prostratin eine ähnliche Masse und auch eine ähnliche Spitzenretentionszeit wie das Referenz-Prostratin (Sigma, St. Louis). Die Untersuchung ergab, dass Prostratin aus Jatropha PEs synthetisiert werden kann. Nichtsdestotrotz müssten an dieser Stelle Folgeuntersuchungen durchgeführt werden um diesen Prozess zu optimieren und Schlüsse auf den Ertrag an Prostratin aus Jatropha PEs ziehen zu können.

Einige der Anforderungen eines erfolgreichen biologischen Insekten- oder Pflanzenschutzschutzmittels sind folgende: Es sollte eine hohe Wirksamkeit gegen den Zielorganismus aufweisen, eine lange Lagerungsbeständig besitzen und im Boden biologisch Abbaubar sein. Noch dazu sollte es in hohen Mengen verfügbar, einfach zu gewinnen und nachhaltig zur Verfügung stehen. Die PEAF kann diesen Anforderungen gerecht werden. Das Vorkommen und die Neuentdeckung der PEs in Jatropha Spezies könnte eine neue Quelle für die Pharmaindustrie werden. Wenn man den geschätzten Öl Ertrag von 26 Millionen Tonnen jährlich bis zum Jahr 2015 (GEXSI, 2008) betrachtet, könnten durchaus riesige Mengen an Ausgangsmaterial für sowohl die Biodieselproduktion als auch die Pharmaindustrie zur Verfügung stehen. PEs in Form der PEAF könnten entweder als Insektenschutzmittel dem Landwirtschaftlichen Sektor oder alsAusgangsmaterial für die Prostratinsynthese der Pharmaindustrie dienen.

Thesis structure



1. Setting up the Scenario

The large scale cultivation of plant based feedstocks for bio-energy production has become a reality in a short time. The bio-energy sector is predicted to expand in future. This expansion is triggered mainly by increased petroleum prices, climatic change and energy security concerns. The bio-energy produced from sustainably managed systems could provide a renewable and carbon neutral source of energy. The production of biofuels is an attractive solution for mitigating green house gas emissions, enhancing energy security and providing socio-economic benefits. The leading contributors of biomass for biofuel production are agriculture, forestry and the wood processing industries. However, continuous feedstock supply for biofuel production is intimately linked with issues such as competition with food crops, ecotoxicity, and water and land use (Ruane et al., 2010).

Currently, biofuels are classified as first generation or second generation biofuels based on the feedstock. The first generation biofuels are generally produced from sugars, grains or seeds (Ruane et al., 2010). The major first-generation bio-fuels include ethanol produced from corn/sugarcane or biodiesel produced from edible and nonedible oils. The second generation biofuels are produced from non edible lignocellulosic sources such as bagasse from sugarcane, grasses, cereal straw, crop residues, short rotation forests and other energy crops. The first generation and second generation biofuels, depending on the choice of feedstock and cultivation techniques have the potential to provide benefits from waste residues and waste land. The availability of land and water is an important factor in determining the sustainability of bio-energy. Biofuel sustainability could be improved by critically considering the usage of waste land and management systems for using low quality water. In addition, the feedstock plants can generally be grown on marginal or wastelands and thus, reducing the competition for agricultural land (Ruane et al., 2010).

In a relatively short time, *Jatropha curcas* L., a first generation biofuel plant has gained a lot of momentum for the production of biodiesel on a commercial scale. Among the many tropical and subtropical countries involved in large scale production of Jatropha, India, china and Indonesia are at the forefront. The total worldwide cultivation is expected to increase to 12.8 million hectares by 2015. The current global seed production and projected future seed production is 23 and 43 mMT (GEXSI, 2008). The Jatropha seed oil can be processed into high quality biodiesel, which meets European and American biodiesel standards (Makkar et al., 2009a).

However, considering the relative infancy of the Jatropha biodiesel industry, long term economic sustainability still remains a critical factor. In addition, it should also be noted that most of the reported studies have overstated the impacts of first generation biofuels on land and agricultural markets by ignoring the role of biofuel byproducts and coproducts. Among the many byproducts some, such as oilseed meal, could be utilized in the livestock industry as a source of protein, and coproducts such as bioactive phytochemicals could be utilized in agro-pharmaceutical applications (Devappa et al., 2010ab; Makkar et al., 2009b). The maximum utilization of value added byproducts/ coproducts could increase the sustainability of the Jatropha biodiesel industry while in turn reducing the impact on land and green house gas emissions by avoiding the disposal or burning of wastes obtained during biodiesel production.

The increased expansion of the Jatropha biodiesel industry in future and the current limited knowledge of its byproducts/coproducts and their effective utilization provide the opportunity to investigate their potential for increasing the sustainability of the Jatropha biodiesel industry.

It is therefore the general aim of this dissertation to explore the potential of phytochemicals from Jatropha seed for increasing the sustainability of the Jatropha biodiesel production chain.

1.2. Aim, hypotheses and objectives

The thesis work described herein focuses on a group of natural compounds (phytochemicals) known as phorbol esters. These compounds are most studied for their biochemical and toxicological aspects. The working hypothesis was that phorbol esters present in *Jatropha curcas* oil could be obtained as a coproduct during the biodiesel production and utilized in agro-pharmaceutical applications.

Based on this hypothesis the following specific objectives were set:

1. Optimization of conditions for the extraction of phorbol esters from *Jatropha curcas* oil and evaluation of feedstock quality of residual oil for biodiesel production
2. Evaluation of biological activity of extracted phorbol esters using various bioassays.
3. Evaluation and enhancement of shelf life of Jatropha phorbol esters
4. Assessment of biodegradability of Jatropha phorbol esters in soil
5. Characterization of Jatropha phorbol esters by chromatographic techniques/NMR
6. Evaluation of biological activities of purified Jatropha phorbol esters in *in vitro* and *in vivo* models
7. Exploring agricultural and pharmaceutical potential of Jatropha phorbol esters

This dissertation gives:

- General insight into Jatropha as a source of oil for biodiesel production and the potential of its byproducts and coproducts.
- Information on the methodology for isolating phorbol esters and their bioactivity, shelf life, ecological toxicity and potential agricultural applications.

1.3. Outline of this dissertation

The dissertation is divided into 14 chapters.

Chapter 1 is the general introduction. It reports literature reviews on the agronomical aspects and potential of Jatropha seed oil for production of biodiesel and other value added products, the potential of Jatropha seed proteins and peptides, Jatropha diterpenes, toxicity of Jatropha seed and biochemical aspects of phorbol esters. The general introduction in chapter 1 feeds into the results and discussion (Chapters 2 to 13).

Chapter 2 describes the localisation of major antinutrients/toxic phorbol esters and rapid qualitative identification of toxic phorbol esters in Jatropha seed.

Chapter 3 describes the optimal conditions for the extraction of phorbol esters from Jatropha oil as a phorbol esters enriched fraction (PEEF).

Chapter 4 describes the biodiesel quality prepared from the residual Jatropha oil after phorbol ester extraction.

Chapter 5 describes the screening of extracted phorbol esters for their biological activity using bioassays

Chapter 6 describes shelf life of phorbol esters enriched fraction from Jatropha oil.

Chapter 7 describes the biodegradability of phorbol esters in soil.

Chapter 8 describes agricultural potential of phorbol esters enriched fraction as an insecticide.

Chapter 9 describes purification of phorbol esters, shelf life and their biological activity.

Chapter 10 describes the toxicity of phorbol esters: intragastric, dermal and ocular exposure.

Chapter 11 discusses whether all *Jatropha curcas* phorbol esters are tumour promoters or not?

Chapter 12 describes the pharmaceutical potential of phorbol esters enriched fraction.

Chapter 13 describes overall discussion of thesis.

Conclusion describes the outcome of the investigations undertaken and discusses future prospects.

CHAPTER -1

Introduction

The importance of biofuels such as biodiesel produced from non edible plant sources has attracted considerable interest and many countries have even adopted a policy of blending biofuels with regular petrol or diesel. Biodiesel is both biodegradable and renewable. The most attractive aspect of potential biofuels made from nonedible oil sources (waste vegetable or animal fat, Neem, Karanja, Simarouba, Jatropha, Jojoba etc.) is their long term sustainability which produces both environmental and socio-economic benefits (Devappa et al., 2007). Among potential sources of biofuel, Jatropha is pre-eminent as it is non edible, does not compete with food crops when grown on marginal/waste land, has wide climatic adaptability, requires little water to grow, can provide income for poor farmers and most importantly produces large quantities of seed oil that can be turned into high quality biodiesel (Makkar et al., 2009b). The biodiesel produced from Jatropha oil has been successfully used to fuel cars, buses, trains and even power passenger jets. In recent years, *Jatropha curcas* (*Euphorbiaceae*) has attracted many investments for large scale production. The estimated global Jatropha plantation in 2008 was 0.94 million hectares and projected to reach 12.8 million hectares by 2015 (GEXSI 2008). Although, commercial cultivation of Jatropha has uncovered some shortcomings in matters such as yield, water footprint and ecological impact, progress in systematic scientific investigation will enable the selection of favourable genotypes that will allow Jatropha to realize its full potential. However, the Jatropha based biodiesel industry is still in its infancy and requires effective strategies to utilize raw/processed products which could benefit the production chain, both economically and environmentally. The more these coproducts are utilised, the more efficient and profitable the industry will become.

In the following subsections the agro-botanical features of the Jatropha plant and the industrial potential of its oil, byproducts and coproducts are discussed. In addition, chemistry, biological activity and potential applications of phorbol esters are discussed

1.1. Agro-botanical features of Jatropha curcas

The genus *Jatropha* is native to tropical America and currently distributed in the tropical and subtropical areas of South America, Central America, Asia, Australia and Africa. The oldest

remains of Jatropha were found in geological formations in Peru corresponding to the early tertiary age (upper Eocene or lower Oligocene) (Berry, 1929; Carels, 2009).

The genus *Jatropha* belongs to the tribe *Jatropheae*, order *Malpighiale* in the Euphorbiaceae family and contains more than 170 known species (Wurdack, 2008). The most widespread species around the world is *Jatropha curcas*. Jatropha has wide climatic adaptability and grows where there is rainfall between 250−3000 mm and the temperature between 15−40 °C. It does not thrive at high altitudes and low temperatures. It can grow on a wide range of soils provided they are well drained and aerated (Kumar and Sharma, 2008). If fertilization is not available, fungal mycorrhization can help to sustain growth and development (Achten et al., 2008). The plant can be easily propagated either by seeds, seedlings or cuttings and has a productive life of 30–50 years. The reported seed yield is highly variable and average seed yield is 1−3 tons/hectare. However, the seed yield can be influenced by genotype, planting density, agroclimatic conditions and the health of the plant (Brittaine and Lutaladio, 2010).

The *Jatropha curcas* has characteristically five lobed leaves, one main taproot and four shallow lateral roots. It is monoecious with terminal inflorescences containing unisexual flowers on the same inflorescence (raceme). The inflorescence is a panicle, with fewer female flowers (~10–20%) than male flowers (~80–90%) (Raju et al., 2002; Jongschaap et al., 2007). After pollination, the inflorescence forms a bunch of ovoid green fruit (Kochhar et al., 2008; Carel, 2009). The harvested mature fruits are generally sundried (<8% moisture) and the dried fruits is 30−35% husk and 60−65% seeds. The seeds have a heat of combustion of 4980 cal/g (20.85 MJ/kg) with an oil content of 35%. The energy content of the oil is 9036 cal/g (37.83MJ/kg). The oil can be converted to biodiesel by transesterification with an alkyl ester purity of 95–97% (Augustus et al., 2002; Singh et al., 2008).

Pests which have been reported to attack Jatropha include *Scutellera nobilis* F., *Pempelia morosalis, Pachycoris klugii Burmeister* (Scutelleridae), *Leptoglossus zonatus Dallas* (Coreidae), the blister miner *Stomphastis thraustica*, the Meyrick (*Acrocercops*), the semi-looper (*Achaea janata* L.), the flower beetle (*Oxycetonia versicolor* Fabr), the Empoasca leafhoppers (*Empoasca* sp.), the broad mites (*Polyphagotarsonemus latus* Banks), the capsule-borers (*Pachycoris torridus* Scopoli), and thrips. The fungi antracnose (*Colletotrichum gloeosporioides* Penzig and *Colletotrichum capsici* Syd.) and fusariose have also been found (Saturnino et al., 2005; Freire and Parente, 2006).

1.2. Jatropha as a source of fuel, byproducts and secondary metabolites

The fruits are manually collected and transported to the oil processing unit. On arrival the fruits undergo various processes: (a) separation of husk from nut, (b) de-shelling (separating shell from the kernel) (c) drying, and (d) oil extraction. In order to improve the technological aspects of these processes a better understanding of the physiochemical properties of the Jatropha fruit is needed. The physical parameters of the seeds vary with the moisture content and weight of the seeds (Karaj et al 2008; Garnayak et al., 2008). The number of seeds in the Jatropha fruits varies. For example Makkar et al (2008a) have reported the distribution of the number of seeds in the fruit of a non toxic variety as - 3 seeds (58.3%) > 2 seeds (23.4 %) > 4 seeds (10.4%) > 1 seeds (8.1%) and in the toxic variety as - 3 seeds (52%) > 2 seeds (25.8 %) > 1 seeds (14.6%) > 4 seeds (7.6%). From 1600 kg of dried fruits, 1000 kg of seeds and 600 kg of husk can be separated, and 625 kg of kernel and 375 kg of shell can be obtained from 1000 kg of seeds. The optimal oil content of the Jatropha seeds varies between species, genetic variants, climatic and soil conditions. Achten and Erik Mathijs (2007) have reported that the seed oil content varies from 27−40%. However, for commercially grown Jatropha seeds the average seed oil content is considered to be 30−35% (Makkar et al., 2008a).

1.2.1. Processing of seeds to extract oil

The manually de-husked seeds from the ripe fruits should be cleaned, separated from moulds/contaminants and dried before storage in a ventilated room which is protected against pests. The de-husking and decortication of Jatropha seeds are important to give high yields of good quality oil and to reduce the bulk of material to be processed. However, there is no universal standardized procedure for decortication and oil extraction.

Generally, Jatropha seeds are pressed manually or with a hydraulic press to extract oil. However these methods results in relatively low oil yields. Traditionally, a machine called 'Ghani' is used in Asia but less so in other areas. The 'Ghani' consists of a heavy wooden or metal pestle driven by animal traction or a motor, inside a large metal or wooden mortar. Normally the material to be pressed should contain some fibrous material to allow the oil to escape more freely from the press. 'Ghanis' have relatively high capital and maintenance costs and need skilled operators to achieve high oil yields.

In mechanical pressing such as expeller pressing, heated oilseeds are passed via the feed inlet into one end of a barrel or tube and are conveyed by a rotating worm assembly to the discharge end. This extraction method results in oil yield up to 30−35%, considering the average oil content of Jatropha seeds ranging from 35−40% (Karaj et al., 2009). However, when large quantities

of oilseed cake have to be processed, solvent extraction becomes a commercially-viable option to extract the residual oil left in the cake and to obtain an almost oil-free powder known as oil-seed meal. Generally, solvent extraction plants use hexane as a solvent to extract oil from oilseed cake. These plants are expensive and only suitable for large volumes of material which justify the capital cost of the equipment. The residual cake and meal obtained after oil extraction are potentially useful byproducts. Among the many reported oil extraction methods for *Jatropha curcas* L. seed, the three phase partitioning method has been found promising. In this process, a slurry of Jatropha seed kernels is prepared in *t*-butanol (1:1, v/v) and 30% (w/v) ammonium sulphate, sonicated and enzyme treated (fungal proteases at pH 9) to obtain oil with 97% yield within 2 h (Shah et al., 2005).

Overall, out of 27−40% oil available in the seed, 60−80% and 70−99% could be recovered by mechanical and solvent extraction respectively (Achten et al., 2007). The high variability in oil yield may be attributed to the difference in genotypes of Jatropha seeds and extraction methods used in the studies.

1.2.2. Jatropha oil for biodiesel production

The Jatropha oil is a good quality feedstock for biodiesel production. Detailed information on the high quality transesterification processes of Jatropha oil can be found elsewhere (Makkar et al., 2009a). The general composition of Jatropha oil extracted by different methods was compared with that of the oil from a nontoxic variety in Table 1 (Makkar et al., 2009a).

The biodiesel produced from Jatropha oil meets American and European biodiesel standards (Makkar et al., 2009a). Although Jatropha oil is a good quality feedstock, the presence of toxic factors (phorbol esters) raises concern over whether there are possible ecotoxicity and exposure hazards. During pressing, 70% of the phorbol esters present in the seed are extruded with the oil and 30% remain in the matrix of seed cake, thus making both oil and seed cake nonedible. The solvent extracted and cold pressed toxic oil contains 3.10 mg/g and 3.77 mg/g phorbol esters respectively (Makkar et al., 2009a). During biodiesel processing, the physical refining steps such as stripping which involves exposure of oil to very high temperatures (240−260 °C) lead to complete degradation of phorbol esters and, the biodiesel and glycerol produced are free of phorbol esters (Makkar et al., 2009a). However, phorbol esters were detected in the acid gums and washings resulting from the degumming process of oil. This means that acid gums cannot be used in animal feeds and simply disposing of washings from biodiesel production may pose problems.

Table 1. General composition of different crude Jatropha oil samples (Data adopted from Makkar et al., 2009a)

Parameters	Solvent extracted oil	Cold pressed oil	Nontoxic oil
Water (ppm)	197	731	735
Free fatty acids (% C18:1)	6.87	5.34	3.00
Fatty acid composition (% w/w)			
C14:0	0.1	0.1	0.2
C16:0	15.8	15.3	12.0
C16:1	0.9	0.9	0.6
C17:0	0.1	0.1	0.1
C17:1	0.0	0.1	0.1
C18:0	6.7	6.8	6.4
C18:1 t	0.0	0.1	0.1
C18:1 c	42.1	42.0	36.7
C18:2 t	0.0	0.1	0.1
C18:2 c	34.1	34.4	43.5
C18:3 t	0.0	0.0	0.0
C18:3 c	0.2	0,.2	0.1
C20:0	0.0	0.1	0.1
Total saturated	22.8	22.3	18.8
Total mono unsaturated	42.9	43.1	37.5
Total poly unsaturated	34.3	34.6	43.7
Elements (ppm)			
P	87.9	35.5	54.9
Ca	51.1	21.6	32.8
Mg	23.9	23.0	N.D.
Na	13.3	6.44	1.48
K	15.3	28.7	6.57
Fe	8.31	0.29	0.07

1.2.3. Jatropha coproducts and constraints in their utilization

During biodiesel production from Jatropha oil, many value added byproducts/coproducts could be obtained. The pressed cake obtained after oil extraction has a significant potential as biomass. When seed cake is used to produce biogas it produces more energy on fermentation than cattle dung (Carels, 2009). Alternatively, the seed cake could be used as microbial substrates to produce enzymes such as proteases and lipases, and the nitrogen rich seed cake could also be used as a fertilizer or spread on fields as a mulch (Sharma et al., 2008). The briquettes produced from seed cake could be used for domestic or industrial fuel. One kilogram of briquettes undergoes complete combustion within 35 min at 525–780 °C (Singh et al., 2008; Vyas

and Singh, 2007). Jatropha wood and husks/shells could also be used for fuel. The shells can be used for making plywood. The glycerine and oil could be used to produce soap (Makkar et al., 2009b). In addition, the genus Jatropha is a rich source of phytochemicals that exhibit many biological activities both *in vitro* and *in vivo*. The plant contains phytochemicals such as alkaloids, lignans, cyclic peptides, and terpenes.

Brief information about the Jatropha proteins, peptides, diterpenes and toxicity of Jatropha based products are provided in the following subsections.

1.2.4. Jatropha proteins and peptides

Jatropha proteins have interesting nutritional and biochemical properties. The detailed information on the potential of Jatropha proteins and peptides are discussed in our published review (Devappa et al., 2010b). The cross references could be accessed from this review article. In nutritional context, they are at least comparable to if not better than soybean proteins. Due to its high protein content, high protein digestibility and good amino acid composition (Table 2) make Jatropha proteins a promising supplemental source in the diets of ruminant and monogastric animals including fish. The presence of antinutritional factors/toxic components has restricted the maximum utilization of Jatropha proteins in animal nutrition. The toxic principles include phorbol esters and curcin. However, recently a detoxification process was developed in our laboratory which will pave the way for using Jatropha kernel meal and protein isolates in livestock nutrition. In addition, the plant also produces protein having physiological role such as aquaporins and betaine aldehyde dehydrogenase, which are necessary to sustain environmental pressures such as drought and arid conditions; esterase and lipases involved in triglyceride hydrolysis; the bioactive proteins such as curcin and curcain has a potential to be used as a successful immunoconjugate in chemotherapy and wound healing medications respectively. Furthermore, several cyclic peptides present in Jatropha seeds have potential clinical significance and show their potential in pharmacy (Table 3).

Table 2. Comparison of amino acid composition from Jatropha curcas defatted kernel meal, protein concentrate/isolate with essential amino acid requirement of fish, chicks and pigs

Amino acids	Cape Verde genotype[1]	Nicaragua genotype[1]	Nontoxic Mexican genotype[1]	Indian genotype[2]	Protein isolate[3]	EAA requirement in fish[4]	EAA requirement in chick[5]	EAA requirement in young pig[5]	Soybean[1]	FAO reference protein[1]
Lysine	4.28	3.74	3.4	4.17	3	4.1-6.1	6.1	4.7	6.08	5.80
Leucine	6.94	7.03	7.5	7.86	7.08	2.8-5.3	6.7	4.6	7.72	6.60
Isoleucine	4.53	4.46	4.85	5.39	4.47	2.0-4.0	4.4	4.6	4.62	2.80
Methionine	1.91	1.56	1.76	1.3	1.66	2.2-6.5[a]	4.4[#]	3.0[#]	1.22	2.50
Cystine	2.24	1.76	1.58	1.1	1.34	5.0-6.5	-	-	1.70	
Phenylalanine	4.34	4.52	4.89	5.04	5.42	5.0-6.5[b]	7.2*	3.6*	4.84	6.30
Tyrosine	2.99	2.79	3.78	1.42	3.2	-	-	-	3.39	
Valine	5.19	5.24	5.3	5.98	5.18	2.3-4.0	4.4	3.1	4.59	3.50
Histidine	3.3	3.2	3.08	2.75	3.51	1.3-2.1	1.7	1.5	2.50	1.90
Threonine	3.96	3.71	3.59	2.89	3.56	2.0-4.0	3.3	3	3.76	3.40
Serine	4.8	4.88	4.82	3.22	5.23	-	-	-	5.67	-
Glutamic acid	14.68	15.4	15.91	19.26		-	-	-	16.90	-
Aspartic acid	9.49	9.73	9.92	9.37	12.5	-	-	-	11.30	-
Proline	4.96	5.27	3.8	4.65	5.45	-	-	-	4.86	-
Glycine	4.92	4.66	4.61	5.14	5.1	-	-	-	4.01	-
Alanine	5.21	5.04	4.94	4.92	5.47	-	-	-	4.23	-
Arginine	11.8	13.2	12.9	14.22	14.16	3.3-5.9	6.1	1.5	7.13	-

[a] requirement varies depending on the amount of cystine in the diet

[b] requirement varies depending upon the amount of tyrosine in the diet

[#] in the absence of cystine

*in the absence of tyrosine

[1]Makkar et al., 1998; [2]Devappa et al., 2008a ; [3]Makkar et al., 2008b ; [4]Hasan, 2001 ;[5]Halver, 1978.

Table 3. Biological activity of Jatropha proteins and peptides (adopted from Devappa et al. 2010b).

Functional proteins	Biological activity
Aquaporins	Drought resistence
Betaine aldehyde dehydrogenase	Drought resistance
Esterase and Lipase	Hydrolysis of triglycerides
Curcain	Wound healing property
Curcin	Inhibits protein synthesis and immune toxins
Beta glucanase	Antifungal activity
Cyclic Peptides	
Mahafacyclin	Anti malarial activity
Labditin and Biobollein	Immuno modulatory activity
Jatrophidin	Antifungal activity
Chevaleirins	No biological activity reported
Cycloglossine	No biological activity reported
Podacyclin	No biological activity reported
Pohlianins	Anti malarial activity
Curcacycline	Anti malarial activity, inhibits cell proliferation and classical pathway of human complement
Integerrimides	Anti proliferative activity against tumour cells

Note: the table is adopted from our published review, Devappa et al. (2010c). For cross reference please refer the review article.

1.2.5. Jatropha diterpenes

Since long time, Jatropha species are used in ethno medicines. This has led to identify the responsible bioactive molecules and finding possible pharmaceutical or agricultural applications. However, only few Jatropha species are explored for bioactive compounds, such as diterpenes, among others. Most of the diterpenes isolated were in search of new bio-control agents and their definite natural roles remain yet to be discovered. The detailed information on the potential of Jatropha diterpenes is discussed in our published review (Devappa et al., 2010a). The cross references could be accessed from this review article. In brief, more than 65 diterpenes have been

isolated from Jatropha and they exhibit diverse biologically activities *in vitro* (Table 4). The diterpenes such as jatrophone, Jatrophatrione, spruceanol, cleistanthol, curcasones (A and B) and japodagrol possess antitumour activities. The hydroxy derivatives of jatrophones, jatropholones, curcasones, multifidone, Jatrophalactam and faveline are cytotoxic. The caniojane derivatives, jatrogrossidione, hydroxy jatropholones, palmarumycin, jaherin and jatrogrossidentadion exhibited antimicrobial activities. Recent advances in analytical chemistry also led to the identification and comparison of novel chemical structure of these diterpenes, which could also be used as a template for the synthesis of new diterpene derivatives with enhanced functional and physical properties. In addition, phorbol type diterpenes (Jatropha factor C_1−C_6 and Jatropherol) isolated from Jatropha species has rodenticidal, piscicidal, moluscicidal and insecticidal activities, indicating their potential as bio-control agents in agriculture. The abundance and novelty of diterpenes present in Jatropha species could form a new 'stock' for the pharmaceutical industries. Future expansion of Jatropha plantation could generate a huge amount of raw materials for both biodiesel and pharmaceutical industries.

1.2.6. Toxicity of Jatropha

Jatropha plant contains variety of toxic and antinutritional compounds and its concentration varies with different parts of the plant. The detailed information on toxicity of Jatropha plant is discussed in our published review (Devappa et al., 2010a; Devappa et al., 2010c). The cross references could be accessed from this review article. In brief, majority of the toxic effects were studied using aqueous or non aqueous extracts of Jatropha root, bark, leaf, stem, oil, and seed or from direct feeding of plant parts (Table 5). In majority of the organic solvent or aqueous extracts obtained from Jatropha plant contain phorbol esters and curcin respectively as the major toxic phytochemicals. Even though, information on the localization of antinutrients/toxic factors in aerial parts of Jatropha plant is limited, all parts of the plant are found to be toxic to both vertebrates and invertebrates. However, the severity and symptoms of toxicity varied with the extract types, dosage, administrative mode and sensitivity of the animal under investigation. The extracts from Jatropha plant parts or of plant products produced a broad range of biological activities such as moluscicidal, piscicidal, insecticidal, antimicrobial and cytotoxic properties. The oral consumption of unprocessed Jatropha seeds or leaves are found to be toxic or lethal to

Table 4. Biological activity of Jatropha diterpenes (adopted from our published review article, Devappa et al. 2010a).

Sl. No.	Diterpenes	Jatropha species	Biological activities
1	Jatrophone	*J. gossypifolia* *J. elliptica*	Antitumour,Cytotoxic, Molluscicidal, Leishmanicidal, Gastroprotective
2	2α-OH Jatrophone	*J. gossipifolia*	Cytotoxic
3	2β-OH Jatrophone	*J. gossipifolia*	Cytotoxic
4	2β-OH-5,6-isoJatrophone	*J. gossipifolia*	Cytotoxic
5	9β, 13α-dihydroxyisabellione	*J. isabelli*	Cytotoxic
6	Japodagrin	*J. podagrica*	Antibacterial
7	Japodagrone	*J. podagrica*	Antibacterial
8	15-*O*-acetyl japodagrone	*J. multifida*	
9	Jatrophatrione	*J. microrhiza*	Antitumour
10	Jatrophenone	*J. gossypifolia*	Antibacterial
11	Riolozatrione	*J. dioica*	Antibacterial
12	Jatrowedione	*J. wedelliana*	NA
13	Integremmene	*J. integerrima*	NA
14	Citlalitrione	*J. dioica,J. integerrima and J. gossypifolia*	NA
15	Caniojane	*J. grossidentata* *J. integerrima and J. curcas*	Antiplasmodial Cytotoxic
16	1, 11 bisepicaniojane	*J. integerrima*	Antiplasmodial
17	2-epicaniojane	*J. integerrima*	NA
18	Spruceanol	*J. divaricata*	Cytotoxic,Antitumour
19	Cleistanthol	*J. divaricata*	Antitumour
20	*ent*-3β,14α-hydroxypimara-7,9(11),15-triene-12-one	*J. divaricata*	NA
21	*ent*-15(13→8)abeo-8β(ethyl)pimarane	*J. divaricata*	NA
22	Jatrogrossidione	*J. grossidentata*	Leishmanicidal, Trypanocidal
23	Isojatrogrossidion	*J. grossidentata*	NA
24	2-epi-isojatrogrossidion	*J. grossidentata*	NA
25	2-epi-Jatrogrossidione	*J. gaumeri*	Antimicrobial
26	2-Hydroxyisojatrogrossidion	*J. grossidentata, J.wedelliana and J. podagrica*	Antibacterial Antifungal
27	2-epihydroxyisojatrogrossidion	*J. grossidentata, J. wedelliana and J. podagrica*	Antibacterial Antifungal
28	(4E)-jatrogrosidentadione acetate	*J. multifida*	NA
29	(4E)-jatrogrossidentadione	*J. multifida*	NA
30	15-epi-4E-jatrogrossidentadione	*J. grossidentata J. gaumeri*	NA
31	15-O-acetyl-15-epi-(4E)-jatrogrossidentadion	*J. curcas*	NA
32	(14E)-14-O-acetyl-5,6-epoxyjatrogrossidentadion	*J. curcas*	NA
33	3β-acetoxy-12-methoxy-13-methyl-podocarpa- 8,11,13-trien-7-one	*J. curcas*	NA
34	3β,12-dihydroxy-13-methylpodocarpane-8,10,13-triene	*J. curcas*	NA
35	Jatropholone A	*J. isabelli*	Gastroprotection, Cytotoxic,

			Moluscicidal, Antiplasmodial
36	Jatropholone B	*J. isabelli*	Gastroprotective effect moluscicidal
37	α-hydroxyjatropholone	*J. integerrima*	Antibacterial Antiplasmodial
38	2β-hydroxyjatropholone	*J. integerrima*	Antibacterial Cytotoxic
39	Curcasone A	*J. curcas*	Antiinvasive effects in tumour cells
40	Curcasone B	*J. curcas*	Antiinvasive effects in tumour cells
41	Curcasone C	*J. curcas*	Cytotoxic
42	Curcasone D	*J. curcas*	Cytotoxic
43	Jatropherol	*J. curcas*	Insecticidal, Rodenticidal
44	Japodagrol	*J. podagrica*	Antitumour
45	Curculathyrane A	*J. curcas*	NA
46	Curculathyrane B	*J. curcas*	NA
47	(+) Jatrophol	*J. curcas*	NA
48	Multifolone	*J. multifida*	NA
49	Multifidone	*J. multifida*	Cytotoxic
50	Multidione	*J. multifida*	NA
51	Jatropha factor C_1	*J. curcas*	Cytotoxic
52	Jatropha factor C_2	*J. curcas*	Moluscicidal
53	Jatropha factor C_3	*J. curcas*	Rodenticidal
54	Jatropha factor C_4	*J. curcas*	
55	Jatropha factor C_5	*J. curcas*	
56	Jatropha factor C_6	*J. curcas*	
57	Heudolotinone	*J. curcas*	NA
58	Jatrophalactam	*J. curcas*	Cytotoxic
59	Faveline	*J. phyllacantha*	Cytotoxic
60	Deoxofaveline	*J. phyllacantha*	Cytotoxic
61	Faveline methyl ether	*J. phyllacantha*	Cytotoxic
62	Phyllacanthone	*J. phyllacantha*	NA
63	Palmarumycin CP1	*J. curcas*	Antibacterial
64	Palmarumycin JC1	*J. curcas*	Antibacterial
65	Palmarumycin JC2	*J. curcas*	Antibacterial
66	(4Z)-Jatrogrossidentadion	*J. grossidentata, J.wedelliana and J. podagrica*	Antibacterial Antifungal
67	(4Z)- 15-Epijatrogrossidentadion	*J. grossidentata, J.wedelliana and J. podagrica*	Antibacterial Antifungal
68	Jaherin	*J. Zaheyri*	Antibacterial

Note: the table is adopted from our published review, Devappa et al. (2010a). For cross reference please refer the review article.

Table 5. Toxicity of Jatropha plant species (adopted from our published review article, Devappa et al. 2010c).

Species	Plant parts	Test material	Properties
J. curcas	Plant	Chloroform and acetonitrile extracts	Moluscicidal activity
	Fruit	Methanol, chloroform and petroleum ether extract	Toxic to rat (pregnancy terminating effects)
	Seed	Powdered seed	Orally toxic to goat*, sheep*, calves* and humans (oral)**
		Powdered seed mixed in the diet	Toxic to chicken
		Raw or cooked seed	Toxic to rat (oral)
		Aqueous extract	Moluscicidal activity
		Methanol extract	Toxic to rats (intraperitoneal)
		Petroleum ether extract	Insecticidal activity
	Kernel meal	Mixed in the diet	Toxic to rat (oral); toxic to pig (oral)
	Defatted seed cake	Mixed in the diet	Toxic to rat (oral)
	Oil	Methanol and ethanol extracts	Moluscicidal and insecticidal activities; haemolysis in rabbit red blood cells; tumour promoting in mice skin
		Petroleum ether	Toxic to rats and rabbits (topical)
		-	Moluscicidal, ovicidal and insecticidal activities; toxic to rats (oral and topical); pregnancy terminating effects in rats.
	Latex	-	Antiparasitic activity
	Aerial parts	Aqueous extract	Cytotoxic activity
	Leaves	Petroleum ether and benzene extracts	Insecticidal activity
		Methanol extract	Toxic to rats (oral); antischistosomal and cytotoxic activities
		Dichloromethane, methanol and hexane extracts	Antibacterial activity

J. elliptica	Rhizome	Ethanol extract	Moluscicidal activity
	Tubercules	Ethanol extract	Toxic to rat (oral)
J. glauca	Leaves	Acetone extract from fresh leaves	Moluscicidal activity
		Chloroform extract from dry leaves	Moluscicidal activity
		Dry leaves	Toxic to goat (oral)*
	Fruit	Dry fruits	Toxic to goat (oral)*
J. gossypifolea	Bark	Aqueous extract from stem bark	Piscicidal activity
	Leaves	Fresh leaves	Toxic to sheep (oral)*
		Crushed liquid from fresh leaves	Anticoagulant activity
	Aerial parts (leaves and stems)	Ethanol extract	Toxic to mice and rats (intraperitoneal)
	Latex	Lyophilized powder	Piscicidal activity
J. multifida	Seeds	Raw seeds	Toxic to humans (oral)**
	Root (root bark, red root bark and root wood)	Hexane, ethyl acetate, chloroform and methanol extracts	Antibacterial activity
J. aceroides	Leaves	Dry leaves	Toxic to goat (oral)*
	Fruits	Dry fruits	Toxic to goat (oral)*
J. tanoresisi	Leaves	Ethanol (50%) extract	Toxic to rat (oral)
J. podagrica	Roots	Hexane and methanol extracts	Antibacterial activity
	Seed	Aqueous extract	Toxic to rat (intraperitoneal)
J. neopauciflora	Bark	Dichloromethane : methanol extract	Cytotoxic activity
J. macarantha	Sap	Aqueous extract	Toxic to mice (oral)

**force fed through stomach tube **accidental consumption*

Note: the table is adopted from our published review, Devappa et al. (2010c). For cross reference please refer the review article.

animals such as rodents, pig, chicken, sheep, goat and calves. The main affected organs were liver, kidney, spleen, lungs, intestine and heart. Although limited information is available on the toxicity of Jatropha towards humans, the oral, occular or prolonged topical exposure should be avoided. The increased Jatropha cultivation in future and utilization of its agro-industrial products/byproducts may raise the frequency of contact with humans, animals, and other organisms. Thus, any uncontrolled disposal or spread of *Jatropha* plant products either in aquatic or terrestrial environment should be avoided.

1.3. Phorbol esters: Chemistry, Biological activity and potential applications

Phorbol esters are not new to life science research. The phorbol ester has been widely used to understand the molecular mechanisms in cancer research, immunology, toxicology and nutrition. These compounds have fascinated many researchers from basic science to molecular science, resulting in more than 22,000 research articles published over the past 40 years (www.scopus.com; search key: phorbol esters). The complexity and richness of the studies are due to the multiple bioactivities of phorbol esters. The majority of the articles describe phorbol esters as toxic and as tumour promoters. More recent studies also portray phorbol esters as anti-cancer agents or as potential medicinal compounds (Goel et al., 2007; Wender et al., 2008). These apparently contradictory claims often puzzle the lay reader and lead to many questions such as, "What are these phorbol esters?", "Are they really toxic?" "Is it really safe to use them as medicines?". The following sub-sections, provides a brief overview of the chemical and biological properties of phorbol esters.

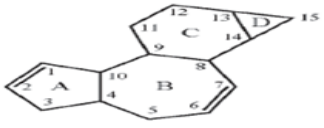
1.3.1. Distribution and chemical structure of phorbol esters

Phorbol and its related compounds are isolated from various natural sources. These esters are widely distributed in plant species of the families Euphorbiaceae and Thymelaceae. Examples of plants from which phorbol compounds have been isolated include among others, *Euphorbia Fischeriana*, *Homalanthus nutans*, *H. acuminatus*, *Neoboutonia melleri*, *Excoecarcia agallocha*, *Croton califonicus*, *Croton tiglium*, *Sapium indicum*, *S. japonicum*, *E. frankiana*, *E. cocrulescence*, *E. ticulli*, *C. spareiflorus*, *C. ciliatoglandulifer*, and *Jatropha curcas* (Beutler et al. 1989; Goel et al., 2007; Haas et al., 2003). Zayed et al. (1977) have also reported the presence of phorbol epoxides with the structure R2 (see Figure 1). In addition to 12-*O*-tetradecanoylphorbol-13-acetate (TPA), various phorbol derived compounds are commercially available (Table 2). The isolation of phorbol compounds from natural sources often requires great skill.

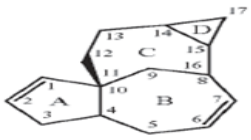
The term ‘phorbol’ refers to a group of compounds belonging to closely related families of diterpenes with polycyclic structural formulas (as shown in Figure 1). The ring ABCD can be attached to one or more substituents. Generally, substituents include hydroxyl, heteroalkyl, alkoxy, alkyl, arylalkoxy, hydroxyalkyl, acyloxy, aldehyde groups or combinations thereof. The numbering of atoms in the basic polycyclic structure is shown in the following Figure 1. Phorbol is more correctly described as a diterpene having a tigliane skeleton. The tigliane skeleton consists 5-membered ring A on the left trans linked to the 7-member ring B. Ring

Table 6. Commercially available synthetic and natural phorbol esters (sigma website)

- Phorbol
- 4α-Phorbol
- Phorbol 12,13,20-triacetate
- Phorbol 12,13-diacetate
- Phorbol 12,13-dibenzoate
- Phorbol 12,13-dibutyrate
- Phorbol 12,13-didecanoate
- Phorbol 12,13-didecanoate 20-homovanillate
- Phorbol 12,13-dinonanoate 20-homovanillate
- Phorbol 12-acetate
- Phorbol 12-decanoate
- Phorbol 12-myristate
- Phorbol 12-myristate 13-acetate
- Phorbol 12-myristate 13-acetate 4-O-methyl ether
- Phorbol 12-phenylacetate 13-acetate 20-homovanillate
- Phorbol 12-retinoate 13-acetate
- Phorbol 12-tigliate 13-decanoate
- Phorbol 13,20-diacetate
- Phorbol 13-butyrate
- Phorbol 13-decanoate
- 4α-Phorbol 12,13-didecanoate
- 4α-Phorbol 12-myristate 13-acetate
- 16-Hydroxyphorbol 12-palmitate 13-acetate



Structure R1 (tigliane)



Structure R2 (tigliane epoxide)

Figure 1. Basic skeleton of phorbol esters.

C is 6-membered and cis linked to the cyclopentane ring D. The compound phorbol was first isolated from *Croton tiglium* seed oil in 1934 (Bohm et al., 1934). Based on the generally accepted nomenclature system, phorbol is 4,9,12,13,20-pentahydroxy-1,6-tigliadien-3-one. The term phorbol ester applies to diversely oxygenated and hydroxylated tiglianes in different esterified forms. The placement of an OH group in 7 membered ring (at C4 position) makes the phorbol an active (ß) or inactive (α) type, which results in spatial re-arrangement of ring D and precludes the activation of PKC (Protein kinase C) and other structurally similar phorbol ester receptors. The inactive 'α' phorbol esters have similar physicochemical properties, especially lipophilicity, as the active 'ß' phorbols, but are unable to activate PKC due to conformational shifts (Figure 2) (Silinsky and Searl, 2003).

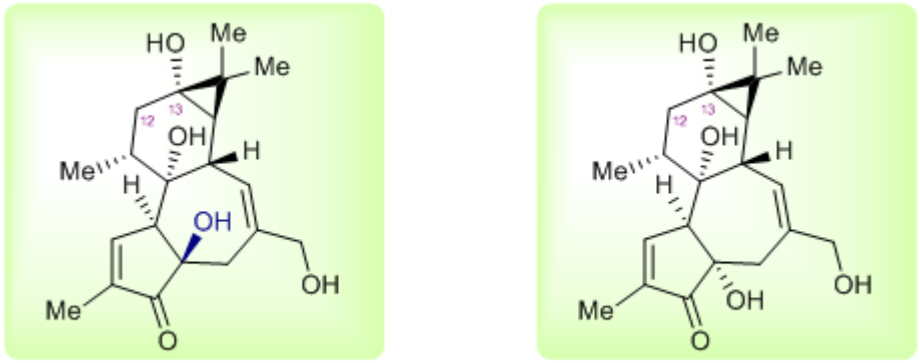


Figure 2. Phorbol esters and their confirmation responsible for bioactivity.

The most studied phorbol ester is phorbol 12-myristate-13 acetate (PMA) (synonym: 12-*O*-tertradeconoylphorbol-13-acetate (TPA)). PMA has been isolated from *croton tiglium* oil. Phorbol esters are easily soluble in most organic solvents such as ethanol, methanol, dichloromethane, and dimethyl sulfoxide, among others (our laboratory observation) and are found to be sensitive to oxidation (Schmidt and Hecker, 1975). PMA is the most potent tumour promoter known to date and is widely used in scientific research. Phorbol esters can be extracted by alcohol/organic solvent extraction or partition methods. Further purification can be carried out using chromatographic techniques, including, HPLC, gel exclusion chromatography. MS and NMR techniques are also frequently used for characterization. Similarly, many synthetic methods for producing parent phorbol compounds and their derivatives have been reported. Although more than 60 different types of natural phorbol esters have been reported, very few have been studied in detail with respect to their biological activity. The chemical structures of phorbol esters are described in detail by Haas et al. (2003).

1.3.2. Mechanisms of phorbol ester bioactivity

Phorbol esters exhibit multiple biological activities including the promotion of tumours. The most studied activity of the phorbol is its activation of PKC (Protein kinase C), which plays an important role in signal transduction pathways and regulates cell growth and differentiation (Clemens et al., 1992; Nishizuka, 1992). Phorbol esters are analogues of diacylglycerol (DAG), which is a secondary messenger in one of the main cellular signal transduction pathways (Figure 3a and 3b). In brief, the enzyme phospholipase C (PLC) (a membrane-bound enzyme) catalyses the hydrolysis of phospholipid PIP2 (phosphatidyl inositol-bisphosphate) to produce diacylglycerol (DAG) and inositol trisphosphate (IP3). The inositol trisphosphate (IP3) diffuses into the cytosol, but DAG due to its hydrophobic properties remains bound in the plasma membrane. These IP3 molecules stimulate the smooth endoplasmic reticulum to release calcium ions which act as a cofactor and facilitate the translocation of PKC from the cytosol to the plasma membrane. The DAG activates the enzyme PKC which catalyses the phosphorylation of other proteins that are involved in signal transduction.

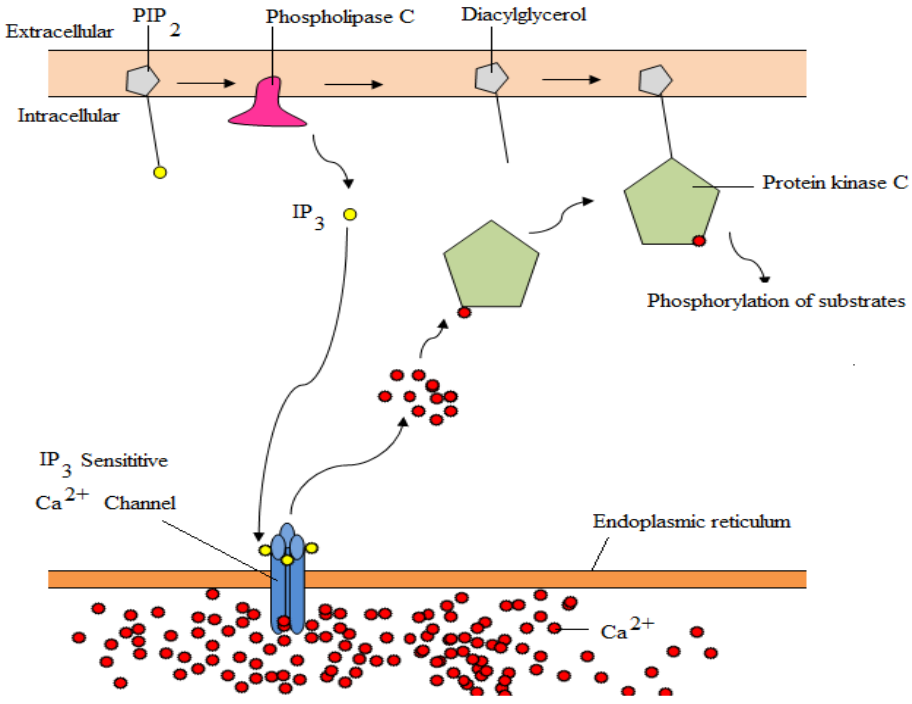


Figure 3a. Signal transduction pathway involving protein kinase C

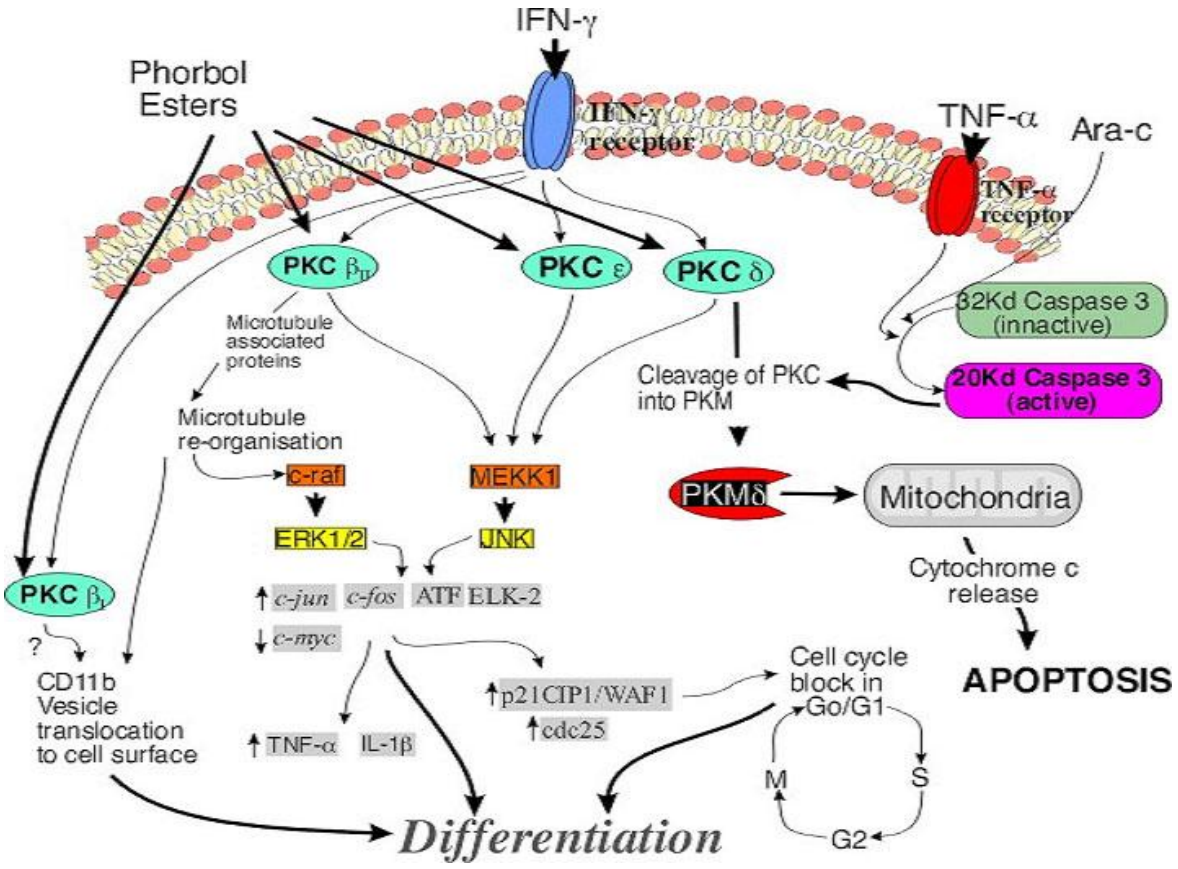


Figure 3b. Molecular effects of phorbol esters in cell signal transduction

(Source: http://www.spacedu.com/protein_kinase_c_experiments.htm).

The biological activities of phorbol esters are structure dependent. The translocation of PKC to the cell membrane depends on the hydrophobicity of the phorbol ester side chain and its ability to incorporate itself into the membrane (Bertolini et al., 2003). The data obtained from phorbol 12- myristate-13 acetate (PMA), its derivatives and analogues revealed that initial binding of a phorbol ester takes place at C1 domain of PKC. The C1 domain contains two β sheets separated by water-filled cavity (Figure 4). Phorbol esters displace water and fit into this cavity so that the phorbol esters become attached to the PKC via the oxygens present at the C3, C4 and C20 of the phorbol ester. The binding of phorbols in the C1 domain provides hydrophobicity to the complex and allows it to get inserted and anchored into the membrane without conformational change. After insertion into the membrane, the catalytic domain of PKC is activated and phosphorylation takes place after binding of appropriate substrates to the C4 domain (see Figure 4). The hyper activation of PKC by phorbol esters (DAG analogue) results in uncontrolled differentiation, thus amplifying efficacy of carcinogens.

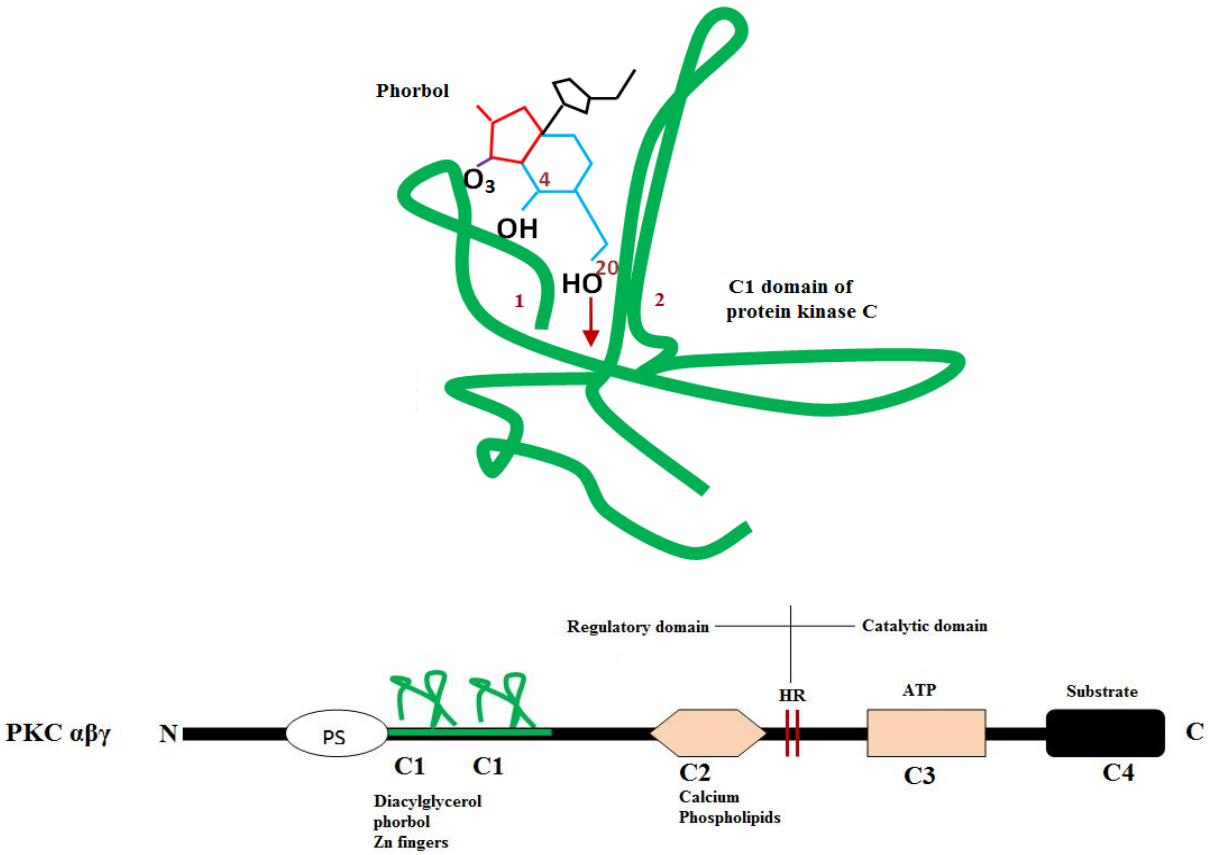


Figure 4. Mechanism of phorbol ester binding to protein kinase C domain.

1.3.3. Biological activity of phorbol esters

The phorbol esters exhibit biological activities such as tumour promotion, platelet aggregation, apoptosis, cell differentiation and other metabolic effects. Among these, tumour promotion by phorbol esters have been most studied (Goel et al., 2007). A tumour promoter is a compound that in classical studies of carcinogenesis is able to increase the likelihood of tumour formation after the application of a primary carcinogen. However, it does not induce tumour formation when applied alone. Non-tumour promoting phorbol esters all have at least one of the biological activities of phorbol compounds in general such as binding to phorbol receptors, but do not have tumour promoting properties. Non-tumour promoting phorbol compounds include 12-deoxyphorbol 13-acetate (prostratin), 12-deoxyphorbol 13-propanoate and 12-dexoxy phorbol 13-phenylacetate (Xu et al., 2009).

Toxic phorbol esters are skin irritants and exhibit mammalian skin inflammatory response releasing increased histamine, prostaglandins and cytokines (Figure 5). Weinstein et al. (1979) have reported that phorbol esters exhibit generalized cellular membrane changes such as changes in membrane morphology, cell surface fucose glycopeptides, cell adhesion properties and membrane fluidity. There are also increased levels of ornithine decarboxylase and plasminogen acti-

vator. The phorbol esters block the G2 phase of the cell cycle due to their effect on multiple targets within a single cell causing cellular differentiation (Figure 3; Kinzel et al., 1984). Phorbols also affect terminal cellular differentiation, which cumulatively results in production of tumours.

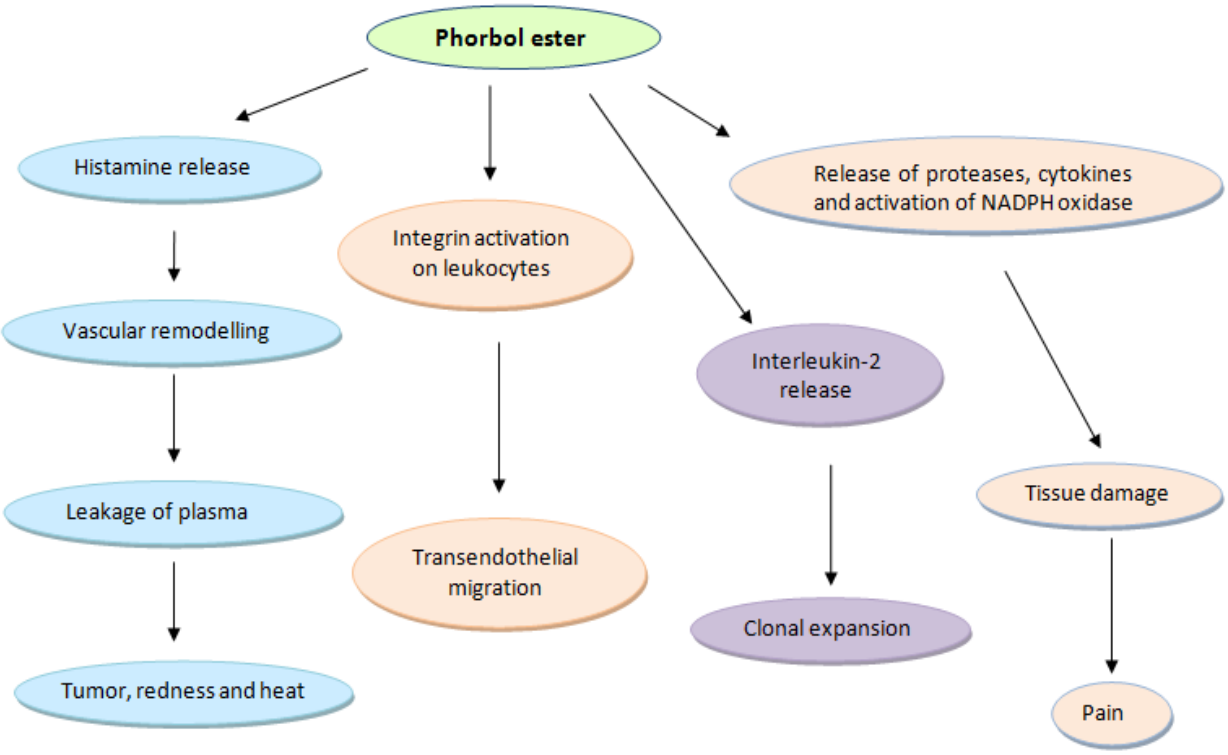


Figure 5. Major biological activity of phorbol esters (adopted from Goel et al., 2007).

Phorbol esters are also potent human blood platelet aggregators exhibiting aggregation at exceptionally low levels (0.3 µM=50% aggregation). The platelet aggregation assay has been suggested to be a choice for initial screening of tumour promoting substances (Brynes et al., 1980; Zucker et al., 1974). The phorbol esters affect many enzyme activities by interacting with PKC such as reduction in phosphoenol pyruvate carboxykinase in H4IIE cell lines, a key enzyme in gluconeogenesis (Chu and Granner, 1986). The phorbol esters are also reported to exhibit non-PKC mediated biological effects such as neurotransmitter secretion. For detailed information on biological activities observed *in vitro* and *in vivo* studies see review articles by Goel et al. (2007) and Silinsky et al. (2003). The non PKC enzyme receptors (Figure 6) include (a) chimaerins, (b) RasGRP, (c) *Caenorhabditis elegans*, Unc-13 and mammalian Munc13s.

(a) Chimaerins are GTPase activating protiens for Rac (a small GTP-binding protein depends which has important role in the regulation of actin cytoskeleton, cell cycle progression, and malignant transformation (Ron and Kazanietz, 1999). The chimaerin isoforms (α1- or n-, α2-

, β1-, and β2-chimaerin) possess approximately 40% homology of their C1 domains to those in PKC isozymes. The chimerins binding to PEs depends on acidic phospholipids (phosphotydil serine) and binds with the same affinity (compared to PKC) at nano molar concentration range. (Areces et al., 1994 and Caloca et al., 1997).

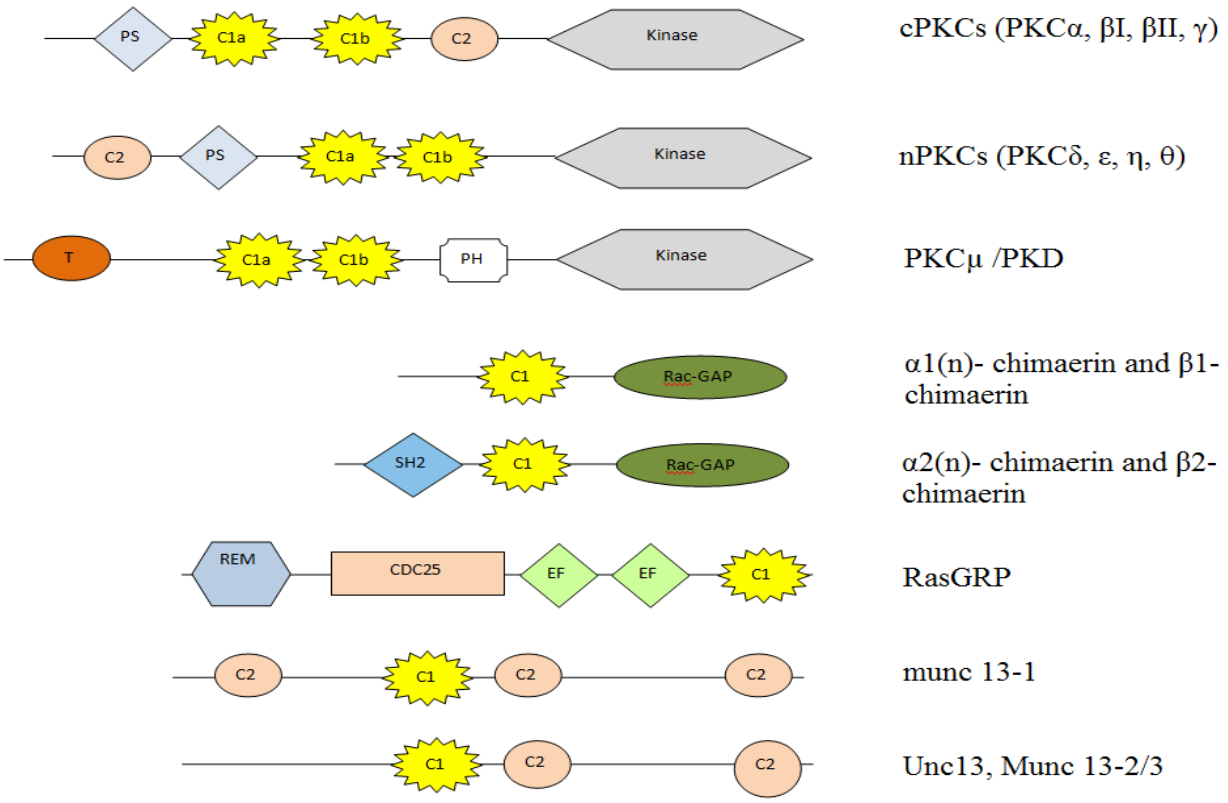


Figure 6. Structures of phorbol ester receptors (adopted from Kazanietz, 2000). The binding of phorbol esters and related analogues takes place at the C1 domain in PKCs and novel receptors. PS, pseudosubstrate domain; C1, cysteine-rich domain; C2, phospholipid binding domain (also a calcium binding domain in cPKCs); T, transmembrane domain, PH, PH domain; SH2, SH2 domain; Rac-GAP, Rac GTPase-activating protein domain; REM, Ras exchange motif; CDC25, region with homology to guanine exchange factor domain of Cdc25 and Sos; EF, EF hand.

(b) RasGRP (a Ras exchange factor) is another novel phorbol ester receptor. The RasGRP enhances the dissociation of GDP and favours the association of resulting GTP to Ras which leads to its activation. This nucleotide exchanging activity on Ras leads to the activation of downstream effectors of Ras (e.g. the MAPK cascade) and malignant transformation. However, the oncogenic potential of RasGRP is dependent on its C1 domain and phorbol ester activation (Ebinu et al., 1998 and Tognon et al. 1998).

(c) The *C. elegans* protein, Unc-13 and its related human homologs Munc13–1, Munc13–2, and Munc13–3 (Ron and Kazanietz, 1999; Maruyama and Brenner, 1991; Betz et al., 1998) is

also profound phorbol ester receptors. These proteins act as scaffold structures for many exocytotic proteins, including syntaxin, Doc2, and synaptotagmin (Duncan et al., 1999). Munc13 is also a pro-apoptotic protein when expressed in kidney cells (Song et al., 1999). Unc-13 and Munc13 isozymes have a single C1 domain with a consensus motif identical to those of PKCs, chimaerins, and RasGRP. The Munc13, or related proteins which are found in the mammalian brain acts as a phorbol ester receptors and mediate stimulatory effects of phorbol esters on neurotransmitter secretion. For detailed information on biological activities observed *in vitro* and *in vivo* studies see review articles by Goel et al. (2007), Kazanietz et al. (2000) and Silinsky et al. (2003).

1.3.4. Phorbol esters: *The good, the bad and the ugly*

As with any other chemical, the toxicity of the compound depends on dosage, mode, and duration of exposure. Generally the toxicity increases with the concentration of the toxic compound to which an organism is exposed. However, at lower nontoxic dosages these chemicals can sometimes exhibit beneficial properties for example cytotoxic, antitumour or anti HIV properties (Goel et al., 2007). Similarly, phorbol esters also possess toxic and beneficial biological activities, acting as a double edged sword. At high doses they exhibit toxicity that can be observed in microorganisms and even in higher animals. The over dosage of phorbol esters are found to be lethal towards higher animal either by oral or topical exposure (Gandhi et al., 1997).

Therefore, the presence of phorbol esters in feed ingredients is deleterious. The toxic effects of the phorbol esters found in Jatropha were discussed earlier (subsection 2.2.2). However, at lower dosages phorbol esters exhibit antitumour properties *in vitro* thereby acting as potentially beneficial compounds (Wender et al., 2008). Not all phorbol esters are toxic. Their activity and potency vary from one type of phorbol ester to another. In addition, these chemicals in a crude form (extracts) are effective in controlling microbes and pests of agricultural interest, suggesting that they may have application as biological control agents. The purified phorbol esters could also be converted or transformed chemically into nontoxic compound with beneficial activities such as prostratin. Prostratin has been found to be a promising anti-HIV agent. Thus, the beneficial effects of phorbol esters could be exploited depending on the application.

1.3.5. Phorbol esters from Jatropha curcas

Depending on the presence or absence of phorbol esters, Jatropha species are classified as toxic and nontoxic genotypes respectively. The most studied Jatropha species, *J. curcas*, contains phorbol esters about 1−3 mg/g in defatted kernel meal or 3−6 mg/g in oil. There are 6 dif-

ferent types of phorbol ester reported in Jatropha seed (Haas et al., 2002). The chemical structure is shown in Figure 7. The biological activities reported for purified Jatropha phorbol esters are scarce. However, the biological activities of phorbol esters containing Jatropha extracts or Jatropha products are discussed in section 2.2.6 (see also our published review article Devappa et al. (2010a and 2010b).

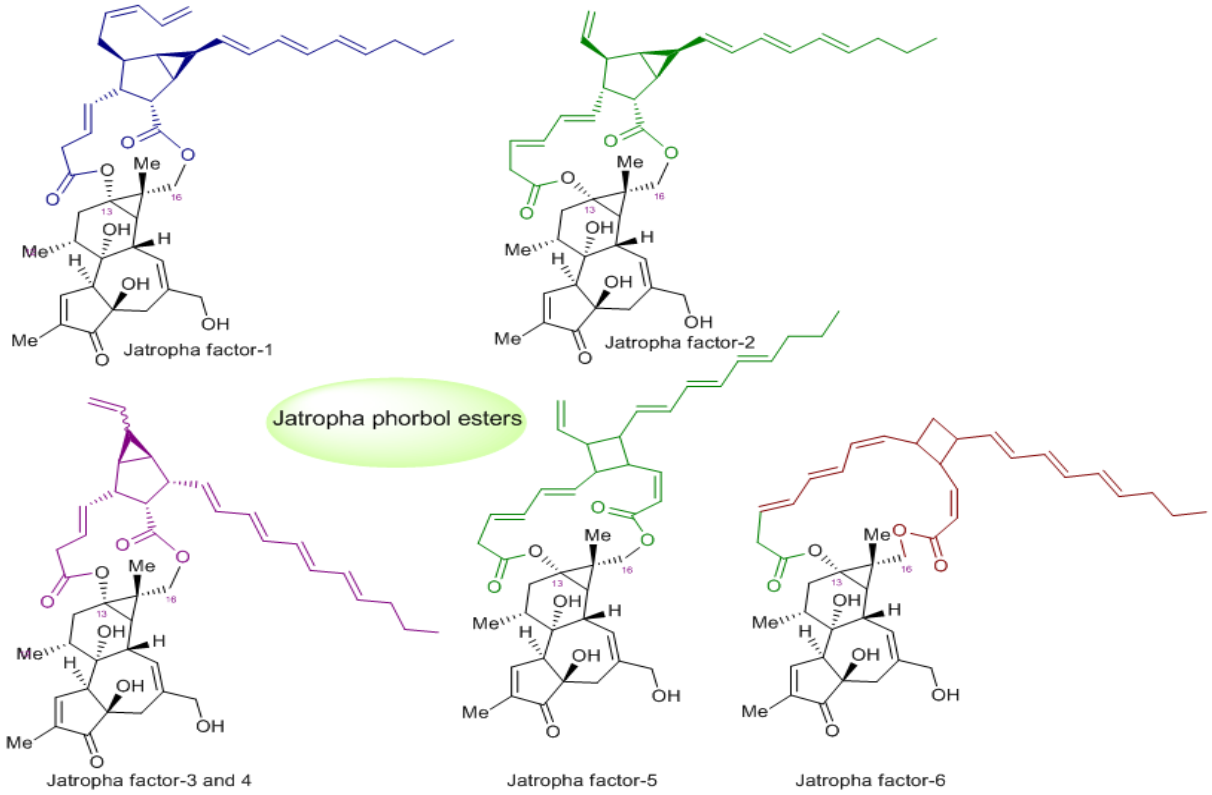


Figure 7. Phorbol esters from Jatropha curcas oil

When choosing natural products from the many byproducts available, it is not only important to choose a complex structure which demonstrates favourable biological activity, but also one which, through its novelty, will attract the interest of researchers, the chemical community and industry. The phorbol esters chosen in our study were found to be potentially satisfying these requirements. The presence of the many and varied biological activities of Jatropha phorbol esters prompted us to further explore its feasibility as a potential agro-pharmaceutical compound.

CHAPTER - 2

Localisation of antinutrients and qualitative identification of toxic components in *Jatropha curcas* seed

Rakshit K. Devappa, Harinder P.S. Makkar* and Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany.

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Localisation of antinutrients and qualitative identification of toxic components in *Jatropha curcas* seed

Rakshit K Devappa, Harinder PS Makkar* and Klaus Becker

Abstract

BACKGROUND: *Jatropha curcas* seed oil is a promising feedstock for biodiesel production. The seeds contain major toxic (phorbol esters, PEs) and antinutritional (phytate and trypsin inhibitor) factors. In the present study the localisation of antinutrients and a rapid qualitative method for detecting the presence of PEs were investigated.

RESULTS: Kernels were separated into cotyledon, hypocotyl, kernel coat and endosperm. The majority of phytate (96.5%), trypsin inhibitor (95.3%) and PEs (85.7%) were localised in the endosperm. Based on PEs, a qualitative method was developed to differentiate between toxic and non-toxic *Jatropha* genotypes. In this method, PEs were easily detected by passing methanol extracts of kernels (*Jatropha* toxic and non-toxic genotypes) through a solid phase extraction (SPE) column and measuring the absorption of the resulting eluates at 280 nm. For raw kernels, SPE eluates with absorbance ≥0.056 were considered as toxic and those with absorbance ≤0.032 as non-toxic. For defatted kernel meals, SPE eluates with absorbance ≥0.059 were considered as toxic and those with absorbance ≤0.043 as non-toxic.

CONCLUSION: The majority of antinutrients/toxic compounds are localised in the endosperm of the kernel. The qualitative method developed for rapid identification of toxic PEs could be useful in screening the toxicity of *Jatropha*-based products in the biodiesel industry. Further confirmation of PEs should be established by high-performance liquid chromatography.

Keywords: *Jatropha curcas*; phorbol esters; identification; localisation

INTRODUCTION

Jatropha curcas (Euphorbiaceae) is a tropical and subtropical plant. Owing to its oil-rich seeds, it is recognised as a promising feedstock for biodiesel production.[1] *Jatropha* grows as a small tree or shrub of 3–5 m but can attain up to 8–10 m under favourable conditions. The plant is resistant to drought and adapted to arid/semiarid conditions and low-nutrient soil.[2,3] The leaves are alternate, palmate, petiolate, stipulate and three–five lobed with spiral phyllotaxis. The inflorescence is formed in the leaf axil. The plant is monoecious and its flowers are unisexual (occasionally hermaphrodite flowers occur) and pollinated by insects, especially honey bees. The average ratio of male to female flowers is 29 : 1. The sexual system facilitates geitonogamy and xenogamy. The ability to self-pollinate through geitonogamy is considered to be adaptive for colonisation.[4] Fruits are generally produced in winter, but the plant may produce two–three fruit harvests during a year under favourable conditions. Each inflorescence yields a bunch of ten or more void fruits. The seeds become mature when the capsule changes from green to yellow with three bivalved cocci and fleshy exocarp.[5] Although the seed is rich in oil (300–350 g kg^{-1}) and protein (170–200 g kg^{-1}), its edible use is limited by the presence of toxic and antinutritional factors.[2] Most plant secondary metabolites with antinutritional/toxic properties are produced as defence mechanisms against predators and are also considered to protect the plant against environment vagaries as well as cold and other harsh conditions. During the long course of evolution, plants have developed enough defence mechanisms to be able to survive and animals/micro-organisms have adapted their digestive and detoxification abilities to find enough food. It is therefore not surprising that most animal and micro-organism species are able to forage only on a limited number of plant foods.[6] The attractiveness of *Jatropha* biodiesel production is stalled by the toxicity of the seeds. The majority of *in vitro* and *in vivo* studies conducted so far indicate that the toxicity of *J. curcas* (whole plant or plant parts, including seeds) is mainly due to diterpenes (phorbol esters, PEs).[7,8] Considering the rapid increase in worldwide *Jatropha* plantations and the toxicity of the plant parts, several scientists have raised concerns over their ecotoxicity and health hazards.[9–11] The problem can be overcome by recovering or removing PEs from the oil and using them for various agroindustrial applications, by cultivating PE-free *J. curcas* plants obtained by plant-breeding techniques or by cultivating the non-toxic *J. curcas* or *Jatropha platyphylla* plants available in Mexico.[12–14] Roasted non-toxic *J. curcas* and *J. platyphylla* seeds are reported to be consumed by humans in Mexico, apparently

* *Correspondence to: Harinder PS Makkar, Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany. E-mail: Harinder.Makkar@fao.org*

Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, D-70593 Stuttgart, Germany

without any side effects.[2,13] The toxic and non-toxic genotypes of *J. curcas* are classified based on the PE concentration in seeds. The inclusion of non-toxic *J. curcas* defatted kernel meal in fish diets increased the growth performance of fish without any biochemical changes, suggesting its potential as a fish feed.[15,16] Thus less investigated non-toxic varieties, if cultivated on a large scale, could be as effective as toxic varieties with respect to high oil yield and nutritional quality of by-products such as seed cake or meal. Recently, many programmes have been initiated to cultivate non-toxic varieties of *Jatropha* and investigate their seed yield and disease susceptibility.

The present widespread commercial cultivation of toxic varieties of *J. curcas* and the upcoming cultivation of non-toxic varieties clearly highlight the need for rapid, cost-effective and accurate differentiation between toxic and non-toxic genotypes of *Jatropha* species. Currently, PEs are determined using high-performance liquid chromatography (HPLC), which is time-consuming, expensive and requires special equipment and skills. In addition, in spite of several reports on the structural elucidation, toxicity and detoxification of antinutrients/toxic compounds present in *Jatropha*, very little is known about the distribution of major antinutrients/toxic compounds within the kernel part of the seed. In this study an attempt has been made to (a) investigate the distribution and localisation of toxic and major antinutritional compounds in the kernel and (b) rapidly differentiate between toxic and non-toxic genotypes using a combination of solid phase extraction (SPE) and UV–visible spectrometry.

MATERIALS AND METHODS

Distribution and localisation of antinutrients in *Jatropha* kernel

Physical characteristics of seeds

Toxic *J. curcas* seeds were obtained from Jaipur, Rajasthan (India). Ten seeds were randomly selected and the average seed weight was calculated by dividing the total weight of the seeds by the total number of seeds.

Preparation of samples for analysis

The seeds were manually cracked to separate shells and kernels and their weight was recorded. The kernel parts (hypocotyl, cotyledon, kernel coat (tegmen) and endosperm) were manually separated under a magnifying lens and their weights were recorded. Care was taken to separate the kernel coat from the endosperm. All samples were stored at 4 °C until further analysis. The kernel parts were powdered and used as such for the analyses, whereas the endosperm was defatted prior to analysis. Oil content in the endosperm was determined using petroleum ether (b.p. 40–60 °C) in a Soxhlet apparatus at 60 °C. PEs, trypsin inhibitor and phytate in the kernel parts were determined as described below.

Antinutrients and toxic compounds

All methods used for the measurement of antinutrients and PEs were according to Makkar *et al.*[17] The methods are briefly described below.

Phytate content

Phytate content was determined by extracting the sample with HCl (35 mL L^{-1}) and using Wade reagent. The pink colour of Wade reagent is due to the reaction between ferric ion and sulfosalicylic acid with an absorbance maximum at 500 nm. In the presence of phytate the iron becomes bound to the phosphate ester and is unavailable to react with sulfosalysilic acid, resulting in a decrease in pink colour intensity. Results were expressed as g phytic acid equivalent kg^{-1} sample.

Trypsin inhibitor activity

Trypsin inhibitor activity was determined indirectly by inhibiting the activity of trypsin. Benzoyl-dl-arginine-*p*-nitroanilide hydrochloride was subjected to hydrolysis by trypsin to produce yellow-coloured *p*-nitroanilide. The degree of inhibition of yellow colour production by the plant extract was recorded at 410 nm using a spectrophotometer. This measures the trypsin inhibitor activity (TIA), and results were expressed as mg trypsin inhibited g^{-1} sample.

Phorbol esters

PEs were determined at least in duplicate based on the method of Makkar *et al.*[2] Briefly, 0.5 g portions of raw kernel meal or defatted kernel meal were weighed into 2 mL Eppendorf tubes and extracted with 1–1.5 mL of solvent (methanol/tetrahydrofuran, 99 : 1 v/v) in a Retsch MM200 mixer mill (Retsch, Haan, Germany) (30 s^{-1}) for 5 min. The tubes were centrifuged (12 500 × *g*, 3 min) and the supernatants were collected. The samples were concentrated using pressurised air. Similarly, the residues were re-extracted three times and the pooled supernatants were concentrated as above and made up to a known volume (1 mL). Suitable aliquots were loaded into a high-performance liquid chromatograph fitted with a reverse phase C_{18} LiChrospher 100 (5 µm, 250 mm × 4 mm i.d.) column (Merck, Darmstadt, Germany). 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^{-1} using gradient elution. The four PE peaks appeared between 25.5 and 30.5 min and were detected at 280 nm. The phorbol 12-myristate-13-acetate (PMA) was used as an external standard, which appeared between 31 and 32 min. The area of the four PE peaks was summed and the concentration was expressed as mg PMA equivalent per kg raw kernel/defatted kernel meal or mg PMA equivalent per mL of the SPE eluate (procedure given in below-mentioned section).[17]

Qualitative identification of phorbol esters from *J. curcas*

Toxic and non-toxic *Jatropha* seeds were collected from different parts of the world. The seeds were transported to Germany and stored in airtight bags in a dark, cool and dry place until further analysis. All chemicals and solvents used were of analytical grade. The seeds were cracked to separate the kernels. The kernels were powdered to get raw kernel meal, which was defatted with petroleum ether (b.p. 40–60 °C) in a Soxhlet apparatus at 60 °C. The resulting kernel meal was air dried to get defatted kernel meal (<8 g oil kg^{-1}). Both raw kernel and defatted kernel meals were used for the study.

Solid phase extraction of phorbol esters

Based on the nature of the column used in the HPLC method for the detection of PEs, a commercially available SPE column (Chromabond C_{18} EC, 3 mL per 500 mg; Art. No. 730 013, Macherey Nagel, Düren, Germany) was selected. It was preconditioned by washing with 1 mL of 200 mL L^{-1} acetonitrile. Elution was done using a vacuum pump (MZ 2C/1,7, Vavuubrand, Wertheim, Germany) at 650 mbar. The raw kernel meal and defatted kernel

meal were extracted with methanol as described above. Aliquots (150 µL) of the methanol extracts were pipetted into the SPE column. The elution gradient was chosen to resemble that used in the HPLC method. The column was washed with 4 mL of 600 mL L^{-1} acetonitrile and the eluate was discarded. The column was eluted again with 1 mL of absolute acetonitrile and the resulting SPE eluate was used for both HPLC analysis and UV spectrophotometry (220–340 nm) measurement. This SPE eluate was also used for the detection of PEs on thin layer chromatography (TLC) plates by UV light.

Qualitative methods for identification of phorbol esters

The methanol extracts and SPE eluates (8–10 µL) were spotted on TLC plates (Silica gel 60 F_{254}, Merck, Germany) and detected under UV light (255 and 366 nm).

The SPE eluates (8–10 µL) were spotted on TLC plates and sprayed with different staining reagents (http://tinyurl.com/3w4cofk), namely vanillin reagent, bromocresol green, potassium permanganate and dinitrophenylhydrazine. The dinitrophenylhydrazine reagent was prepared by mixing 2,4-dinitrophenylhydrazine in concentrated sulfuric acid (60 mL) with water (80 mL) and dissolving the mixture in absolute ethanol (200 mL). The vanillin reagent was prepared by mixing vanillin (15 g) with absolute ethanol (250 mL) and 720 mL L^{-1} sulfuric acid (2.5 mL). The bromocrescol green reagent was prepared by mixing bromocrescol green (40 mg) with absolute ethanol (100 mL) and adding a 0.1 mol L^{-1} solution of aqueous sodium hydroxide dropwise until a light blue colour appeared in the solution. The potassium permanganate reagent was prepared by mixing potassium permanganate (1.5 g), potassium carbonate (10 g) and 100 g L^{-1} sodium hydroxide (1.25 mL) with water (200 mL).

The SPE eluates were screened for UV absorption in the spectrophotometer (220–340 nm) against absolute acetonitrile. The absorbance (280 nm) was recorded for the SPE eluates from toxic and non-toxic genotypes.

RESULTS AND DISCUSSION

Distribution and localisation of antinutrients in *Jatropha* kernel

Jatropha seed kernel mostly stores oil and protein in a living endosperm laterally attached to the cotyledons, which are joined at the base by the hypocotyl. The kernel is covered by a fibrous kernel coat, which in turn is attached to the shell, thus keeping the kernel intact within the shell. The average seed weight was 0.68 ± 0.04 g and the kernel/shell ratio was 59.5 : 40.5. The kernel is reported to contain 580–600 g kg^{-1} oil and 250–300 g kg^{-1} protein.[1–3] The weight distribution of cotyledon, hypocotyl, kernel coat and endosperm was 37.4, 8.6, 17.1 and 937.0 g kg^{-1} respectively (Fig. 1). All these samples were analysed for major antinutrients and toxic compounds. The oil content of the endosperm was 526.0 g kg^{-1}. In the present study, major antinutritional and toxic factors such as phytate, PEs and trypsin inhibitor were investigated (Table 1).

Phytic acid

Cosgrove and Irving[18] have reported some important physiological roles for phytate in plants, such as: (1) phosphate reserve; (2) energy store; (3) a competitor for ATP during its biosynthesis near maturity, when metabolism is inhibited and dormancy is induced; (4) an immobiliser of divalent cations needed for the

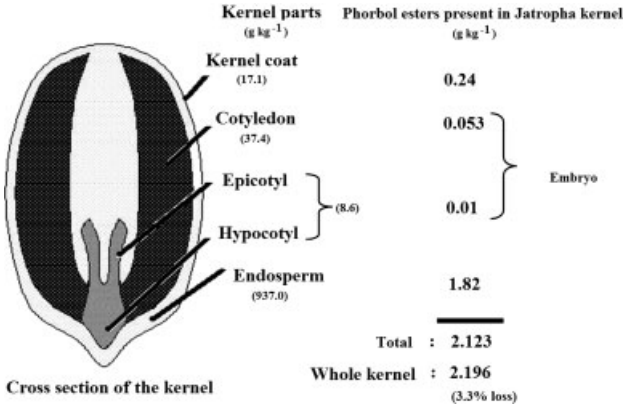

Figure 1. Distribution of phorbol esters in *Jatropha curcas* kernel.

Table 1. Distribution of major antinutrients/toxic factors in *Jatropha curcas* kernel

Sample	Seed part ratio in whole kernel (g kg^{-1})	Phytate[a] (g kg^{-1})	Trypsin inhibitor activity (mg g^{-1})
Kernel coat	17.1	0.84	0.55
Endosperm	937.0	78.1	24.17
Hypocotyl	8.6	0.27	0.002
Cotyledon	37.4	1.7	0.635
Total	1000.1	80.91	24.862

[a] As phytic acid equivalent.

control of cellular processes and released after germination; and (5) a regulator of inorganic phosphate (P_i) in seeds. The present study showed that phytate concentration was highest in the endosperm at 78.1 g kg^{-1}, constituting 96.5% of the total phytate present in the whole kernel, whereas the cotyledon, hypocotyl and kernel coat contained 1.7, 0.27 and 0.84 g kg^{-1}, accounting for 2.1, 0.33 and 1.04% of the total phytate respectively (Table 1), suggesting that the major supply of phosphate during germination for metabolic activities is contributed by phytate present in the endosperm. In most oilseeds the localisation of phytate differs among different parts, concentrating within subcellular inclusions called globoids that are distributed throughout the kernel.[19] The location of phytate within the seed and its chemical associations with other nutrients influence nutrient availability.[20] However, in soybean seeds there appears to be no specific location for phytate.[21] The high phytate content found in protein concentrate prepared from *Jatropha* seed cake indicates that phytate is strongly bound to protein in *Jatropha* kernel and also has high affinity towards protein at low or high pH.[22–24] The calculated value of phytate for defatted *Jatropha* kernel meal (89 g kg^{-1}) was within the range (72–101 g kg^{-1}) reported for various toxic and non-toxic varieties of *J. curcas*, but about 5.9 times higher than that for defatted soy (15 g kg^{-1}).[1–3] For nutritional purposes the high phytate content in *Jatropha* meal is antinutritional, especially when fed to different fish species.[25]

Although many chemical and physical methods have been reported to remove phytate from the meal, enzymatic (phytase) treatment could be beneficial owing to its high specific activity towards phytate. Phytase treatment could improve the nutritional value of *Jatropha* meal as a feed for monogastrics and would also lower phosphorus inclusion in their diets,[8,26] whereas ruminants

Table 2. UV absorption (280 nm) and phorbol ester concentration of SPE eluates from different toxic and non-toxic genotypes of *Jatropha curcas* raw kernel ($n = 3$)

Toxic varieties			Non-toxic varieties		
Genotype	Phorbol esters (mg/mL)	Absorbance at 280 nm	Genotype	Phorbol esters (mg/mL)	Absorbance at 280 nm
Lab sample-5624	0.285	1.028	Lab sample-109	ND	0.055
Cape Verde-6383	0.214	0.663	Lab sample-110	ND	0.053
Paraguay-5472	0.210	0.576	Mexico-5971	ND	0.047
Ghana-5461	0.209	0.465	Mexico-5973	ND	0.047
Puerto Rico (Hatillo)-7709	0.206	0.379	Mexico-6123	ND	0.032
Cape Verde-5929	0.204	0.378	Mexico-7700	ND	0.031
Lab sample-6048	0.195	0.374	Mexico (Veracruz)-7120	ND	0.030
Lab sample-4729	0.189	0.343	Mexico (Veracruz)-7114	ND	0.028
Mexico (Veracruz)-7122	0.182	0.321	Mexico (Veracruz)-7111	ND	0.026
Mexico (Veracruz)-7116	0.139	0.294	Mexico (Veracruz)-7117	ND	0.026
India-5855	0.133	0.277	Mexico (Veracruz)-7124	ND	0.025
Mexico (Veracruz)-7118	0.113	0.217	Mexico (Huatulco)-7708	ND	0.025
Mexico (Oaxaca)-7110	0.102	0.208	Mexico (Veracruz)-7107	ND	0.024
Mexico (Chiapas)-7112	0.092	0.173	Mexico (Veracruz)-7108	ND	0.022
Mexico (Veracruz)-7119	0.083	0.171	Mexico (Veracruz)-7106	ND	0.021
Lab sample-5258	0.074	0.166	Mexico (Veracruz)-7128	ND	0.021
Mexico (Veracruz)-7125	0.072	0.150	Mexico-7701	ND	0.018
Mexico (Veracruz)-7115	0.069	0.130	Mexico (Jonotla)-7707	ND	0.018
Mexico (Oaxaca)-7123	0.052	0.111	Mexico (Xonalpu)-7706	ND	0.017
Mexico (Veracruz)-7109	0.021	0.056	Mexico (Veracruz)-7127	ND	0.016
Mexico (Veracruz)-7104	0.012	0.049	Mexico (Veracruz)-7129	ND	0.016
Mexico (Veracruz)-7120	0.004	0.038	Mexico (Huexta)-7704	ND	0.016
Mexico (Veracruz)-7126	0.003	0.038	Mexico (Chilocco)-7705	ND	0.015
Mexico (Veracruz)-7113	0.002	0.036	Mexico (Huixtla)-7703	ND	0.014
Mexico (Veracruz)-7105	0.002	0.033	Mexico-7702	ND	0.012

ND, not detectable when analysed by HPLC.

are considered to utilise phytate through the action of phytase enzymes produced by ruminal microbes.[27] The presence of phytate in the kernel coat was also found to inhibit aflatoxin B1 production by *Aspergillus flavus*,[28] thus helping in postharvest storage of dry seeds.

Trypsin inhibitor activity

In plants the role of proteinase inhibitors has been related to defence purposes. Generally, defence proteins are stored in dry seeds in anticipation of possible attack from predators. In addition, pathogen or virus attack can induce the synthesis of protease inhibitors, which inhibit the growth of larvae and insects and delay pupation, thus defending against insects that feed on mature dry seeds.[29] In soybean, trypsin inhibitors are supposed to possess a role as storage proteins, regulators of endogenous proteinases, protective against insects and microbial pathogens.[30–33] It is interesting to note that the trypsin inhibitor in *Jatropha* was present in all parts of the kernel. The concentration of trypsin inhibitor in the endosperm was 24.17 mg g^{-1}, contributing nearly 95.32% TIA of the whole kernel, whereas the cotyledon, hypocotyl and kernel coat contained 0.635, 0.002 and 0.55 mg g^{-1}, contributing $<2.5\%$, <0.01 and 2.17% TIA respectively. The calculated value of TIA for defatted *Jatropha* kernel meal (25.36 mg g^{-1}) was within the range (18.4–24.3 mg g^{-1}) reported for various toxic and non-toxic varieties of *Jatropha*, but higher than that for defatted soy bean meal (3.9 mg g^{-1}).[3] In a nutritional context, heat treatment of meal has been found to be highly effective in reducing TIA.[34]

Phorbol esters

PEs constitute a group of compounds that are reported to be co-carcinogenic. Oral consumption of PE-containing material has been found to be toxic in mice, rats, sheep, pigs, chickens and humans.[8] In *Jatropha* seed the first line of defence is the hard shell, which contains a large amount of lignin (~450–480 g kg^{-1}), followed by the kernel coat protecting the endosperm. The shell does not contain PEs. However, the high concentration of PEs in the kernel coat (14.16 g kg^{-1}) suggests that these compounds act as a potent second line of defence in *Jatropha* seed. The results also show that 85.72% of PEs are present in the storage region of the endosperm, providing a defensive environment for the developing embryo during germination (Fig. 1). The kernel coat, hypocotyl and cotyledon contribute 11.3, 0.5 and 2.5% respectively to the total pool of PEs present in the whole kernel. PEs have been shown to have insecticidal and antimicrobial properties, and toxicity studies in carp showed that the threshold level causing adverse effects was 15 mg kg^{-1} diet.[8,15,35] Severe toxicity of these diterpenes has been observed in animals under both natural and laboratory conditions.[8,35] The biosynthesis/distribution of these toxic components in dry seeds seems to be for self-defence against predators and pathogens.

Table 3. UV absorption (280 nm) and phorbol ester concentration of SPE eluates from different toxic and non-toxic genotypes of *Jatropha curcas* defatted kernel meal ($n = 3$)

Toxic varieties			Non-toxic varieties		
Genotype	Phorbol esters (mg/mL)	Absorbance at 280 nm	Genotype	Phorbol esters (mg/mL)	Absorbance at 280 nm
India-5859	0.146	0.297	Mexico (Culiacan)-5681	ND	0.057
Ghana-5462	0.152	0.274	Mexico (Culiacan)-5680	ND	0.043
Ghana-5463	0.113	0.259	Lab sample-5964	ND	0.042
Paraguay-5472	0.096	0.254	Lab sample-5965	ND	0.040
Lab sample-6048	0.095	0.218	Lab sample-5790	ND	0.038
Cape Verde-5929	0.077	0.208	Lab sample-5789	ND	0.036
Ghana-5461	0.087	0.201	Lab sample-5970	ND	0.033
Cape Verde-6383	0.073	0.181	Mexico (Veracruz)-7117	ND	0.027
Paraguay-5629	0.048	0.123	Lab sample-5963	ND	0.027
Mexico (Oaxaca)-7110	0.042	0.109	Mexico (Veracruz)-7104	ND	0.025
India-4260	0.034	0.103	Mexico (Veracruz)-7105	ND	0.023
Mexico (Veracruz)-7118	0.041	0.097	Mexico (Veracruz)-7126	ND	0.023
Ghana-5258	0.040	0.083	Mexico (Veracruz)-7124	ND	0.022
Mexico (Oaxaca)-7123	0.019	0.065	Mexico (Veracruz)-7107	ND	0.021
Mexico (Chiapas)-7112	0.020	0.064	Mexico (Veracruz)-7113	ND	0.021
Mexico (Veracruz)-7119	0.022	0.063	Mexico (Veracruz)-7128	ND	0.021
Mexico (Veracruz)-7125	0.020	0.059	Mexico (Veracruz)-7114	ND	0.017
Mexico (Veracruz)-7115	0.017	0.055	Mexico (Veracruz)-7121	ND	0.017
			Mexico (Veracruz)-7120	ND	0.016
			Mexico (Veracruz)-7109	ND	0.015
			Mexico (Veracruz)-7108	ND	0.013

ND, not detectable when analysed by HPLC.

Qualitative identification of phorbol esters from *J. curcas*

Chromatographic and spectrometric techniques offer simple and fast ways to analyse various analytes. The PE contents in the kernel and defatted kernel meal of toxic and non-toxic *Jatropha* genotypes were determined by HPLC analysis. The samples in which PEs were not detectable by HPLC analysis were assigned as non-toxic, while those that contained PEs were assigned as toxic. The non-toxic genotypes are commonly consumed by local people in Mexico, while those containing PEs are not.[3,13] The methanol extracts from *Jatropha* kernel and defatted kernel meal from both toxic and non-toxic genotypes were spotted on TLC plates and exposed to UV light (366 nm). The spots of toxic genotypes exhibited higher fluorescence than those of non-toxic genotypes. However, no clear distinction could be made to differentiate between the two genotypes. This may be due to the crude nature of the samples, which contained an array of interfering terpenes, other related compounds and their derivatives. In addition, the methanol extracts were passed through a solid phase column to get SPE eluates, and these eluates were spotted on TLC plates. The spots sprayed with vanillin reagent showed a grey colour, and the intensity of the colour was higher for toxic samples than for non-toxic samples. However, no clear distinction could be made between them. Similarly, the results were not distinct when the spots on the TLC plates were sprayed with bromocrescol green, potassium permanganate or dinitrophenylhydrazine reagent (data not shown), suggesting that none of these reagents could be used to differentiate non-toxic and toxic genotypes.

Detection of phorbol esters by UV spectrometry

The PE concentrations in the SPE eluates (from toxic and non-toxic genotypes) were determined by HPLC analysis (Tables 2 and 3). Of the total PEs present in samples, 70% were present in the absolute acetonitrile SPE eluates (data not shown). This indicates that PEs are not completely eluted during SPE owing to their strong binding affinity to the matrix of the solid phase column. However, passing the methanol extracts through the solid phase column helps in the exclusion of impurities. Further, the absorbances of the absolute acetonitrile SPE eluates of toxic and non-toxic genotypes were read between 220 and 340 nm to obtain a spectrum. The results showed that PEs had maximum absorption at 280 nm. At 280 nm the absorption of the SPE eluates increased with increasing PE concentration (Fig. 2, Tables 2 and 3). Overall, the SPE eluates from toxic varieties exhibited higher absorbance than those from non-toxic varieties for both the raw kernel and the defatted kernel meal. The differences in absorbance intensity between and among SPE eluates (raw toxic and non-toxic kernels and defatted toxic and non-toxic kernel meals) are largely due to differences in genotype and PE content (Tables 2 and 3). A linear relationship was observed when the absorbance of SPE eluates from raw kernels and defatted meals was plotted against their PE concentration determined by HPLC analysis (Fig. 2). From the results of this study an absorbance cut-off could be defined to differentiate between toxic and non-toxic varieties. For raw kernels, SPE eluates with absorbance ≥ 0.056 were considered as toxic and those with absorbance ≤ 0.032 as non-toxic; however, SPE eluates with absorbance between 0.056 and 0.032 should be confirmed as toxic or non-toxic by HPLC analysis of their PEs (Table 2). Similarly, for defatted kernel meals, SPE eluates with absorbance ≥ 0.059 were considered as toxic and those with absorbance ≤ 0.043 as non-toxic; however, SPE eluates with absorbance between 0.059 and 0.043 should be confirmed as toxic or non-toxic by HPLC analysis of their PEs (Table 3). These studies suggest that the PEs of *J. curcas* raw kernel/defatted kernel

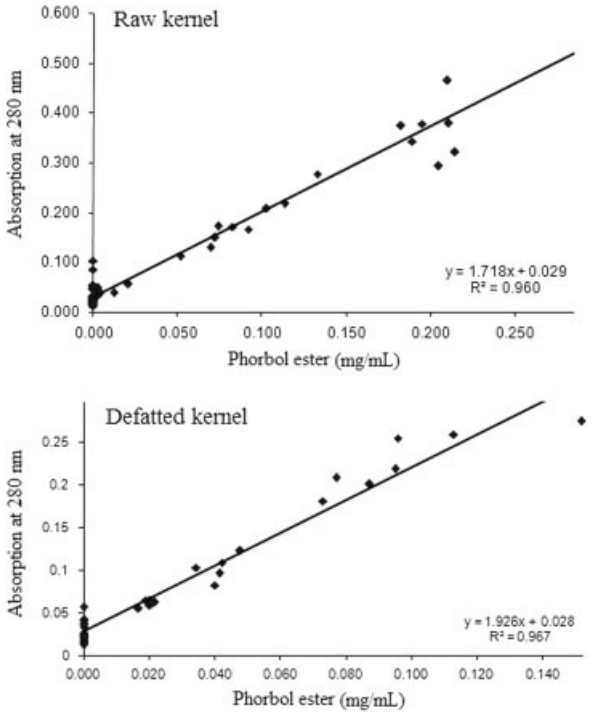

Figure 2. Linear relationship between absorption at 280 nm and phorbol ester concentration of SPE eluates from different toxic and non-toxic genotypes of *Jatropha curcas* kernel ($n = 3$).

meal can be qualitatively assessed by the method outlined here. The time required for analysing one sample by this HPLC method is 45 min, and, during this time, more than 10–15 samples could be qualitatively screened using the method developed in this study.

CONCLUSIONS

Major antinutrients such as phytate, trypsin inhibitor and PEs are localised in the endosperm of the kernel of *Jatropha* seed. As PEs are the major toxic component, a new qualitative method has been developed for the rapid screening and differentiation of toxic and non-toxic genotypes of *J. curcas*. Other advantages of the developed method are that it is easy, affordable and cost-effective and a large number of samples can be analysed in a short time. This qualitative method could find applications in the biodiesel industry for screening the toxicity of products, by-products or co-products obtained from *Jatropha*. However, confirmation of toxic and non-toxic genotypes, especially for food/feed applications, must be done using the already established HPLC-based method for identification and quantification of PEs.

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REFERENCES

1 Makkar HPS, Becker K, Sporer F and Wink M, Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. *J Agric Food Chem* **45**:3152–3157 (1997).
2 Makkar HPS, Aderibigbe AO and Becker K, Comparative evaluation of nontoxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. *Food Chem* **62**:207–215 (1998).
3 Makkar HPS, Becker K and Schmook B, Edible provenances of *Jatropha curcas* from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. *Plant Foods Hum Nutr* **52**:31–36 (1998).
4 Raju AJS and Ezradanam V, Pollination ecology and fruiting behaviour in a monoecious species, *Jatropha curcas* L. (Euphorbiaceae). *Curr Sci* **83**: 1395–1398 (2002).
5 Kochhar S, Singh SP and Kochhar VK, Effect of auxins and associated biochemical changes during clonal propagation of the biofuel plant – *Jatropha curcas*. *Biomass Bioenerg* **32**:1136–1143 (2008).
6 Harborne JB, *Introduction to Ecological Biochemistry*. Academic Press, London (1977).
7 Goel G, Makkar HPS, Francis G and Becker K, Phorbol esters: structure, biological activity and toxicity in animals. *Int J Toxicol* **26**:279–288 (2007).
8 Devappa RK, Makkar HP and Becker K, *Jatropha* toxicity – a review. *J Toxicol Environ Health B* **13**:476–507 (2010).
9 Acda MN, Toxicity, tunneling and feeding behavior of the termite, *Coptotermes vastator*, in sand treated with oil of the physic nut, *Jatropha curcas*. *J Insect Sci* **6**:1–8 (2009).
10 Achten WMJ, Verchot L, Franken YJ, Mathijs E, Singh VP and Aerts R, *Jatropha curcas* bio-diesel production and use. *Biomass Bioenerg* **32**:1063–1084 (2008).
11 Gressel J, Transgenics are imperative for biofuel crops. *Plant Sci* **174**:246–263 (2008).
12 Devappa RK, Makkar HPS and Becker K, Optimization of conditions for the extraction of phorbol esters from *Jatropha* oil. *Biomass Bioenerg* **34**:1125–1133 (2010).
13 Makkar HPS, Kumar V, Oyeleye OO, Akinleye AO, Angulo-Escalante MA and Becker K, *Jatropha platyphylla*, a new nontoxic *Jatropha* species: physical properties and chemical constituents including toxic and antinutritional factors of seeds. *Food Chem* **125**:63–71 (2011).
14 Divakara BN, Upadhyaya HD, Wani SP and Laxmipathi Gowda CL, Biology and genetic improvement of *Jatropha curcas* L.: a review. *Appl Energ* **87**:732–742 (2010).
15 Becker K and Makkar HPS, Effects of phorbol esters in carp (*Cyprinus carpio* L.). *Vet Hum Toxicol* **40**:82–86 (1998).
16 Makkar HPS and Becker K, Nutritional studies on rats and fish (carp *Cyprinus carpio*) fed diets containing unheated and heated *Jatropha curcas* meal of a nontoxic provenance. *Plant Foods Hum Nutr* **53**:182–292 (1999).
17 Makkar HPS, Siddhuraju P and Becker K, *A Laboratory Manual on Quantification of Plant Secondary Metabolites*. Humana Press, Totowa, NJ (2007).
18 Cosgrove DJ and Irving GCJ, *Inositol Phosphates: Their Chemistry, Biochemistry and Physiology*. Elsevier/North Holland, New York, NY (1980).
19 Erdman JW, Oilseed phytates: nutritional implications. *J Am Oil Chem Soc* **56**:736–741 (1979).
20 Angel R, Tamim NM, Applegate TJ, Dhandu AS and Ellestad LE, Phytic acid chemistry: influence on phytin-phosphorus availability and phytase efficacy. *J Appl Poultry Res* **11**:471–480 (2002).
21 Ravindran V, Bryden WL and Kornegay ET, Phytates: occurrence, bioavailability, and implications in poultry nutrition. *Poultry Avian Biol Rev* **6**:125–143 (1995).
22 Makkar HPS, Francis G and Becker K, Protein concentrate from *Jatropha curcas* screw-pressed seed cake and toxic and antinutritional factors in protein concentrate. *J Sci Food Agric* **88**:1542–1548 (2008).
23 Cheryan M, Phytic acid interactions in food systems. *CRC Crit Rev Food Sci Nutr* **13**:297–334 (1980).
24 Okubo K, Myers DV and Iacobucci GA, Binding of phytic acid to glycinin. *Cereal Chem* **53**:513–524 (1976).
25 Kumar V, Makkar HP, Devappa RK and Becker K, 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.). *Food Chem Toxicol* **49**:2144–2156 (2011).
26 Devappa RK, Makkar HPS and Becker K, Nutritional, biochemical, and pharmaceutical potential of proteins and peptides from jatropha: review. *J Agric Food Chem* **58**:6543–6555 (2010).

27 Duffus CM and Duffus JH, Introduction and overview, in *Toxic Substances in Crop Plants*, ed. by D'Mello FJP, Duffus CM and Duffus JH. Royal Society of Chemistry, Cambridge, pp. 1–21 (1991).
28 Chen D, Ling X and Rong Y, Phytic acid inhibits the production of aflatoxin B1. *J Food Process Preserv* **19**:27–32 (1995).
29 Ryan CA, Protease inhibitors in plants: genes for improving defence against insects and pathogens. *Annu Rev Phytopathol* **28**:425–449 (1990).
30 Wilson KA, The release of proteinase inhibitors from legume seeds during germination. *Phytochemistry* **19**:2517–2519 (1980).
31 Halim AH, Wassom CE, Mitchel HL and Edmunds LK, Suppression of fungal growth by isolated trypsin inhibitors of corn grain. *J Agric Food Chem* **21**:1118–1119 (1973).
32 Mosolov VV, Loginova MD, Fedurkina NV and Benken II, The biological significance of proteinases inhibitors in plants. *Plant Sci Lett* **7**:77–80 (1976).
33 Mosolov VV, Loginova MD, Malova EL and Benken II, A specific inhibitor of *Colletotrichum lindemuthianum* protease from kidney bean (*Phaseolus vulgaris*) seeds. *Planta* **144**:265–269 (1979).
34 Aderibigbe AO, Johnson COLE, Makkar HPS, Becker K and Foidl N, Chemical composition and effect of heat on organic matter and nitrogen degradability and some antinutritional components of *Jatropha* meal. *Anim Feed Sci Technol* **67**:223–243 (1997).
35 Wink M, Koschmieder C, Sauerweien M and Sporer F, Phorbol esters of *J. curcas* – biological activities and potential applications, in *Biofuel and Industrial Products from* Jatropha curcas, ed. by Gubitz GM, Mittelbach M and Trabi M. DBV Verlag, Graz, pp. 160–166 (1997).

CHAPTER - 3

Optimization of conditions for the extraction of phorbol esters from Jatropha oil

Rakshit K. Devappa, Harinder P.S. Makkar* and Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany.

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Optimization of conditions for the extraction of phorbol esters from Jatropha oil

Rakshit K. Devappa, H.P.S. Makkar, K. Becker*

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany

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ABSTRACT

The production of *Jatropha curcas* seeds as a biodiesel feedstock is expected to reach 160 Mt by 2017. The present study aims at extracting phorbol esters (PEs) as a co-product from Jatropha oil before processing it to biodiesel. The conditions were optimized for extraction of PEs in organic solvents by using a magnetic stirrer and an Ultra turrax. The extent of reduction in PEs was >99.4% in methanol using any of the stirring tools. However, the extraction using Ultra turrax affected considerably the colour of the remaining oil. Therefore, further solvent:oil ratio, time and temperature were optimized using a magnetic stirrer to get PE rich fraction-I (48.4 mg PEs g^{-1}) and virtually PE-free oil. PEs were 14 fold higher in this fraction than the control oil. PEs, extracted in methanol from the untreated Jatropha oil, at 1 mg L^{-1} produced 100% mortality in snails (*Physa fontinalis*). The methanol extract from virtually PE-free oil when concentrated 20 and 25 time the untreated Jatropha oil (equivalent of 20 mg L^{-1} and 25 mg L^{-1} PEs in the control oil) was nontoxic to snails. PE rich fraction-I, obtained as a co-product, can be used in agricultural, medicinal and pharmaceutical applications and the remaining oil can be used for biodiesel preparation. The remaining oil will be friendly to the environment and workers.

1. Introduction

In recent years, biodiesel is receiving considerable attention as a renewable source of energy. Biodiesel can be produced by transesterification of plant oils or animal fats. One of the non-edible feedstocks that has received great attention as a source of renewable energy is *Jatropha curcas*. Jatropha is a small tree or shrub distributed in the subtropical and tropical regions of the world. The plant is stress tolerant, drought resistant, grows in semi arid and marginal lands. The plant produces capsulated fruits bearing seeds. On an average seed weight ranges from 0.53 to 0.86 g and it contains 30–40% oil [1]. This inedible oil can be easily converted into biodiesel that meets American and European standards [2]. Seeds and vegetative parts of Jatropha are toxic in nature. The seeds contain toxic (phorbol esters, PEs) and antinutritional factors (trypsin inhibitor, phytate, lectin and curcin) [3]. Among them, PEs are the most potent and they exhibit a wide range of biological activities affecting from microorganisms to higher animals.

The PEs are located mainly in kernel portion of the seed and their concentration varies with the genotype ranging from 0.8 to 3.3 mg g^{-1} kernel [4]. Hitherto, six different PEs from *J. curcas* have been characterized [5]. All of them have a tigliane basic skeleton with four rings (A, B, C and D), hydrogenation of this structure at different positions and ester bonding to various acid moieties results in the formation of different PEs. In general, PEs are known to activate protein kinase C by mimicking the diacyl glycerol, which in turn activates cascade of signal transduction reaction causing

* *Corresponding author*. Tel.: +49 71145923640; fax: +49 71145923702.
E-mail address: makkar@uni-hohenheim.de (H.P.S. Makkar).
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tumor promotion. PEs are cocarcinogens, they cause tumor promotion only in presence or following exposure to sub-carcinogenic dose of carcinogens [6]. PEs are hydrophobic in nature, oil soluble and heat stable when present in oil or seed cake [7].

Traditionally, the oil has been used to treat skin diseases and to soothe pain such as that caused by rheumatism. It is also used as a purgative [8]. Various aqueous and organic solvent extracts of the seeds have a wide range of activities from microorganism to higher animals. For example, organic solvent extracts from seeds, oil and vegetative parts have moluscicidal activity against *Biomphalaria glabrata* (which cause schistosomiasis), insecticidal activity against mosquitoes like, *Aedes aegypti* L. (which cause dengue fever) and *Culex quinquefasciatus* (lymphatic filariasis vector), and anti-birth activity against houselfies (*Monodelphis domestica*) [6,9]. They also exhibit toxicity in higher animals (mice, rat, sheep, goat, pig and chicken) and have antibacterial, fungicidal and rodenticidal properties [10–16]. In majority of the organic solvent extracts tested, PEs are considered to be the active principle.

The International Jatropha Organization has claimed that in 2017 there will be around 330,000 km^2 of land cultivated worldwide producing 160 Mt of seeds and 95% of its total production will be concentrated in Asia. The total projected annual Jatropha oil production in Asian countries will be 47 Mt, with India and China together playing a major role [17]. This indicates huge feedstock supply in future for the Jatropha based biodiesel industry.

The seeds are mechanically pressed or solvent extracted to get oil. The oil cannot be used without detoxification for nutritional purposes making it attractive for biodiesel production. During the mechanical/solvent extraction, majority of PEs present in the seeds comes in the oil fraction. The oil is a rich source of PEs. Studies in our laboratory show that PE concentration in the oil from different genotype varies from 2 to 8 mg g^{-1} (unpublished). In the process of producing biodiesel, the oil is further subjected to many treatments such as degumming, stripping and esterification. In all these stages, the PEs undergo partial or complete destruction depending on the deodorisation conditions [18]. Instead of losing the PEs, if a suitable method can be adopted to extract these esters before the oil is taken to biodiesel production, which does not change the biodiesel quality, the PEs could be a valued co-product that would contribute to enhance economic viability and sustainability of Jatropha oil based biodiesel production chain. The PEs can find various applications in agriculture, medicine and pharmaceutical industries.

During the last decade, increased consumer preference along with global appeal for using renewable natural sources as biocontrol agents in conventional agricultural practices has propelled the search for new raw materials. PEs obtained could meet these requirements for some of the agricultural applications. It may be noted that PEs are easily degradable in soil [19].

The objective of the study was to comprehensively evaluate the effects of mechanical extractions combined with organic solvents on the yield and biological activity of extracted PEs.

2. Experimental

2.1. Materials

J. curcas seeds were collected in November 2007 from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. These were transported to Germany and stored in airtight bags in dark, cool and dry place until further analysis. Phorbol 12-myristate-13-acetate (PMA; CAS number 16561-29-8) was obtained from Sigma (St. Louis, USA) and all other chemicals/solvents used were of analytical grade.

2.2. Preparation of Jatropha oil

J. curcas seeds were mechanically pressed using a screw press to obtain oil. The oil was centrifuged at 3150 × gravity for 20 min to remove residues and the supernatant was collected by gravity separation and stored in a refrigerator (4 °C) until further use.

2.3. Extraction of phorbol esters

In order to optimize the conditions, two different mechanical approaches were used: (1) high shear mixer (Ultra turrax), and (2) a magnetic stirrer. Initially efficiency of different organic solvents for extraction of PEs was investigated. Methanol was found to be the best solvent. Subsequently extraction conditions in this solvent were optimized. Below, the optimized procedure has been presented. The approaches and steps used to arrive at this optimized procedure are illustrated in the following sections.

2.3.1. The optimized procedure

The Jatropha oil was mechanically extracted with methanol (1:2 w/v) using magnetic stirrer (300 rpm) at 60 °C for 5 min. The resulting mixture was gravity separated. The upper methanol layer was rotaevaporated to get the PE rich fraction.

2.3.2. Selection of solvents

Jatropha oil was extracted using ethanol, methanol, 2% dichloromethane (DCM) in methanol (v/v) and 2% 1:1 (DCM: Tetra hydro furan, THF, v/v) in methanol (v/v). The extraction of PEs was carried out by following Approach 1 mentioned below for methanol.

In all the approaches, the starting weight of the oil was 20 g. Each experiment was done at least in duplicate.

2.3.3. Approach 1

Jatropha oil was mixed with methanol (1:1, w/v) in a capped container and the contents were stirred at room temperature (23 °C) for 15 min using a magnetic stirrer (300 rpm). Thereafter, the mixture was centrifuged at 3150 × gravity for 5 min to get upper methanolic and lower oily layers. Both the layers were separated. The oily layer was re-extracted 3 more times with the fresh solvent (1:1, w/v) as stated above.

2.3.4. Approach 2

Jatropha oil mixed with methanol (1:1, w/v) was homogenized using a high shear mixer (ULTRA TURRAX-T25, Janke and

Kunkel, IKA-labortechnik, 600 W, 8,000–24,000 rpm) at 9500 rpm for 2 min at room temperature (23 °C). Rest of the procedure was similar to that described in Approach 1 except that the oily layer was extracted 3 times using the Ultra turrax (2 min each) instead of using a magnetic stirrer.

2.3.5. *Approaches 3 and 4*

Approach 3 was carried out using the Ultra turrax (as in Approach 2; 9500 rpm, 2 min each) at methanol:oil ratios of 1:1, 1.5:1 or 2:1; v/w (fresh methanol added after each extraction). Instead of centrifugation, gravity separation method (60 °C, 15 min) was used to recover methanolic layer from the oily layer.

In Approach 4 additional two speeds of the Ultra turrax (13,000 and 20,500 rpm) were also used, and the extraction was carried out at methanol:oil ratios of 1:1, 1.5:1 or 2:1 (v/w). For each methanol:oil ratio, extraction was carried out for a total of 8 min (4 × 2 min each; 1 min interval in between) and without changing methanol.

Since drastic change in oil colour was observed in treatments using Ultra turrax (see Results and Discussion), subsequent approaches were carried out using a magnetic stirrer.

2.3.6. *Approach 5*

The method of extraction was similar to that described in Approach 1, except that different ratios of methanol:oil (see Table 2) were used, the temperature of extraction was 60 °C and gravity separation method (60 °C, 15 min) was used to separate methanol from oil. The use of this non distillation method for recovering solvent after extraction requires 25–30% less energy than centrifugation. The extraction was done in a capped container kept in a water bath adjusted at 60 °C.

2.3.7. *Approach 6*

The optimum methanol:oil ratios arrived at from the results of Approach 5 were taken and the extraction was carried out in a water bath adjusted at different temperatures (35 °C, 45 °C, 55 °C, 60 °C, 65 °C and 75 °C) for 15 min. Gravity separation method was used to recover methanolic layer from the oily layer. The aim was to determine the optimum temperature.

2.3.8. *Phorbol esters analysis*

PEs were determined at least in duplicate [4,20]. Briefly, 0.5 g of oil sample was extracted four times with methanol. A suitable aliquot was loaded into a high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 × 4 mm id, 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 $cm^3\ min^{-1}$ using a gradient elution. The four phorbol ester peaks containing six PEs were detected at 280 nm and appeared between 25.5 and 30.5 min. The spectra were taken using Merck-Hitachi L-7450 photodiode array detector. PMA was used as an external standard (appeared between 31 and 32 min). The area of the four phorbol ester peaks was summed and converted to PMA equivalent by taking its peak area and concentration.

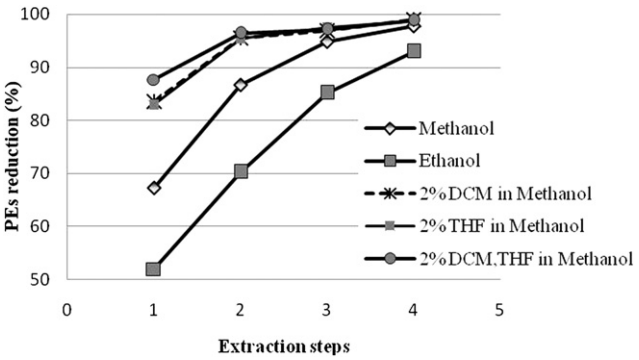

Fig. 1 – Effect of various solvents on extraction of phorbol esters (PEs).

PEs were analyzed in methanol fractions isolated from the control (untreated) Jatropha oil, virtually PE-free oil, low-PE oil, and PEs rich fraction-I (for virtually PE-free oil, low-PE oil and PEs rich fraction-I see Results and Discussion).

2.3.9. *Bioassay for toxicity in snails*

Snails are highly susceptible to phorbol esters. Tests with *Physa fontinalis* were performed according to the method Rug and Ruppel [21]. All tests were carried out in deionized water at room temperature (23 °C). Stock solutions of extracts were prepared in methanol and further diluted in water. Groups of 10 snails were placed in glass containers with 400 ml of water containing the test substance (methanol extract diluted in water). Snails were prevented from crawling out of the containers by a fine stainless steel mesh suspended just above the water surface. After 24 h of incubation the snails were transferred to deionized water and maintained for another 48 h. Death of the snails was determined by absence of movement and lack of reaction to irritation of the foot with a needle. Control experiments were performed with the same quantity of methanol in water as used for the test preparations and no mortality was recorded in the control containers. All tests were independently repeated three times. Toxicity is expressed as LC_{100}, referring to concentrations killing 100% of the snails.

3. Results and discussion

In the present study, the initial concentration of PEs was 2.53 mg g^{-1} oil. This level was lower than that reported earlier

Table 1 – Percent of phorbol esters (PEs) reduction in Approaches 1 and 2.

Extraction steps	Total solvent consumed (volume/weight of oil)	Approach 1[a] (%)	Approach 2[a] (%)
1	2	69.4	72.1
2	4	88.5	88.2
3	6	95.7	96.2
4	8	98.5	98.8

The values are average of three determinations.
a Temperature: 23 °C.

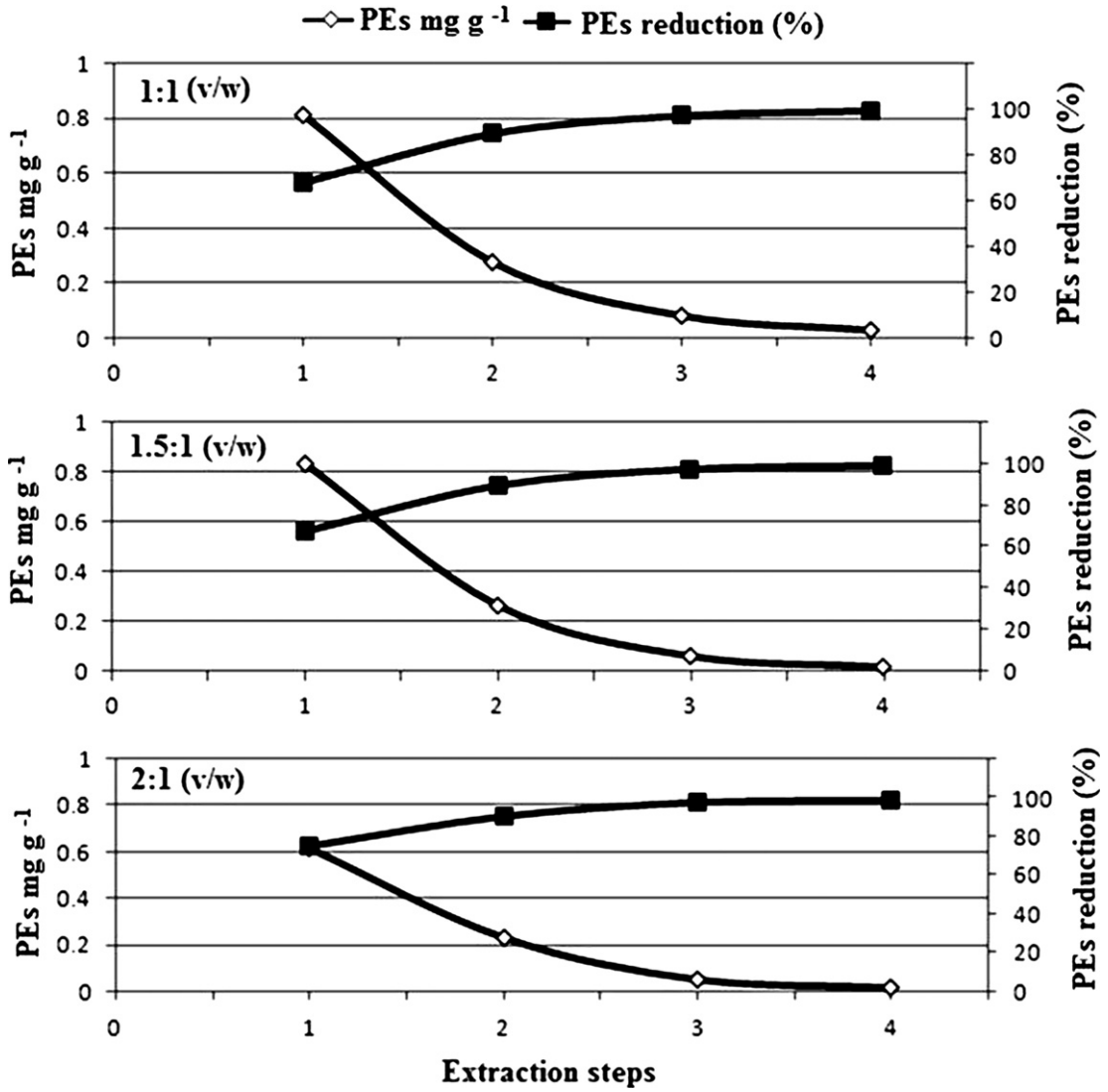

Fig. 2 – Extraction of phorbol esters (PEs) at different methanol:oil (v/w) ratios using Approach 3.

(2–8 mg g^{-1}) from our laboratory for different genotypes (unpublished observation). This oil was subjected to two different approaches with the intention of obtaining PE-free oil, which can be transesterified to produce biodiesel and simultaneously recovering the PEs in the organic solvent phase for potential use in agricultural, medicinal and pharmaceutical industries.

3.1. *Selection of the solvent*

The criteria for selection of suitable solvents include its ability to extract the desired compound, low cost of solvent and of energy used in the extraction, and product yield. In addition, the solvent should be easily recoverable, nontoxic, stable, non reactive with the sample and with the equipment, and adequately available with high purity and at low price, and should have low flammability. The ability to fullfill these requirements depends in part upon physical and chemical properties of the solvent. Commercial solvents used for extraction include hydrocarbon naphthas, halogenated hydrocarbons, alcohols, aldehydes, ketones or mixed solvents [22]. In the present study, percent PE reduction when extracted with 2% dichloromethane in methanol, 2% tetrahydrofuran in methanol, 2% (1:1) DCM: THF in methanol, methanol and ethanol at room temperature is shown in Fig. 1. In this study, mixed solvents were chosen to compare the efficacy of PEs extraction against the single solvent extraction. PE reduction at first extraction was maximum (87%) with 2% (1:1) DCM:THF in methanol, which was followed by 2% DCM in methanol (83.5%), 2% THF in methanol (83%), methanol (67.3%) and ethanol (52%). Although, the mixed solvent systems were effective in reducing the PE content in the oil, they are less suitable for industrial use. At large scale use, this could enhance complexity of the extraction process and make the solvent recovery difficult. This ultimately would increase cost of the treatment. Considering the above factors, the single solvent system was preferred. Since the extent of PE extraction was better in methanol than in ethanol, methanol was selected for further optimization.

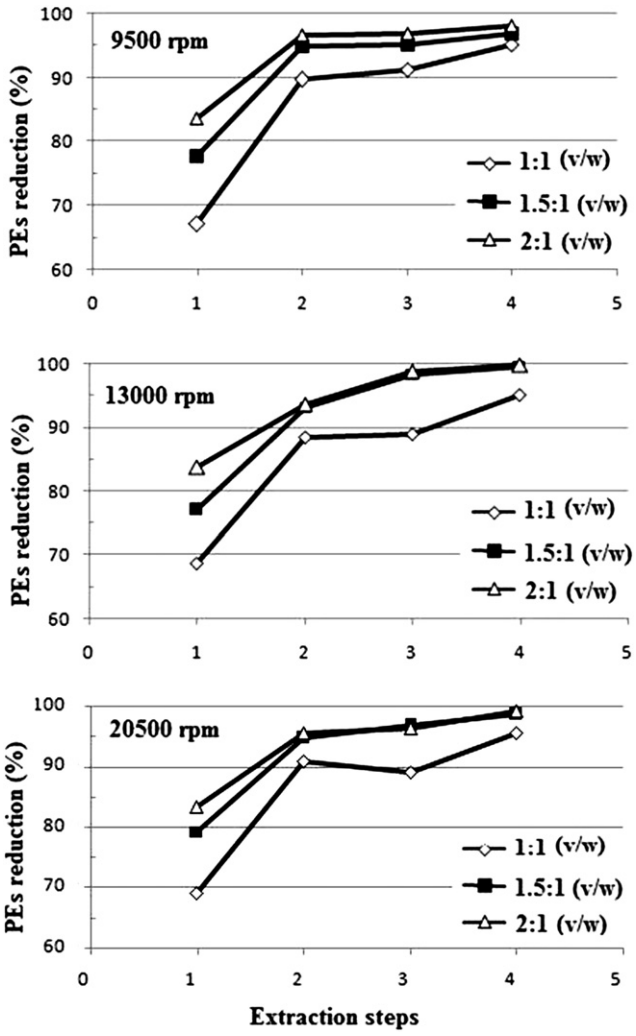

Fig. 3 – Extraction of phorbol esters (PEs) using Ultra turrax at different speeds (Approach 4).

3.2. *Approaches 1 and 2*

The oil was subjected to two different extraction systems with methanol as the solvent. The result showed that for both Approach 1 (using magnetic stirrer) and Approach 2 (Ultra turrax), the reduction in PEs increased with increase in the extraction steps (Table 1). In Approach 1 and Approach 2, 69% and 72% reduction, respectively, after the first extraction step was observed. However, in both the approaches maximum reduction of PEs was observed (>99%) only after fourth extraction. Although, both approaches are effective in reducing 99% of PEs, they consumed eight litres of methanol for one kg of oil, and the total extraction time was 60 min for Approach 1 and 8 min for Approach 2.

In another study, Approaches 1 and 2 were repeated in the presence of UV light (6 W, distance between lamp and top layer: 10 cm), with the contention that UV light might accelerate the extraction of PEs. However, no difference was observed in presence or absence of UV light (results not shown).

3.3. *Approach 3*

The extraction using Approach 2 had an advantage over Approach 1 with respect to time of extraction. So, further optimization was done using Approach 2 (use of Ultra turrax), by changing methanol to oil ratio. The extent of PE extraction at the first step increased with increase in the ratio of solvent to oil. At the first extraction step the percent PE reduction was 75.5, 67.8, 67.2 for 2:1, 1.5:1, 1:1 (methanol:oil ratio; v/w). With increase in the extraction steps, the extent of PE extraction increased and reached >99% after the fourth extraction at all the methanol to oil ratios (Fig. 2). From these results it is evident that higher is the initial solvent volume, higher the extraction of PEs at first step.

Total volume of methanol used in all the four steps was 8, 6 and 4 L for methanol:oil ratio of 2:1, 1.5:1 and 1:1 (v/w), respectively.

3.4. *Approach 4*

Further, studies were carried out to reduce the solvent utilization, without changing the solvent at each extraction step and at different extraction speed. The reduction of PE increased with increase in solvent to oil ratio and with increase in Ultra turrax speed. Although PE was reduced (>99%) at all the extraction speeds after four extraction steps, PE extraction at first step was highest at 2:1 methanol:oil ratio, followed by 1.5:1 and 1:1 ratios. At 2:1 ratio of methanol:oil, the extent of PE extraction was similar at 13,000 rpm and 20,500 rpm, but was lowest at 9500 rpm. So, extraction at 2:1 of methanol:oil ratio at 13,000 rpm was chosen as the best amongst these speeds (Fig. 3). It was observed that as the PE extraction increased with increase in speed and time of the extraction and in methanol: oil ratio, colour of the oil drastically changed from golden yellow to greyish black. We also observed that as the speed increased the temperature of the extraction medium increased (9,500 rpm, 24 °C; 20,500 rpm, 43.8 °C; temperatures are after fourth extraction). Although, the Ultra turrax treatment (Approach 4) was effective in reducing PEs, milder treatment (magnetic stirring) might be able to maintain the quality of the oil better for biodiesel production.

3.5. *Approach 5*

Based on the results of Approach 4, further optimizations were carried out using a magnetic stirrer (continuation of Approach 1). Each extraction was done for 15 min using different methanol:oil ratios as shown in Table 2. In Approach 5, for all the methanol:oil ratios, reduction in PEs was >98% after four extraction steps (Table 2). Among them, Approach 5*c* and 5*f* were promising with 78% reduction in PEs at first step of the extraction. Methanol consumed in approaches 5*c* and 5*f* was 8 and 5.5 times, respectively, the weight of the oil. Considering the total solvent used for the extraction, Approach 5*f* was chosen as the best. So, Approach 5*f* was further optimized for the extraction temperature.

3.6. *Approach 6*

Approach 6 was carried out using Approach 5*f* at temperatures 35 °C, 45 °C, 55 °C, 60 °C, 65 °C and 75 °C. Although methanol

Table 2 – Effect of methanol to oil ratio on the extraction of phorbol esters (PEs) from Jatropha oil (Approach 5).

Approach	Extraction steps	Treatment[a] (with methanol change)	PEs μg g^{-1} oil	Percent reduction	Total time (min)	Methanol used (volume/weight of oil)
5a	1	1:1	950	64.2	15	1
	2	1:1	304	88.5	30	2
	3	1:1	111	95.8	45	3
	4	1:1	40	98.5	60	4
5b	1	1:1.5	650	75.5	15	1.5
	2	1:1.5	187	92.9	30	3
	3	1:1.5	58	97.8	45	4.5
	4	1:1.5	12	98.5	60	6
5c	1	1:2	578	78.2	15	2
	2	1:2	134	94.9	30	4
	3	1:2	26	99.0	45	6
	4	1:2	4.7	99.8	60	8
5d	1	1:1.5	719	72.8	15	1.5
	2	1:1	134	94.9	30	2.5
	3	1:1	94	96.4	45	3.5
	4	1:1	41	98.4	60	4.5
5e	1	1:1.5	641	75.8	15	1.5
	2	1:1.5	176	93.3	30	3
	3	1:1	70	97.3	45	4
	4	1:1	29	98.6	60	5
5f	1	1:2	588	77.8	15	2
	2	1:1.5	185	93.0	30	3.5
	3	1:1	56	97.9	45	4.5
	4	1:1	15.5	99.4	60	5.5

The values are average of three determinations.
a Treatment time for all was 15 min.

has high boiling point (64.7 °C), higher temperatures (65 °C and 75 °C) were also taken for the study. The results indicated that the percent reduction in PEs increased with increase in temperature, reaching maximum at 55 °C (87%) and then started decreasing with further increase in temperature (Fig. 4). The decrease in PEs extraction at higher temperature may be due to evaporation and change in methanol:oil ratio. Although, at 55 °C, 60 °C and 65 °C PE extraction was almost similar, 55 °C was chosen, because at higher temperature more solvent is lost due to evaporation and moreover more energy is required, which would increase the cost of PEs extraction.

3.7. *Pilot scale extraction*

A pilot scale extraction was carried out using Approach 6 at 55 °C with fresh batch of Jatropha oil which had a phorbol ester concentration of 3.45 mg g^{-1}. Jatropha oil (1215 g) was extracted as shown in the flow diagram (Fig. 5). After four extraction steps, the oil obtained was almost free of PEs (PE < 23 μg g^{-1} oil), termed as virtually PE-free oil (Fig. 6a and b). The material balance showed the recovery of 7.1% of PE rich fraction-I and approximately 92.3% of the almost PE-free oil. There was a negligible loss of material (approximately 0.6%) during the extraction. Total solvent used for the extraction was 6.45 L, from which 98% was recovered. The concentration of PEs in PE rich fraction-I was 48.4 mg g^{-1}. This fraction was enriched for PEs by approximately 14 times compared to the starting oil.

3.8. *Evaluation of bioactivity using snail*

Further, the virtually PE-free oil along with the control (untreated) Jatropha oil and PE rich fraction-I was evaluated for toxicity in snails. The control oil and PE rich fraction-I exhibited LC_{100} at 1 mg L^{-1} PEs. Mortality of snails at 0.1, 0.2, 0.5 and 1 mg L^{-1} PEs was 6.7%, 30%, 63% and 100%, respectively. Whereas, methanol extract of virtually PE-free oil when concentrated 20 and 25 time the untreated Jatropha oil (equivalent of 20 mg L^{-1} and 25 mg L^{-1} PEs in the control oil)

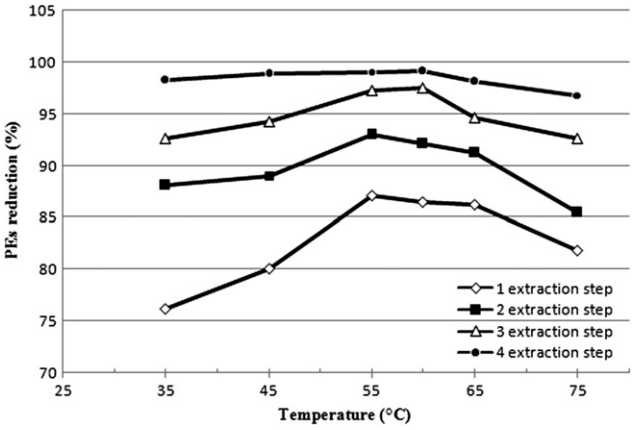

Fig. 4 – Effect of temperature on the extraction of phorbol esters (PEs) (Approach 6).

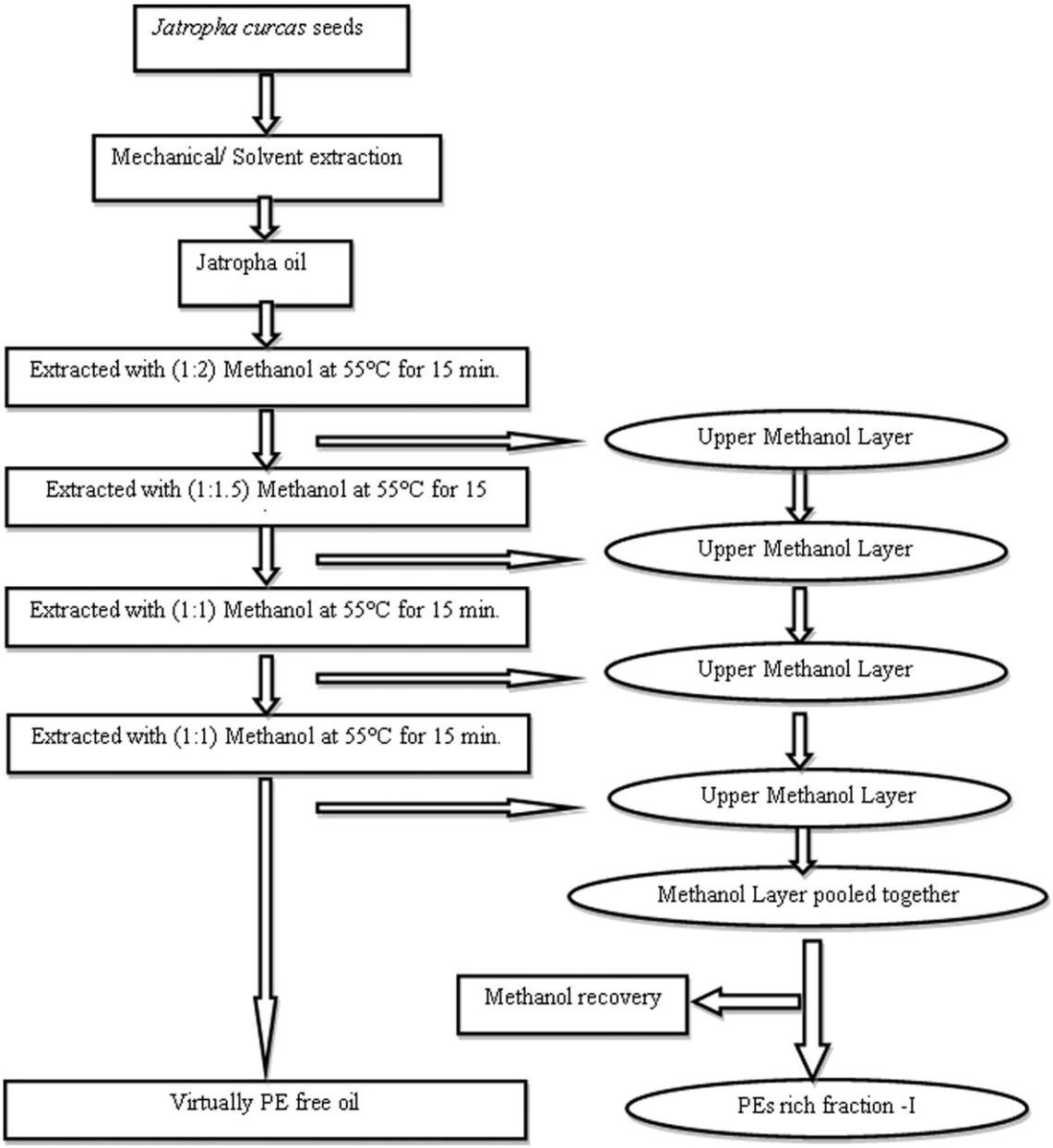

Fig. 5 – Flow diagram for the preparation of virtually phorbol ester free oil and phorbol ester rich fraction-I.

did not show any sign of toxicity. However, at higher concentrations (30 and 40 times concentrated) (equivalent of 30 mg L^{-1} and 40 mg L^{-1} PEs in the control oil) produced 6.7 and 13.3% mortality, respectively. This may be due to the residual phorbol esters present in virtually PE-free oil (<23 μg g^{-1}). By taking this concentration, PE level present in 30 and 40 times concentrated virtually PE-free oil corresponds to 0.28 and 0.37 mg L^{-1} (Fig. 6c). The mortality observed at 0.2 mg L^{-1} of PEs (6.7%) from control oil was almost similar to that observed by 30 times concentrated virtually PE-free oil (equivalent of 30 mg L^{-1} PEs in the control oil). No toxicity to snails even after 25 fold concentration of the virtually PE-free oil indicates that recovering PEs from Jatropha oil before processing it to biodiesel would make it innocuous.

3.9. *Practical considerations*

Complete removal of PEs from the oil takes 60 min, which might be considered long for the industry. So, studies were continued to extract maximum PEs in the shortest possible time. The extraction was carried out only once at methanol:oil ratio of 2:1 (v/w) at temperatures 55 °C, 60 °C and 65 °C for 15 min using a magnetic stirrer. The experiment was carried out in quadruplets. At these temperatures it was possible to extract 78%, 79% and 77%, respectively, of PEs. As shown above, the remaining PEs (21–23%) can be extracted with successive extraction steps, but might not be worth spending energy and time for recovering the remaining PEs. Further studies showed similar extent of PE extraction after 5, 10 and

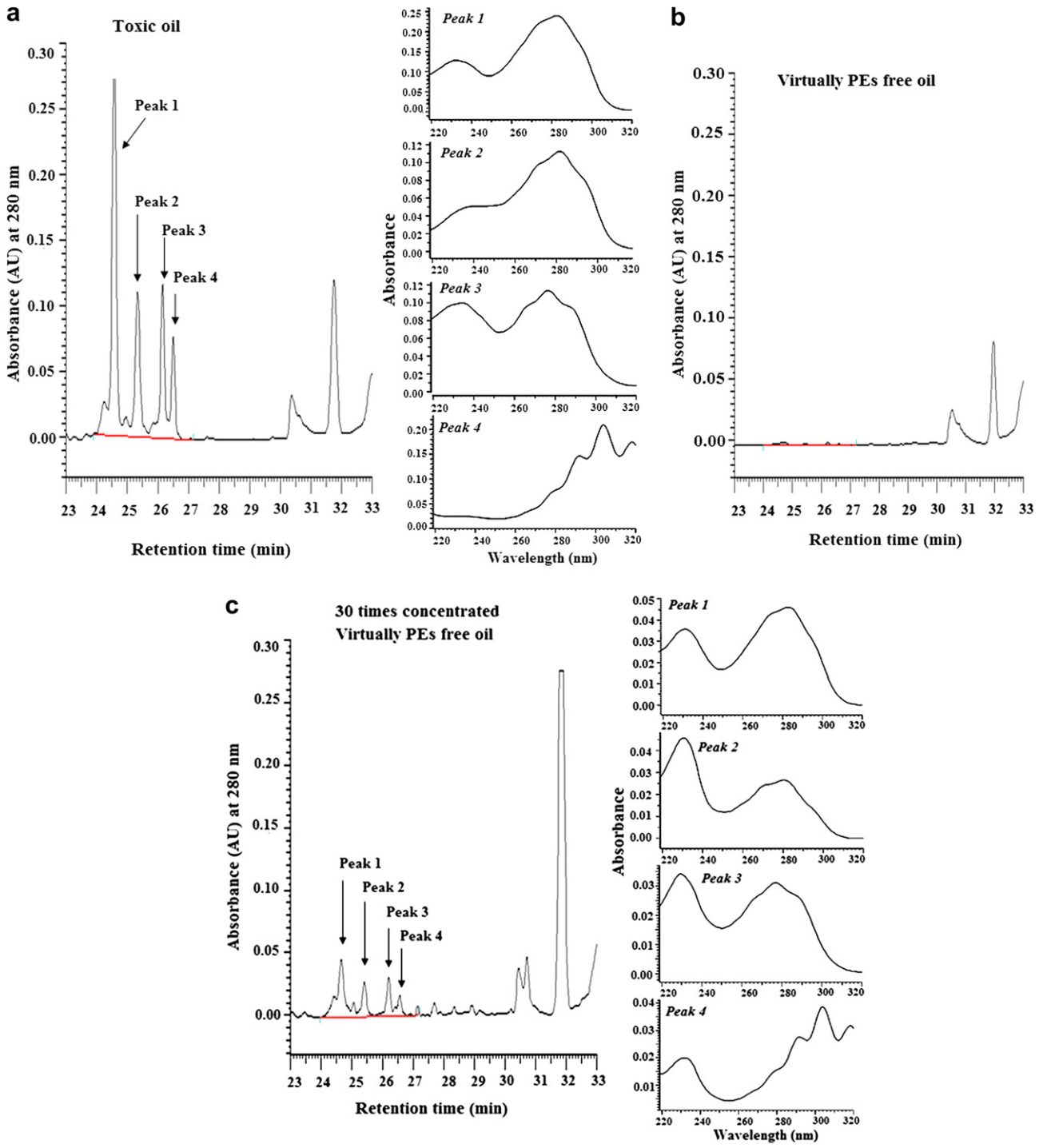

Fig. 6 – HPLC chromatogram and absorption spectra of (a) toxic Jatropha oil, (b) virtually PEs-free oil and (c) concentrated virtually PE-free oil.

15 min of extraction at 60 °C; however, on comparing PE extraction for 5 min at 55 °C and 60 °C, the extent of PE extraction was slightly lower at 55 °C (results not shown). Hence it is suggested that the extraction is done at 60 °C for 5 min. This will result in approximately 80% removal of PEs. The quality of biodiesel produced from low-PE Jatropha oil obtained from this treatment was evaluated. The quality of the biodiesel produced did not differ significantly from that produced from the untreated oil [23].

4. Conclusion

Phorbol esters have a wide range of biological activities. Approximately 78–80% of phorbol esters present in the crude Jatropha oil obtained after mechanical or solvent extraction can be extracted at methanol:oil ratio of 2:1 (v/w) and stirring on a magnetic stirrer at 60 °C for 5 min. The remaining oil can be used for biodiesel preparation. Upon methanol recovery,

the PE rich fraction can be used for various agricultural, medicinal and pharmaceutical applications. In addition to producing a valued co-product in the form of PEs, the extraction process will make the process of biodiesel preparation friendly for both the workers and environment. Studies on shelf life of the phorbol ester rich fractions at −80 °C, −20 °C and 22–23 °C are in progress.

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Conflict of interest

The authors declare that there are no conflicts of interest.

REFERENCES

[1] Makkar HPS, Aderibigbe AO, Becker K. Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem 1998;62(2):207–15.
[2] Azam MM, Waris A, Nahar NM. Prospects and potential of fatty acid methyl esters of some nontraditional seed oils for use as biodiesel in India. Biomass and Bioenerg 2005;29(40):293–302.
[3] Makkar HPS, Becker K, Schmook B. Edible provenances of *Jatropha curcas* from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. Plant Foods Hum Nutr 1998;52:31–6.
[4] Makkar HPS, Becker K, Sporer F, Wink M. Studies on Nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 1997;45:3152–7.
[5] Haas W, Sterk H, Mittelbach M. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J Nat Prod 2002;65:1434–40.
[6] Goel G, Makkar HPS, Francis G, Becker K. Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 2007;26:279–88.
[7] Makkar HPS, Francis G, Becker K. Protein concentrate from *Jatropha curcas* screw-pressed seed cake and toxic and antinutritional factors in protein concentrate. J Sci Food Agric 2008;88(9):1542–8.
[8] Gubitz GM, Mittelbach M, Trabi M. Exploitation of the tropical oil seed plant *Jatropha curcas* l. Bioresource Tecnol 1999;67:73–82.
[9] Solsoloy AD, Solsoly TS. Pesticidal Efficacy of Formulated *Jatropha curcas* Oil on pests of selected field crops. In: Gubitz GM, Mittelbach M, Trabi M, editors. Biofuels and industrial products from *Jatropha curcas*. DBV, Graz; 1997.
[10] Adam SEI. Toxic effects of *Jatropha curcas* in Mice. Toxicology 1974;2:67–76.
[11] Amin MA, Daffalla AA, Abdel Moneim O. Preliminary report on the molluscicidal properties of Habat El Mulluk, Jatropha Sp. Trans R Soc Trop Med Hyg 1972;66(5):805–6.
[12] Becker K, Makkar HPS. Effects of phorbol esters in carp (*Cyprinus carpio* L). Vet Hum Toxicol 1998;40(2):82–6.
[13] Chivandi E, Makuza SM, Erlanger KH, Mtimuni JP, Read JS, Tivapasi M. Effects of dietary *Jatropha curcas* on the haematology of weaned pigs. Zimbabwe Vet J 2000;31(4):83–91.
[14] Devappa RK, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya S. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. Food Chem Toxicol 2008;46:3621–5.
[15] Karmegam J, Sakthivadivel M, Daniel T. Indigenous plant extracts as larvicidal agents against *Culex quinquefasciatus* say. Bioresource Technol 1996;59:137–40.
[16] Rahuman AA, Gopalakrishnan G, Venkatesan P, Geetha K. Larvicidal activity of some euphorbiaceae plant extracts against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: *Cluicidae*). Parasitol Res 2008;102:867–73.
[17] Siang CC. *Jatropha curcas* L.: development of a new oil crop for biofuel. Available from, <http://eneken.ieej.or.jp/en/data/pdf/467.pdf>; 2009.
[18] Makkar HPS, Jeroen M, Becker K. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* Oil. J Am Oil Chem Soc 2009;86(2):173–81.
[19] Devappa, RK, Makkar, HPS, Becker, K. Fate of Jatropha curcas phorbol esters in soil. In: 13th Annual Green Chemistry and Engineering Conference, college Park, June 2009, 23–25, Maryland, USA.
[20] Makkar HPS, Siddhuraju P, Becker K. A laboratory manual on quantification of plant secondary metabolites. New Jersey: Humana Press; 2007. p. 130.
[21] Rug M, Ruppel A. Toxic activities of the plant *Jatropha curcas* against intermediate snails and larvae of schistosomes. Trop Med Int Health 2000;5:423–30.
[22] Johnson LA, Lusas EW. Comparison of alternative solvents for oils extraction. J Am Oil Chem Soc 1983;60(2):229–41.
[23] Devappa, RK, Maes, J, Makkar, HPS, de Greyt, W, Becker, K. Isolation of phorbol esters from Jatropha curcas oil and quality of produced Biodiesel. In: 2nd International Congress on Biodiesel: The Science and the Technologies, November 2009, 15–17, The Westin Grand Arabellapark, Munich, Germany.

CHAPTER - 4

Quality of biodiesel prepared from phorbol ester extracted *Jatropha curcas* oil

Rakshit K. Devappa [a], Jeroen Maes [b], Harinder P.S. Makkar [a] *, Wim De Greyt [b] and Klaus Becker [a]

[a] *Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany.*

[b] *Desmet-Ballestra Group, Minervastraat 1,1930 Zaventem, Belgium*

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ORIGINAL PAPER

Quality of Biodiesel Prepared from Phorbol Ester Extracted *Jatropha curcas* Oil

Rakshit Kodekalra Devappa · Jeroen Maes · Harinder Paul Singh Makkar · Wim De Greyt · Klaus Becker

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Abstract *Jatropha curcas* seeds are rich in oil (28–32%), which can be converted to high quality biodiesel. The oil is non-edible due to the presence of toxic compounds, namely, phorbol esters (PEs). PEs have a number of agricultural/medicinal/pharmaceutical applications and hence their recovery generates a value added co-product towards the biodiesel production chain. This study aims to assess the effects of PE extraction on quality of both the residual oil and the biodiesel production from it. Two Approaches (1, use of an Ultra-turrax; and 2, use of a magnetic stirrer) were used with an effective treatment time of 2 and 5 min, resulting in 80 and 78% extraction of PEs, respectively. The phosphorus content was reduced by 70.2 and 75.8%, free fatty acids by 55.3 and 55.6%, and the fatty acid composition did not change in the residual oils. The peroxide value increased from 2.69 (untreated oil) to 3.01 and 3.49 mequiv O_2/kg in the residual oils following Approach 1 and Approach 2, respectively. The biodiesel prepared from both residual oils met European (EN 14214:2008) and American biodiesel standard (ASTM D6751-09) specifications. Oxidative stability indices for both the biodiesels were well within the permitted limit. It is concluded that PEs could be isolated in active forms for various applications by either of the two methods with a high yield and the residual oil can be processed to produce high quality biodiesel.

R. K. Devappa · H. P. S. Makkar (✉) · K. Becker
Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart, Germany
e-mail: makkar@uni-hohenheim.de

J. Maes · W. De Greyt
Desmet-Ballestra Group, Minervastraat 1, 1930 Zaventem, Belgium

Keywords Jatropha oil · Biodiesel · Phorbol esters · Co-product · Bioactivity

Introduction

Recently, biofuels have been getting considerable attention because of global emphasis on reducing greenhouse gases, conserving the environment and energy security. The use of biodiesel to (partially) replace fossil diesel has a significant potential for reducing pollution and creating socioeconomic benefits for farmers. Plant oils are usually converted into biodiesel by transesterification with short-chain alcohols, such as methanol, to bring their combustion properties closer to those of conventional fuels or mineral diesel. Biodiesel is currently being produced from grease, vegetable oils or animal fats. The use of edible plant oils for biodiesel production is under discussion as they compete with food crops for scarce agricultural land and water. Therefore, the focus is now moving towards the use of non-edible crops as feedstocks for biodiesel production, such as Jatropha and algae oil, used cooking oils, low-quality animal fats and side-streams from oil refining.

Jatropha curcas belongs to the Euphorbiaceae family and is commonly known as physic nut. It is believed to have originated in Central America and now it is widespread all over the tropical and subtropical world. The plant is perennial, drought resistant, and can grow in marginal lands, rocky lands, and even in saline soils. The seeds and kernels contain 22–31% and 51–59% oil, respectively, varying with different genotypes [1, 2]. The seeds are crushed and the oil is extracted using mechanical pressing. The press cake can then be solvent (hexane) extracted to optimize production yields. Both seed cake

and oil are non-edible due to the presence of toxic compounds, namely, phorbol esters (PEs) [2–8].

PEs are diterpenes with a tigliane skeleton. They are hydrophobic, heat stable and oil soluble [8]. Six different types of PEs have been characterized from *J. curcas* oil. The concentration of PEs varies with genotype, ranging from 2 to 8 mg/g (unpublished data from our laboratory). PEs are potent inducers of a range of biological effects, including cocarcinogenicity and tumor growth [9]. PEs are used as a pharmacological tool for the investigation of biochemical processes such as carcinogenesis and also in many agricultural applications such as pesticides, molluscicides, insecticides, bacteriocides, and fungicides [8, 10, 11]. Isolated PEs could have a high commercial retail value ($2000 per gram according to Balandrin et al. [12]). Jatropha oil is a rich source of PEs and extraction of these esters as a co-product can increase the revenues for biodiesel industries. The extracted PEs could be used in various agricultural and pharmaceutical applications. The hypothesis in the present study was that the residual oil left after the extraction can be processed to high quality biodiesel, and that the isolated PEs are biologically active.

In this study, we demonstrate the quality of the residual Jatropha oil and the produced biodiesel, after extracting PEs from the crude oil, using optimized conditions as reported by Devappa, et al. [13], and the biological activity of the extracted phorbol esters.

Materials and Methods

Materials

J. curcas seeds (toxic Indian variety) were obtained from Jaipur in India. The phorbol 12-myristate-13-acetate standard was purchased from Sigma (St. Louis, USA). All other chemicals and solvents used in this study were of analytical grade.

Preparation of Jatropha Oil

J. curcas seeds were mechanically pressed in a screw press to obtain crude press oil. The oil was centrifuged at 3,150*g* for 20 min to remove solid residues and the supernatant was collected and stored in a refrigerator at 4–6 °C until further use.

Extraction of Phorbol Esters

The optimization of the conditions for the extraction of PEs has been reported earlier by Devappa et al. [13]. The main purpose of study was to evaluate quality of the residual oil obtained after extraction of PEs and of the biodiesel produced from the residual oil. The process for extraction of PEs and biodiesel production is schematically presented in Fig. 1. The procedures adopted are described briefly.

Approach 1

Jatropha oil was mixed with methanol (1:2, w/v) at 23 °C and high shear mixed (T25 Ultra-turrax from IKA-Werke GmbH & Co, Staufen, Germany) at 13,000 rpm for 2 min. Thereafter, the mixture was centrifuged at 4,000*g* for 5 min to obtain upper methanolic and lower oily layers. After decantation, the extracted methanolic layer was recovered in a rotary evaporator and methanol removed under vacuum (300 mbar) at 55 °C to get a PE enriched fraction (PEEF-U). The oily layer was also rotary evaporated at 55 °C to remove residual methanol, and the oil was stored in a refrigerator (4–6 °C) until further use.

Approach 2

Jatropha oil was mixed with methanol (1:2, w/v) in a capped container and the contents were stirred at room temperature (23–25 °C) for 5 min using a magnetic stirrer (300 rpm). Thereafter, the mixture was centrifuged at 4,000*g* for 5 min to get upper methanolic and lower oily layers. After separation of the methanolic layer, methanol was removed under vacuum as stated in "Approach 1" to obtain a PE-enriched fraction (PEEF-M). The oily layer was also rotary evaporated at 55 °C to remove residual methanol, and the oil was stored in a refrigerator (4–6 °C) until further use.

Biodiesel Production

The residual oils obtained after extraction using approaches 1 and 2 were pre-treated (acid degumming, silica treatment and stripping) and transesterified according to the procedures described earlier by Makkar et al. [14].

Analytical Methods

The extracted oil and biodiesel samples were characterized using the following AOCS official methods and recommended practices [15]: water content (Ca 2e–84), free fatty acid content (Aa 6–38), peroxide value (Cd 8–53), para-anisidine value (Cd 18–90), fatty acid composition (Ce 1b–89 combined with Ce 1e–91), content of micro-elements (phosphorus, calcium, magnesium, sodium, potassium, and iron) using ICP (iCAP 6000 series, Thermo Scientific, Zellik, Belgium), acid value (Cd 3d–63) and oxidative stability index (ADM OSI, Omnion Inc., Rockland, MA, USA).

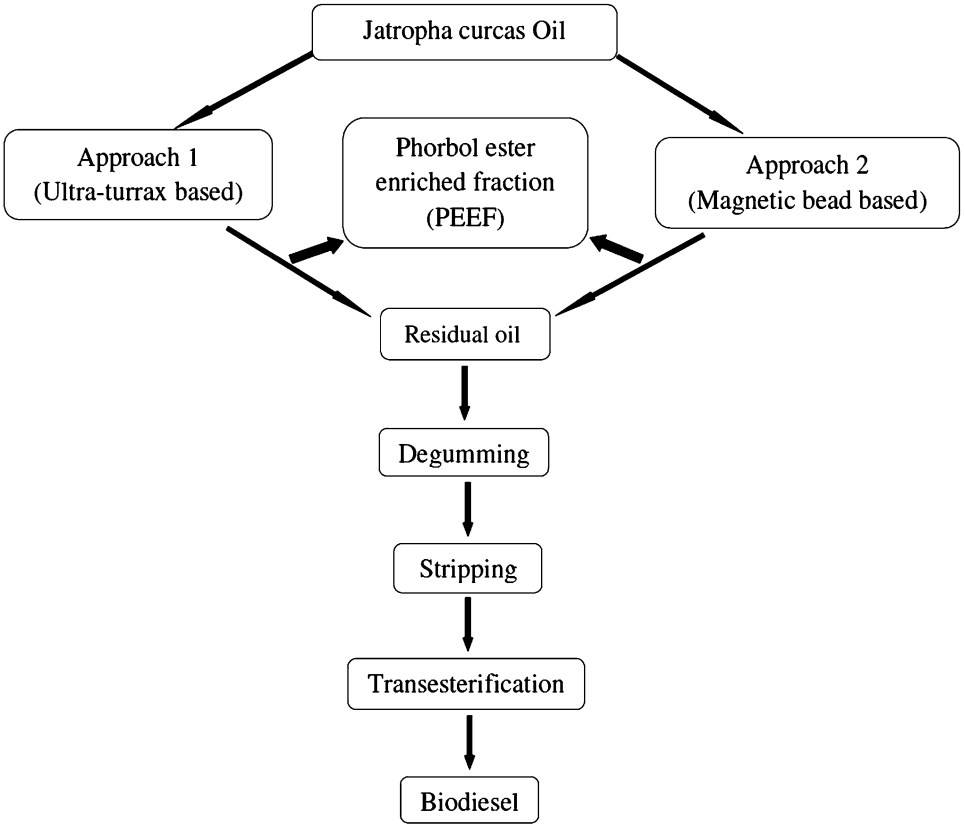

Fig. 1 Flow diagram for the extraction of phorbol esters and preparation of biodiesel from residual oil

Phorbol Esters Analysis

PEs were determined at least in duplicate [2, 16]. Briefly, 0.5 g of oil sample was extracted four times with methanol. A suitable aliquot was loaded into a high-performance liquid chromatograph (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 × 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. [16]. The four phorbol ester peaks containing six PEs were detected at 280 nm and appeared between 25.5 and 30.5 min. Phorbol-12-myristate 13-acetate (PMA) was used as an external standard (appeared between 31 and 32 min). The area of the four phorbol ester peaks was summed and converted to phorbol-12-myristate 13-acetate equivalent by taking its peak area and concentration. PEs were analyzed in the residual oil obtained from approaches 1 and 2, respectively.

The PEEF-U and PEEF-M obtained following approaches 1 and 2 were taken further for the bioassay, based on snail toxicity (see below). For evaluation of the bioactivity of PEs present in these two fractions, PEs purified from untreated Jatropha oil were used as a positive control. The purification of PEs was done as described in Li et al. [9]. In brief, the untreated oil was extracted for PEs and subjected to HPLC as described by Makkar et al. [16] and PEs peaks were carefully collected at the retention time of 25.5 and 30.5 min. The collected fractions were in approximately 90% acetonitrile. The fractions were pooled and frozen at −20 °C. The top acetonitrile layer was separated from the frozen water layer to avoid any oxidation and the acetonitrile layer was further rotaevaporated to collect a colorless oily fraction designated as the purified phorbol esters fraction (PPEF). This fraction (PPEF) was redissolved in methanol and checked for purity and concentration by HPLC. The PPEF and PEEFs from Approaches 1 and 2 were evaluated for snail toxicity. The concentration of purified PEs and PEs in the PEEFs was expressed as equivalent to PMA.

Evaluation of Bioactivity using Snails

The bioactivity of PEEFs was assessed using snails (*Physa fontinalis)* as described by Devappa et al. [13]. In brief, all tests were carried out in deionized water at room temperature (23 °C). Stock solutions of PEEFs and PPEF were prepared in methanol and further diluted by water. Groups of ten snails were placed in glass containers with 400 ml of water containing the test substance. After 24 h of incubation with test sample, the snails were transferred to

deionized water and maintained for another 48 h. Mortality was determined by the absence of movement and lack of reaction to irritation of the foot with a needle. The experiments were performed with the same quantity of methanol (negative control) in water as used for the test preparations and no mortality was recorded. All tests were independently repeated three times. Toxicity is expressed as LC_{100}, referring to concentrations killing 100% of the snails.

Statistical Analysis

All bioassay 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

Evaluation of Phorbol ester Extraction Procedures

The initial concentration of PEs in the oil was 3.15 mg/g. Two effective tools, an Ultra-turrax (Approach 1) and a magnetic stirrer (Approach 2) were used for the maximum possible extraction of PEs with a minimum consumption of solvent and time (13). Approach 1 and Approach 2 reduced PE content in the oils by 80 and 78%, respectively. However, on using Approach 1, the color of the residual oil changed from golden yellow to greyish yellow color. Since both the approaches were effective in reducing PEs, the residual oils obtained were selected for biodiesel production.

Suitability of Extracted Residual Oil as Biodiesel Feedstock

The untreated Jatropha oil and the residual oils from both Approach 1 and Approach 2 were analyzed for feedstock quality before subjecting them to pre-treatment and transesterification procedures for biodiesel production. Various components could affect the transesterification process and final biodiesel quality. Higher concentration of metallic elements, especially phosphorous, can result in poor separation of biodiesel and glycerine after transesterification. There are no regulations for the presence of metals in feedstock materials, but these analyses are done to ensure that manufacturing process proceeds smoothly. The elemental content of the residual oils from both Approach 1 and Approach 2 are shown in Table 1. Both the approaches reduced the content of major elements by several fold. Phosphorus content was reduced by 70 and 76%; calcium by 13 and 16%; magnesium by 55 and 58%; sodium by 76 and 75%; and potassium by 93 and 97% for Approach 1 and Approach 2, respectively. The residual oil from Approach 1 had a higher iron content (increased by 3.7 times), which might be due to leaching out of rusted iron from the Ultraturrax probe used during extraction. However, the overall excessively high element content makes a degumming step prior to transesterification necessary.

The anisidine value (AnV) is a measurement of the secondary oxidation, and essentially reflects how oil has been handled and stored, while the peroxide value (PV) represents the status of the primary oxidation. For both AnV and PV, a lower number is better. Both the approaches for PE extraction did not substantially increase these values. The residual oils from Approach 1 and Approach 2

Table 1 Feedstock quality of Jatropha untreated oil (control) and residual oils (Approach 1 and Approach 2)

Parameters	Control untreated oil	Residual oil from Approach 1	Residual oil from Approach 2
Phorbol esters (PEs) (mg/g oil)	3.15	0.69	0.63
Water (ppm)	222	37	ND
Free fatty acids (weight % as C18:1)	2.93	1.31	1.30
Peroxide Value (mequiv O_2/kg)	2.69	3.01	3.49
Anisidine Value (−)	0.89	1.01	ND
Element content (ppm)			
P	133	39.6	32.2
Ca	21.3	18.6	18.0
Mg	12.1	5.46	5.13
Na	5.78	1.40	1.45
K	19.6	1.29	0.87
Fe	0.06	0.22	0.06

ND not detectable, *Approach 1* ultra-turrax based, *Approach 2* magnetic stirrer based

had 1.11 and 1.29 times higher PV than the control oil. AnV increased in Approach 1 (1.13 times) compared to the control oil and decreased to an undetectable level in Approach 2 (Table 1).

In oil, water is naturally present in small amounts. The control oil had a water content of 222 ppm, which was reduced by 83.3% in Approach 1 and was not detectable in the residual oil obtained from Approach 2 (Table 1). The substantial decrease in water content could be due to (a) solubility of water in methanol during the extraction of PEs, which was removed by phase separation in the subsequent step, and (b) removal of moisture together with methanol in the evaporation step (rotaevaporatory system, 55 °C, 270 mbar; generally this step took 30 min). For a complete transesterification reaction, a free fatty acid (FFA) value lower than 0.3% is needed. As the acidity of the oil increases, the efficiency of transesterification decreases, and moreover, the presence of FFA in biodiesel can do harm to the engine oil endangering the engine's lubrication. The control oil had an FFA content of 2.93 (% as C18:1). After extraction of PEs, the FFA content was reduced by 55% in both Approach 1 and Approach 2 (Table 1). However, the residual FFA content of 1.3% was still too high and should be reduced by stripping prior to transesterification.

The fatty acid profile is another important characteristic for the evaluation of changes that occur during the extractions. The fatty acid composition of the control oil was compared with those of the residual oils from Approach 1 and Approach 2 (Table 2). The predominant fatty acids in Jatropha oil consist of monounsaturated (44.9%), followed by polyunsaturated (33.4%) and saturated (21.6%) fatty acids, which were similar for the control oil and the residual oils. The major fatty acids in Jatropha oil are oleic (44%), linoleic (33.3%), palmitic (14.7%) and stearic (6.7%) acids. The fatty acid composition observed in this study was similar to those observed in our earlier studies [14]. When compared to the control Jatropha oil: (a) the fatty acid composition of residual oil from both Approach 1 and Approach 2 did not change, and (b) there was no influence of the extraction of PEs on the fatty acid composition.

Quality of Oil after Pre-treatment

After degumming and stripping, the residual oil quality was excellent for producing biodiesel. During degumming, the phosphorous content was reduced from 32 to 40 ppm to below 2 ppm. Also the levels of Ca, Mg, Na, K, and Fe were reduced to acceptably low levels. Stripping resulted in FFA contents of the residual oils of below 0.05%. The water content after stripping was <110 ppm in both samples (Table 3).

Quality of Produced Biodiesel

The quality of the produced biodiesel depends on several factors such as genotype of the plant, soil type, plant health, maturity of seeds at harvest, seed storage conditions and duration, degree of unsaturation and fatty acid

Table 2 Fatty acid composition of untreated Jatropha oil (control) and residual oils (Approach 1 and Approach 2)

Fatty acid composition (%)	Control untreated oil	Residual oil from Approach 1	Residual oil from Approach 2
C14:0	ND	ND	ND
C16:0	14.7	14.7	14.6
C16:1t	ND	ND	ND
C16:1c	0.7	0.7	0.7
C17:0	0.1	0.1	0.1
C17:1	0.1	0.1	0.1
C18:0	6.7	6.7	6.7
C18:1t	0.0	0.0	0.0
C18:1c	44.0	44.1	44.2
C18:2t	0.0	0.0	0.0
C18:2c	33.3	33.2	33.2
C18:3t	0.0	0.0	0.0
C18:3c	0.1	0.1	0.1
C20:0	0.1	0.1	0.1
Total saturated	21.6	21.7	21.6
Total mono-unsaturated	44.9	44.9	45.1
Total Poly-unsaturated	33.4	33.4	33.3
IV calculated (-)	96.7	96.6	96.6

ND not detectable, *Approach 1* ultra-turrax based, *Approach 2* magnetic stirrer based

Table 3 Effect of degumming and stripping on quality of residual oils (Approach 1 and Approach 2) for biodiesel production

	Degumming		Stripping	
Parameters	Approach 1	Approach 2	Approach 1	Approach 2
Free fatty acids (wt% C18:1)	1.3	1.3	0.02	0.04
P (ppm)	1.4	1.5	1.2	1.1
Na + K (ppm)	1.5	2.4	0.22	0.86
Ca + Mg (ppm)	0.2	0.2	0.34	0.19
Fe (ppm)	0.00	0.00	0.01	0.01
Water (ppm)	ND	ND	50	106

ND not detectable, *Approach 1* ultra-turrax based, *Approach 2* magnetic-stirrer based

Table 4 Biodiesel quality after transesterification of residual oils from Approach 1 and Approach 2

Parameters	EN14214:2008[b]	ASTM D 6751-09[b]	Solvent extracted[a]	Cold pressed[a]	Approach 1	Approach 2
Water (ppm)	Max. 500	Max. 500[c]	290	<50	151	55
Acid Value (mg KOH/g)	Max. 0.5	Max. 0.5	0.16	0.11	0.08	0.25
Oxidative stability at 110 °C (h)	Min. 6	Min. 3	5.9	8.7	11.3	7.28
Elements (ppm)						
P	Max. 4	Max. 10	1.0	0.07	0.05	0.06
Ca + Mg	Max. 5	Max. 5	ND	ND	0.03	ND
Na + K	Max. 5	Max. 5	0.05	ND	0.67	0.38
Free and total glycerol (%)						
Free glycerol	Max. 0.02	Max. 0.02	0.005	0.008	0.005	0.012
Total glycerol	Max. 0.25	Max. 0.24	–	–	0.225	0.194
Monoglycerides	Max. 0.80	–	0.72	0.62	0.67	0.65
Diglycerides	Max. 0.20	–	0.21	0.16	0.27	0.09
Triglycerides	Max. 0.20	–	0.06	0.09	0.1	0.09

ND Not Detectable, *Approach 1* Ultra-turrax based, *Approach 2* Magnetic-stirrer based, *Max.* Maximum, *Min.* Minimum

[a] Data taken from Makkar et al. [14]

[b] European and American biodiesel standards [21, 22]

[c] Measured as free water content (ASTM D2709)

composition of oil, as well as the refining and transesterification process [14, 17]. These parameters are interrelated with several other fuel quality criteria, such as sulfated ash content, carbon residue, water content, FFA content, quantities of the chemicals used in the processing stages such as those of sodium/potassium hydroxides (catalysts) and alkaline earth metals (absorbents) [18–20]. The quality parameters of biodiesel prepared from both feedstocks (Approach 1 and Approach 2) were well within the limits of European Union and the United States biodiesel standards (EN 14214:2008 and ASTM D 6751-09) [21, 22]. The high quality biodiesel prepared from the residual oils obtained from both the approaches had low acid value, phosphorus, Na + K, Ca + Mg, and water content (Table 4).

The monoglycerides (0.67 and 0.65%), triglycerides (0.1 and 0.09%), free glycerol (0.005 and 0.012%) and total glycerol (0.225 and 0.194%) levels in the biodiesels meet the specifications of the European Union and United states biodiesel standards (Table 4). However, the biodiesel produced by following Approach 1 had a slightly higher diglyceride content (0.27%) than the European and United States biodiesel standards (0.2%) permit. The biodiesel prepared from residual oils obtained following Approach 1 and Approach 2 had higher OSI values (11.3 and 6.7 h, respectively). The reason for the higher OSI obtained for Approach 1 is not clear. However, both these values meet the EN 14214:2008 and ASTM D 6751-09 specifications [21, 22].

Bioactivity of PEEFs

Snails (*Physa fontinalis*) have been found to be highly sensitive to PEs (our unpublished data). In snails, both purified phorbol esters and methanol extract of PEEFs obtained from Approaches 1 and 2 were highly and equally

Table 5 Bioactivity of purified phorbol esters (PPEF) and of phorbol esters present in PEEFs (PEEF-U and PEEF-M) using snails (*Physa fontinalis*). The data are expressed as percent mortality

Concentration in ppm	Purified phorbol esters (PPEF)	PEEF-U from Approach 1	PEEF-M from Approach 2
0.01	$13.3 \pm 4.7^{a, A}$	$3.3 \pm 5.8^{a, A}$	$5.8 \pm 5.8^{a, A}$
0.1	$46.7 \pm 4.7^{a, B}$	$30.0 \pm 10^{b, B}$	$26.7 \pm 5.8^{b, B}$
0.2	$66.7 \pm 4.7^{a, C}$	$56.7 \pm 5.8^{ab, C}$	$53.3 \pm 5.8^{b, C}$
0.5	$80.0 \pm 8.2^{a, D}$	$76.7 \pm 5.8^{a, D}$	$73.3 \pm 15.3^{a, D}$
1.0	$100 \pm 0.0^{a, E}$	$100 \pm 0.0^{a, E}$	$100 \pm 0.0^{a, E}$

Values are means ($n = 3$) $\pm$ standard deviation

Mean values in the same row with different superscript (small letters) differ significantly ($P < 0.05$)

Mean values in the same column with different superscript (capital letters) differ significantly ($P < 0.05$)

lethal at a concentration of 0.5 and 1 ppm (LC_{100}) indicating no influence of the extraction approaches on the activity of PEEFs (Table 5). The results suggest that PEEFs extracted either using Approach 1 or Approach 2 are biologically active and can be used as a suitable bio-control agent in agricultural and pharmaceutical applications. In addition, it may be noted that PEs present in PEEFs are completely biodegradable in soil (get degraded within 9 days (23% moisture and 32 °C), and the degradability increases with increases in temperature and moisture levels in the soil [23].

Conclusions

Approximately 80 and 77.7% of the PEs present in Jatropha oil could be extracted in 2 and 5 min using an Ultra-turrax (13,000 rpm, 23 °C) and a magnetic stirrer (300 rpm, 60 °C), respectively. The PEs bioactivity was not affected by the extraction procedures and thus, PEEFs could be used as a potential bio-control agent in various agricultural and pharmaceutical applications. The residual oil obtained after extraction of PEs is of good quality and could, after pre-treatment, be processed into high quality biodiesel, meeting the European and American biodiesel specifications.

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References

1. Makkar HPS, Aderibigbe AO, Becker K (1998) Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem 62(2):207–215
2. Makkar HPS, Becker K, Sporer F, Wink M (1997) Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 45:3152–3157
3. Makkar HPS, Becker K, Schmook B (1998) Edible provenances of *Jatropha curcas* from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. Plant Food Hum Nutr 52:31–36
4. Devappa RK, Bhagya S (2008) Biochemical and nutritional evaluation of Jatropha protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J Sci Food Agric 88:911–919
5. Becker K, Makkar HPS (2008) *Jatropha curcas*: a potential source for tomorrow's oil and biodiesel. Lipid Technol 20:104–107
6. Makkar HPS, Becker K (2009) Challenges and opportunities for using byproducts from the production of biodiesel from Jatropha oil as livestock feed. Proceedings of Animal Nutrition Association World Conference (14–17 Feb). New Delhi. pp 168–170
7. Devappa RK, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya S (2008) Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. Food Chem Toxicol 46(12):3621–3625
8. Makkar HPS, Becker K (2009) *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J Lipid Sci Technol 111:773–787
9. Li CY, Devappa RK, Liu JX, Makkar HPS, Becker K (2010) Toxicity of *Jatropha curcas* phorbol esters in mice. Food Chem Toxicol 48(2):620–625
10. Solsoloy AD, Solsoloy TS (1997) Pesticidal efficacy of formulated *J. curcas* oil on pests of selected field crops. In: Gübitz GM, Mittelbach M, Trabi M (eds) Biofuels and industrial products from *Jatropha curcas*. DBV, Graz, pp 216–226
11. Goel G, Makkar HPS, Francis G, Becker K (2007) Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 26:279–288
12. Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH (1985) Natural plant chemicals: sources of industrial and medicinal materials. Science 228:1154–1160
13. Devappa RK, Maes J, Makkar HPS, Greyt WD, Becker K (2009) Isolation of phorbol esters from *Jatropha curcas* oil and quality of produced biodiesel. 2nd International Congress on Biodiesel: the science and the technologies, Munich, Germany
14. Makkar HPS, Maes J, De Greyt W, Becker K (2009) Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J Am Oil Chem Soc 86:173–181

15. AOCS (1990) Official methods and recommended practices of the American Oil Chemists Society, 4th edn. AOCS Press, Champaign
16. Makkar HPS, Siddhuraju P, Becker K (2007) A laboratory manual on quantification of plant secondary metabolites. Humana press, New Jersey, p 130
17. Tate RE, Watts KC, Allen CAW, Wilkie KI (2006) The viscosities of three biodiesel fuels at temperatures up to 300 °C. Fuel 85:1005–1010
18. Mittelbach M (1996) Diesel fuel derived from vegetable oils, VI: specifications and quality control of biodiesel. Bioresour Technol 56:7–11
19. Mittelbach M (2000) Chemische und motortechnische Untersuchungen der Ursachen der Einspritzpumpenverklebung bei Biodieselbetrieb; Bund-Bundesländer-kooperations-projekt
20. Meher LC, Sagar DV, Naik SN (2004) Technical aspects of biodiesel production by transesterification: a review. Renew Sust Energy Rev 3:1–21
21. ASTM (American Society for Testing and Materials) (2008) Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels, ASTM D6751-09. In: ASTM Annual Book of Standards. American Society for Testing and Materials, West Conshohocken. http://www.astm.org)
22. European Committee for Standardization (CEN) (2008) Automotive fuels fatty acid methyl esters (FAME) for diesel engines requirement methods EN 14214:2008. European Committee for Standardization (CEN), Brussels
23. Devappa RK, Makkar HPS, Becker K (2009) Fate of *Jatropha curcas* phorbol esters in soil. 13th Annual green chemistry and engineering conference, Washington DC. (available at: http://acs.confex.com/acs/green09/webprogram/Paper69396.html)

CHAPTER - 5

Activities of *Jatropha curcas* phorbol esters in various bioassays

Rakshit K. Devappa[a], Sanjay K. Rajesh[b], Vikas kumar[a], Harinder P.S. Makkar[a*], Klaus Becker[a]

[a] *Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart, Germany*

[b] *Department of Biotechnology, Sri Jayachamarajendra College of Engineering, Mysore, India*

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Rakshit K. Devappa [a], Sanjay K. Rajesh [b], Vikas Kumar [a], Harinder P.S. Makkar [a,*], Klaus Becker [a]

[a] Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart, Germany
[b] Department of Biotechnology, Sri Jayachamarajendra College of Engineering, Mysore, India

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ABSTRACT

Jatropha curcas seeds contain 30–35% oil, which can be converted to high quality biodiesel. However, *Jatropha* oil is toxic, ascribed to the presence of phorbol esters (PEs). In this study, isolated phorbol ester rich fraction (PEEF) was used to evaluate the activity of PEs using three aquatic species based bioassays (snail (*Physa fontinalis*), brine shrimp (*Artemeia salina*), daphnia (*Daphnia magna*)) and microorganisms. In all the bioassays tested, increase in concentration of PEs increased mortality with an EC_{50} (48 h) of 0.33, 26.48 and 0.95 mg L^{-1} PEs for snail, artemia and daphnia, respectively. The sensitivity of various microorganisms for PEs was also tested. Among the bacterial species tested, *Streptococcus pyogenes* and *Proteus mirabilis* were highly susceptible with a minimum inhibitory concentration (MIC) of 215 mg L^{-1} PEs; and *Pseudomonas putida* were also sensitive with MIC of 251 mg L^{-1} PEs. Similarly, *Fusarium* species of fungi exhibited EC_{50} of 58 mg L^{-1} PEs, while *Aspergillus niger* and *Curvularia lunata* had EC_{50} of 70 mg L^{-1}. The snail bioassay was most sensitive with 100% snail mortality at 1 μg of PEs mL^{-1}. In conclusion, snail bioassay could be used to monitor PEs in *Jatropha* derived products such as oil, biodiesel, fatty acid distillate, kernel meal, cake, glycerol or for contamination in soil or other environmental matrices. In addition, PEs with molluscicidal/antimicrobial activities could be utilized for agricultural and pharmaceutical applications.

1. Introduction

Bioenergy generated from plants will play a key role in future energy supply. Recently, the non-edible *Jatropha curcas* has been hailed as one of the world's most sustainable biofuel crops. The *Jatropha* seed oil has gained tremendous interest as a feedstock for biodiesel production (Makkar and Becker, 2009; Devappa et al., 2010a). In 2015, it is expected to produce 12.8 million tonnes of *Jatropha* oil and the majority of cultivation being done in developing countries (GEXSI, 2008). *Jatropha curcas* plant belonging to Euphorbiaceae family contains a variety of biologically active phytochemicals such as proteins, peptides and diterpenes exhibiting a spectrum of biological activities (Devappa et al., 2010b, 2011). However, the seeds contain toxic phytochemicals called phorbol esters (PEs) (Haas et al., 2002). During the mechanical extraction of oil from seeds, 70–75% of PEs come with the oil and the rest are still retained in the pressed cake, thus making both cake and oil non edible (Makkar et al., 2009, 2008). The PEs are diterpenes having tigliane skeleton. In *J. curcas*, six types of PEs have been reported (Haas et al., 2002). The purified PEs mixture and PEs containing extracts/seeds/leaves are toxic to many animal species including, rat, mice, fish, chicken, goat and cow (Li et al., 2010; Adam and Magzoub, 1975; Ahmed and Adam, 1979; Becker and Makkar, 1998; El-Badwi and Adam, 1992; Gandhi et al., 1995; Devappa et al., 2010c). In majority of the cases, toxicity is attributed to the presence of PEs. The *Jatropha* PEs exhibited an LD_{50} of 27.34 mg kg^{-1} body weight in mice (Li et al., 2010). In addition, PEs are found to be stable during initial processing stages of oil during biodiesel production (Makkar et al., 2009) enunciating the possible occupational exposure. Thus, screening of oil samples is necessary to limit the exposure towards *Jatropha* oil, to distinguish the toxic seeds from nontoxic *J. curcas* seeds and its ecotoxicity. Recently, increased utilization of *Jatropha* plant for oil and co-products has raised concern over the toxicity of PEs in the environment (Achten et al., 2008).

Unfortunately, despite the advances in chromatographic and spectroscopic techniques, majority of the natural product chemists or quality control specialists in developing countries involved in *Jatropha* biodiesel production chain with empirical practices either lack expertise/infrastructure for biological screening or often long waiting time is required for such screening if samples are sent to other laboratories. It is therefore highly desirable to use simple bioassays in laboratories, which are specific, rapid, accurate, valid, reproducible, cost effective, and do not require an expertise knowledge of biochemistry, biology or pharmacology (Gutleb et al., 2004; Van den berghe and Vlietinck,

* Corresponding author. Fax: +49 71145923702.
E-mail address: makkar@uni-hohenheim.de (H.P.S. Makkar).

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1991). Most of the aforesaid requirements for rapid screening of phytochemicals are better met by *in vitro* tests than the time consuming *in vivo* tests. The biological test systems/bioassays may provide useful and rapid screening methods for measuring potency of toxic phytochemicals either in purified form or in mixture/extracts (Gutleb et al., 2004). Generally, the toxicity levels of chemicals to aquatic organisms are classified (Commission of the European Communities, 1996) as 'very toxic' when the EC_{50} (effective concentration) value is $<1\ mg\ L^{-1}$; 'toxic' when EC_{50} is $1–10\ mg\ L^{-1}$, 'harmful' when EC_{50} is $10–100\ mg\ L^{-1}$, and 'nontoxic' when the EC_{50} is $>100\ mg\ L^{-1}$. In addition, the potency of test compounds in bioassays is generally expressed as 50% of lethal concentration (LC_{50})/effective concentration (EC_{50})/effective dose (ED_{50})/lethal dose (LD_{50}).

In the present study, the sensitivity of PEs is evaluated using a battery of bioassays *in vitro* involving lower organisms (snail, daphnia, artemia, and microorganisms), which could easily be applied to evaluate the presence of PEs in the ecosystem interacting with the emerging *Jatropha* biodiesel production chain.

2. Materials and methods

2.1. Materials

J. curcas seeds (toxic Indian variety) were collected from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. Phorbol 12-myristate-13-acetate (PMA), Ampicillin, gentamycin, nystatin, and carbendazim was obtained from Sigma (St. Louis, USA) and all other chemicals/solvents used were of analytical grade.

2.2. Extraction of phorbol ester enriched fraction (PEEF)

J. curcas seeds were mechanically pressed using a screw press to obtain oil. The oil was centrifuged at $3150 \times g$ for 20 min to remove the residues. The extraction of PEEF was carried as reported by Devappa et al. (2010d). The oil was mixed with methanol (1:2, w/v) and mixed at 55 °C for 15 min using a magnetic stirrer (300 rpm). Further, the mixture was centrifuged ($3150 \times g$ for 5 min) to get upper methanolic and lower oily layers. The methanol layer was rotaevaporated to get oily PEEF. The oily PEEF was stored at −80 °C until further analysis.

2.3. Phorbol ester analysis

Phorbol esters were determined at least in duplicate according to Makkar et al. (2007), based on the method of Makkar et al. (1997). Briefly, 0.5 g of test sample was extracted four times with 2% tetra hydrofuran in methanol. A suitable aliquot was loaded into a high-Performance liquid chromatograph (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250×4 mm id, from Merck (Darmstadt, Germany)) column. The column was protected with a head column containing the same material. The separation was performed at RT (23 °C) and the flow rate was $1.3\ mL\ min^{-1}$ using a gradient elution (Makkar et al., 2007). The four phorbol ester peaks (containing 6 PEs) appeared between 25.5 and 30.5 min were detected at 280 nm. The spectra of each peak were taken using Merck-Hitachi L-7450 photodiode array detector. Phorbol-12-myristate 13-acetate was used as an external standard, which appeared between 31 and 32 min. The area of the four phorbol ester peaks was summed and the concentration was expressed equivalent to PMA. The PEs detection limit in the HPLC was 3–4 μg.

2.4. Bioassay for evaluating toxicity in snails

Tests with snails (*Physa fontinalis)* were performed as described by Devappa et al. (2010c). All tests were carried out in triplicate using de-ionized water. Stock solutions of extracts were prepared in methanol and further diluted in water. Groups of 10 snails were placed in glass containers with 400 mL of water containing the test substance (PEEF in methanol was further diluted in water). The glass containers were placed in a water bath adjusted at 26 °C. Snails were prevented from crawling out of the containers by a fine stainless steel mesh suspended just above the water surface. After 24 h of incubation the snails were transferred to deionized water, fed and maintained for another 48 h. Death of the snails was determined by lack of reaction to irritation of the foot with a needle. Control experiments were performed with the same quantity of methanol in water as used for the test preparations and no mortality was recorded in the control containers. All tests were independently repeated three times. Toxicity is expressed as percent mortality referring to concentrations killing 100% of the snails and EC_{50} was calculated (Section 2.8).

2.5. Bioassay for evaluating toxicity in Artemia salina

The assay was carried by slight modification of Kinghorn et al. (1977). The *A. salina* cysts were incubated under a light source (60 W bulb) and constant aeration in a hatching chamber containing artificial seawater. After 48 h, the hatched active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. For toxicity tests, the PEEF was dissolved in DMSO (dimethyl sulfoxide) and serially diluted in saline water in a micro well plate with a final volume of 100 μl. Further, *A. salina* nauplii were diluted to a concentration of 100 organisms per mL in saline water and 100 μl aliquots were transferred into a 96-well polystyrene micro well plate. The plates were incubated at room temperature under illumination. The DMSO was used as an assay blank. Deaths were recorded after 24 h of PEs exposure and the surviving animals were killed by the addition of 100 μl of 5% (v/v) phenol to each well and counted for total number of nauplii. The experiment was carried out in triplicates. Results were expressed as percentage of mortality and EC_{50} was calculated (Section 2.8).

2.6. Bioassay for evaluating toxicity in Daphnia magna

The test was carried out according to conditions mentioned in OECD TG202 (OECD, 2004; Terasaki et al., 2009) total of 20 organisms aged <24 h were used for each of the test concentrations and solvent control. The organisms were placed in 250 mL disposable plastic containers containing 100 mL reconstituted water with test solutions. The reconstituted water was prepared with suitable dilution as mentioned in ISO 6341 (ISO-6341, 1996). The test solution of PEEF was diluted using methanol. The solution was slightly milky as the concentration increased (tried up to $5\ mg\ L^{-1}$) but was completely soluble. The photoperiod was 16:8 light:dark cycle (temperature 22–24 °C). The number of immobilized daphnids was recorded after exposure for 24 h and 48 h. The daphnids that were unable to swim after gentle stirring were considered dead. The EC_{50} was calculated after 48 h of exposure. Results were expressed as percentage mortality and EC_{50} was calculated (Section 2.8).

2.7. Bioassay for evaluating toxicity to microorganisms

2.7.1. Bioassay using bacteria

The bioassay was carried out on six genera of bacteria, by means of disk diffusion method. The bacterial strains were selected based on their ease of availability, laboratory culturing and their broad sensitivity towards phytochemicals. The assay was carried out according to the method of Moreno et al. (1998) with some modifications. The bacterial cultures used *Bacillus subtillis, Pseudomonas putida, Proteus mirabilis, Staphylococcus aureus, Streptococcus pyogenes* and *Escherichia coli* were obtained from culture collection center, Food Microbiology Department, Central Food Technological Research Institute, Mysore, India. A loop full of organisms was pre-cultured in 10 mL nutrient broth for 6 h at 37 °C. The turbidity of the culture was adjusted to 0.5 McFarland optical densities. From the seed culture 0.1 mL of bacterial suspension was inoculated on Muller Hinton agar plates by the spread plated method. Sterilized filter paper disks (Whatman number-42, 6 mm diameter) impregnated with 10 μL of *J. curcas* PEEF from the stock giving a concentration of 0 to 430 $\mu g\ disk^{-1}$. The disks were placed on the seeded agar plates and diameter of inhibitory zones was measured after the plates were incubated at 37 °C for 24 h. Gentamycin ($0–1\ mg\ mL^{-1}$) and Ampicillin ($0–2.5\ mg\ mL^{-1}$) were used as positive controls. The tests were carried out in triplicate.

2.7.1.1. Minimum inhibitory concentration determination. Minimum inhibition concentration of the PEs against bacterial species was determined according to the method of Vairappan (2003). Different concentrations (215, 250, 394, 465 and $537\ \mu g\ mL^{-1}$ of nutrient broth) of PEEF was mixed with the desired bacterial strains cultured in 10 mL of nutrient broths (10^4 colony forming unit) and the tubes were incubated at 37 °C for 24 h. Gentamycin ($0–1\ mg\ mL^{-1}$) and Ampicillin ($0–2.5\ mg\ mL^{-1}$) were used as positive controls. Bacterial growth was monitored at 4, 8, 12, 16, 20 and 24 h and quantified as colony forming unit (CFU) by serial dilution method using plate count agar.

2.7.2. Bioassay using fungi

Seven important fungal strains (*Aspergillus niger*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium moniliforme*, *Aspergillus flavus*, *Curvularia lunata*, and *Penicillium notatum*) were obtained from the culture collection center, Department of Food Microbiology, Central Food Technological Research Institute Mysore, India, were selected for antifungal activity. The antifungal activity of PEEF was carried out according to the method of Saetae and Suntornsuk (2010). The fungi were cultured on potato dextrose agar (PDA) plates at 25 °C for 7 day prior

to use. The commercial fungicides, Nystatin and Carbendazim were used as positive controls. Sterilized PDA was cooled to 50 °C and poured into petri dishes. The agar was mixed well with various concentrations of PEEF (28.6 μg–143.3 μg of PEEF mL^{-1} of culture media). The fungal strains were inoculated in each well of 5 mm diameter (made with a cork borer) with 7-day-old phytopathogen and incubated at 25 °C until it fully grew on a PDA plate. The commercial fungicides Nystatin and Carbendazim were used at a concentration of 0–25 and 0–50 μg mL^{-1} as positive controls and petri plates inoculated with the fungal strains without PEEF were also incubated as control. The diameter of fungal colonies on PDA plates was measured each day to determine the effective inhibitory concentration (EC_{50}). The experiments were done in triplicate for each crude extract concentration. Percentage inhibition of mycelial growth was calculated using the formula:

$$\%\,\text{Inhibition} = \frac{(\text{Colony diameter of control}) - (\text{colony diameter of PEEF})}{\text{Colony diameter of control}} \times 100$$

2.8. Statistical analysis

All data were subjected to an one-way analysis of variance ANOVA and the significance of differences between means was tested using Tukeys test ($P < 0.05$). The software used was SAS, Version 9.1 (Statsoft Inc., Tulsa, USA). The EC_{50} was calculated using the statistic software SPSS (Probit analysis).

3. Results and discussion

The activities of toxic PEs present in PEEF (43.8 mg g^{-1}) were studied using different bioassays, with the aim to detect PEs in the ecosystem.

3.1. Bioassay for evaluating toxicity in snails

Aquatic snails have long been used in bioassay systems such as *Biomphalaria glabrata*, and *Oncomelania hupensis* (Liu et al., 1997; Rug and Ruppel, 2000). The *physa fontinalis* used in our bioassay system, is a fresh water pulmonate commonly found in ponds, rivers, and streams in the tropics, subtropics and temperate conditions. In our study, the PEEF was highly toxic against the snails. The toxicity increased with increase in concentration of PEs in the assay (Fig. 1). The lowest effective concentration of PEs was 0.1 mg L^{-1} at which 6.7% mortality was observed. Further, at a concentration of 0.2 mg L^{-1}, 0.3 mg L^{-1}, 0.5 mg L^{-1} ,and 1 mg L^{-1} snails exhibited mortality of 25%, 43.3%, 61.1%, and 100%, respectively. The calculated EC_{50} was 0.33 mg L^{-1} for PEs. The mortality was positively correlated ($R^2 = 0.96$, $y = 103.8x + 2.128$) with the concentration of PEs. Rug and Ruppel (2000) reported that crude oil and methanolic extract of oil exhibited toxicity against snails (*Biomphalaria glabrata*) with an LC_{50} value of 50 mg L^{-1} and 5 mg L^{-1}, respectively and LC_{100} values of 100 mg L^{-1} and 25 mg L^{-1}, respectively. However, genus *Bulinus* was more sensitive to methanolic extract of *Jatropha* oil than *B. glabrata* with an LC_{50} and LC_{100} values of 0.2 mg L^{-1} and 1 mg L^{-1}, respectively (Rug and Ruppel, 2000). Liu et al. (1997) have reported that methanol extract of seed kernels exhibited 50% mortality at a concentration of 1 mg% (~10 mg L^{-1}) against *O. hupensis* exhibiting more sensitivity than *B. glabrata*. Whereas purified PEs, 4β-phorbol-13-decanoate was toxic at 10 mg L^{-1} against both snails (*B. glabrata and O. hupensis*). In addition, 4α-phorbol, 4β-phorbol and 'α' form of PEs were inactive (Liu et al., 1997). The toxicity in methanolic fraction of *Jatropha* oil is attributed to the presence of PEs (Rug and Ruppel, 2000; Liu et al., 1997). On comparison with the reported studies, it is evident that the sensitivity of snails towards PEs is species specific and also depends on the chemical properties of PEs. In our study, *Jatropha* PEs were found to be highly sensitive to the snails (*P. fontinalis*). As suggested by Rug and Ruppel (2000), we also believe that moluscicidal activity observed towards snails in our study may be due to interference in the cellular signal transduction pathway by PEs. Wherein, they act as agonists of enzyme protein kinase C (PKC), which in turn results in the expression of uncontrolled cellular biological activities, such as apoptosis or cell death.

3.2. Bioassay for evaluating toxicity in Artemia salina

Artemia salina (L. 1758), a crustacean is widely used in bioassay systems as an indicator of toxicity. Ruebhart et al. (2008) have reviewed the importance of valid taxonomic identification of *Artemia* and also reported that the attractiveness of *Artemia* bioassay is due multi-attributes: (a) easy commercial availability of the cysts (b) easy storage, maintenance and hatching of the cysts, and (c) the assay is cost effective, simple, rapid and sensitive (d) less test samples are required, and can be carried out in microplates (96 well), and (e) meets animal ethical guidelines in many countries. In our study, the toxicity of PEEF increased with increase in concentration of PEs in the assay (Fig. 2). The lowest effective concentration of PEs was 2.9 mg L^{-1} at which 11.7% mortality was observed. The mortality increased reaching 72% mortality at 47 mg L^{-1}. Further increase in the concentration was not effective in causing toxicity against *A. salina*. However, 100% mortality was recorded at very high concentration of 6000 mg L^{-1}. The mortality was positively

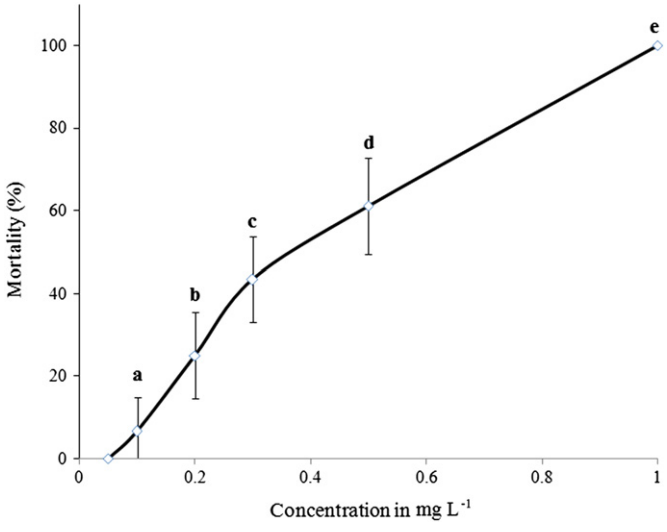

Fig. 1. Activity of phorbol ester enriched fraction (PEEF) containing phorbol esters against snails (*Physa fontinalis*). The mean values ($n = 3$) at different concentrations with different superscript differ significantly ($P < 0.05$).

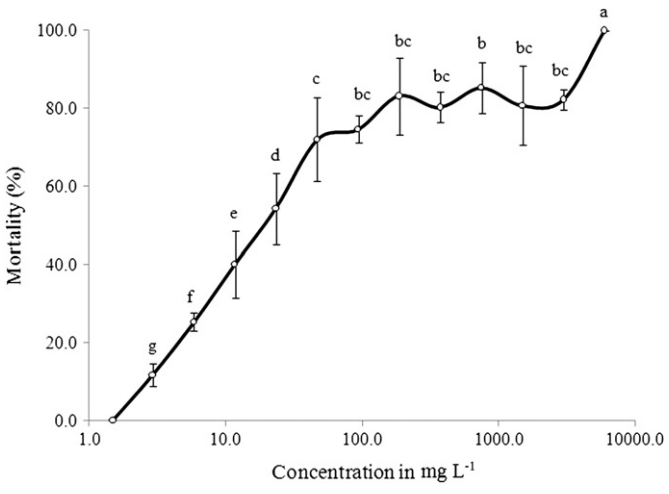

Fig. 2. Activity of phorbol ester enriched fraction (PEEF) containing phorbol esters against *Artemia salina*. The mean values ($n = 3$) at different concentrations with different superscript differ significantly ($P < 0.05$).

correlated ($R^2=0.96$, $y=103.8x+2.128$) with the concentration of PEs. The calculated EC_{50} was at 26.08 mg L^{-1} PEs. Similarly, Kinghorn et al. (1977) have reported that purified PEs such as phorbol 12-tetradconoate 13-acetate, phorbol 12, 13-didecanoate, and phorbol 12, 13-dibenzoate elicited toxicity with an effective mortality dose (ED_{50}) of 3.8, 6.8, and 11.8 mg L^{-1}, respectively. The phorbol and 4α-phorbol 12, 13-didecanoate were relatively nontoxic ($ED_{50,}$ > 1000 mg L^{-1}). From the above reported studies, it is evident that bioactivity of PEs depends on the chemical structure/configuration and purity. Upon comparison with snail bioassay (EC_{50}), the *A. salina* bioassay used in the study is less sensitive towards *Jatropha* PEs with respect to screening the toxicity.

3.3. Bioassay for evaluating toxicity in Daphnia magna

Daphnia magna, a crustacean is also widely used in bioassay systems as an indicator of toxicity. The acute toxicity of PEEF containing PEs toward *D. magna* is presented in Fig. 3. The toxicity increased with increase in concentration of PEs in the assay. The lowest effective concentration of PEs was 0.5 mg L^{-1} at which 26% mortality was observed. The 100% mortality was observed at 3 mg L^{-1}. The calculated EC_{50} of PEEF was 0.95 mg L^{-1} equivalent of PEs. The mortality was positively correlated ($R^2=0.95$, $y=34.0x+7.512$) with the concentration of PEs. To our knowledge, there are no other studies reported on toxicity evaluation of PEs in *Daphnia* species for comparison. Based on comparison of EC_{50} recorded in the snail bioassay, the *D. magna* bioassay was found to be less sensitive towards *Jatropha* PEs.

3.4. Bioassay for evaluating toxicity to microorganisms

3.4.1. Antibacterial activity

Table 1 shows the results of the antimicrobial activity of PEEF against six genera of bacteria. The PEEF showed broad spectrum antibacterial activity. The *P. putida* and *S. pyogenes* were found susceptible towards PEEF with a zone of inhibition 19 and 18 mm, respectively. The PEEF has a moderate inhibition zone on *P. mirabilis, E. coli and S. aureus* species with the diameter of 16, 15, and 14 mm, respectively. Among the tested bacterial species *B. subtilis* was found to be least susceptible (inhibition zone 12 mm). The MIC value of the PEs on six species of bacteria tested is shown in Table 2. The MIC value varies from 215–537 μg mL^{-1} of culture media. The PEEF showed low MIC value for *S. pyogenes* (215 μg mL^{-1}), *P. mirabilis* (215 μg mL^{-1}), *P. putida* (250.7 μg mL^{-1}) and moderate MIC value for *S. aureus* (394 μg mL^{-1}). However, the MIC value for *E. coli* and *B. subtilis* was comparatively higher than the other species (465.7 and 537 μg mL^{-1}, respectively). The MIC value for Ampicilin varied between 125–250 μg mL^{-1} and for Gentamycin it remained same for all the species of bacteria (50 μg mL^{-1}).

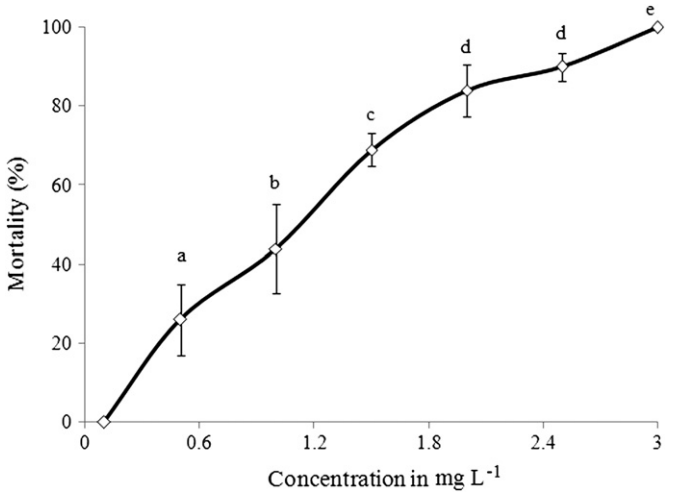

Fig. 3. Activity of phorbol ester enriched fraction (PEEF) containing phorbol esters against *Daphnia magna*. The mean values ($n=3$) at different concentrations with different superscript differ significantly ($P<0.05$).

Table 1
Effect of phorbol ester enriched fraction (PEEF) on different bacterial species.

Bacterial species	Zone of inhibition (mm)		
	PEs[1]	GM[2]	AM[3]
Bacillus subtilis	11.7^{e}	16.0^{c}	NA
Escherichia coli	15.3^{c}	16.7^{abc}	8.3^{b}
Proteus mirabilis	15.7^{c}	17.7^{ab}	10.3^{a}
Pseudomonas putida	18.7^{ab}	18.0^{a}	8.0^{b}
Staphylococcus aureus	13.7^{d}	17.7^{ab}	10.3^{a}
Streptococcus pyogenes	17.7^{b}	16.3^{bc}	9.3^{ab}

[a–c]Mean values ($n=3$) in the same column with different superscript differ significantly ($P<0.05$).
NA-No activity.

[1] Concentration of PEs in PEEF (μg).
[2] GM- Gentamycin (μg).
[3] AM- ampicillin (μg).

Table 2
Minimum inhibitory concentration of phorbol ester enriched fraction (PEEF) on different bacterial species.

Bacterial **species**	PEs [1]	AM [2]	GM [3]
Bacillus subtilis	537.0^{a}	NA	50.0^{a}
Escherichia coli	465.7^{ab}	125.0^{c}	50.0^{a}
Proteus mirabilis	215.0^{c}	180.0^{b}	50.0^{a}
Pseudomonas putida	250.7^{c}	250.0^{a}	50.0^{a}
Staphylococcus aureus	394.0^{b}	180.0^{b}	50.0^{a}
Streptococcus pyogenes	215.0^{c}	180.0^{b}	50.0^{a}

[a–c]Mean values ($n=3$) in the same column with different superscript differ significantly ($P<0.05$).
NA-No activity.

[1] Concentration of PEs in PEEF (μg mL^{-1}).
[2] AM-ampicillin (μg mL^{-1}).
[3] GM-Gentamycin (μg mL^{-1}).

3.4.2. Antifungal activity

Antifungal activity of *Jatropha* PEs on seven pathogenic fungi is shown in Table 3. The PEEF showed inhibitory effect on all seven species tested. The *B. cinerea*, *F. oxysporum*, *F. moniliforme* were found to be most susceptible, wherein 100% inhibition was found at the concentration of 114.6 μg mL^{-1}. On *A. niger, A. flavus*, *C. lunata,* and *P. notatum* 100% inhibition was obtained at the concentration of 143.3 μg mL^{-1}. The antifungal activity of different concentration of commercial fungicides nystatin and carbendazim (25 and 50 μg mL^{-1}) was also tested on the seven species of fungi. Both nystatin and carbendazim showed 100% inhibition on the growth of all the seven species of fungi at the concentration of 50 μg mL^{-1}. The EC_{50} value for *F. oxysporum* and *F. moniliforme* was 44 and 47 μg mL^{-1}, respectively; whereas for *B. cinerea, A. niger, A. flavus*, *C. lunata*, and *P. notatum*, EC_{50} value was 63, 64, 72 and 65 μg mL^{-1}, respectively.

The antifungal and antimicrobial activities of alcoholic/organic solvent extracts from different parts (roots, leaves and seed meal/cake) of *Jatropha* species have been reported and the activity of tested extracts was attributed to the presence of PEs. (Devappa et al., 2010c). The hexane, chloroform, methanol or alcoholic extracts of *J. podagrica*

Table 3
Effect of phorbol ester enriched fraction (PEEF) on different species of fungi (% of inhibition).

Organisms	Concentration of PEs (μg mL^{-1})					Positive Control			
						Nystatin (μg mL^{-1})		Carbendazim (μg mL^{-1})	
	28.6	57.3	86.0	114.6	143.3	25	50	25	50
Aspergillus niger	13.67 ± 0.52 ^{cd}	27.00 ± 0.89 ^{cd}	45.67 ± 1.37 ^{d}	88.67 ± 2.73 ^{c}	120.00 ± 2.73 ^{b}	52.7 ± 3.2 ^{ab}	101.7 ± 3.5 ^{bc}	48.0 ± 2.0 ^{c}	104.0 ± 2.0 ^{ab}
Botrytis cinerea	16.00 ± 0.89 ^{c}	30.33 ± 1.37 ^{bc}	64.67 ± 2.25 ^{c}	99.33 ± 2.73 ^{a}	130.33 ± 1.37 ^{a}	55.3 ± 3.1 ^{a}	100.0 ± 2.6 ^{c}	55.7 ± 1.5 ^{ab}	105.0 ± 2.0 ^{ab}
Fusarium oxysporum	29.67 ± 1.37 ^{a}	55.67 ± 1.37 ^{a}	85.67 ± 2.73 ^{a}	99.67 ± 1.37 ^{a}	134.00 ± 1.79 ^{a}	47.0 ± 3.6 ^{b}	104.7 ± 1.2 ^{abc}	52.0 ± 1.0 ^{b}	109.0 ± 3.6 ^{a}
Fusarium moniliforme	23.67 ± 0.52 ^{b}	58.67 ± 1.86 ^{a}	79.00 ± 1.79 ^{b}	99.33 ± 2.33 ^{a}	128.33 ± 2.25 ^{a}	54.7 ± 2.5 ^{a}	108.3 ± 2.1 ^{ab}	55.3 ± 3.2 ^{ab}	104.7 ± 1.5 ^{ab}
Aspergillus flavus	12.33 ± 1.03 ^{de}	31.33 ± 0.52 ^{b}	62.00 ± 0.89 ^{c}	95.33 ± 2.73^{abc}	118.67 ± 2.73 ^{b}	54.3 ± 1.5 ^{ab}	103.3 ± 3.1 ^{bc}	54.3 ± 2.1 ^{ab}	101.7 ± 2.5 ^{b}
Curvularia lunata	10.33 ± 0.52 ^{e}	25.67 ± 1.37 ^{d}	50.67 ± 1.37 ^{d}	91.33 ± 2.73 ^{bc}	118.67 ± 2.25 ^{b}	52.0 ± 2.0 ^{ab}	110.3 ± 2.1 ^{a}	53.7 ± 2.5 ^{abc}	102.0 ± 2.0 ^{b}
Penecellium notatum	12.00 ± 0.89 ^{de}	27.67 ± 0.52 ^{bcd}	63.67 ± 1.37 ^{c}	96.67 ± 1.37 ^{ab}	115.00 ± 1.15 ^{b}	53.0 ± 2.0 ^{ab}	106.0 ± 2.0 ^{abc}	58.3 ± 1.5 ^{a}	104.7 ± 1.5 ^{ab}

$^{a-c}$Mean values ($n=3$) in the same column with different superscript differ significantly ($P<0.05$).

(roots) and *J. podagrica* (leaves) have been found to inhibit *B. cereuss, B. pumilus, B. subtilis, Bordetella bronchiseptica, S. epidermidis, Klebsiella pneumoniae, S. faecalis, and Candida albicans (*Aiyelaagbe et al., 2000; Kumar et al., 2006). Devappa et al. (2009) have shown that the methanol extract from *J. curcas* meal has antibacterial activity (MIC) in the following order: *Yersinia enterocolitica, Proteus vulgaris*, and *P. aeruginosa* (50 μg mL^{-1}) < *S. pyogenes* (200 μg mL^{-1}) < *S. aureus* (400 μg mL^{-1}). Saetae and Suntornsuk (2010) have reported an effective antifungal activity (580 μg mL^{-1}) from ethanolic extract of *J. curcas* seed cake containing PEs on seven fungi: *F. oxysporum* (2800 μg mL^{-1}), *F. semitectum* (580 μg mL^{-1}), *Colletotrichum capsici* (1400 μg mL^{-1}), *C. gloeosporioides* (4,000 μg mL^{-1}), *Pythium aphanidermatum* (1,500 μg mL^{-1}), *Lasiodiplodia theobromae* (950 μg mL^{-1}), and *C. lunata* (1,000 μg mL^{-1}). It is evident that the bacterial (*S. pyogenes* and *mirabilis*) and fungal species (*F. oxysporum* and *F. moniliforme)* used in our study are sensitive to PEs present in PEEF and could be utilized as bioassay models to test the presence of PEs. However, when compared with snail bioassay, microbial bioassays were found to be less sensitive towards *Jatropha* PEs.

In addition, the large variance in the effective concentrations for different bioassays observed in our study may be due to organism's specific physiological sensitivity towards the test chemical. Therefore, ranking of the assay was based on the toxicity results. In addition, the mechanism of action of PEs in snails, *Artemia*, *Daphnia* and microorganisms may involve hyper activation of PKC, which in turn results in cell apoptosis. Amongst the tested organisms (*Artemia*, *Daphnia* and microorganisms), the snail bioassay was the most sensitive and it could be easily adopted for screening the Jatropha PEs.

4. Conclusions

Considering the rapid growth in Jatropha plantation or utilization of Jatropha based products, there is always a possibility of human/animals coming in contact with *Jatropha* based products containing toxic PEs. When compared with the expensive offsite analysis of PEs (for example using HPLC), the bioassays could be used as a tool to monitor the toxicity. All the bioassays (*P. fontinalis*, *A. salina, D. magna* and micro organisms) used in this study were easy to handle and can be conducted with little resources. The snail bioassay has been found to be the most sensitive. This bioassay could be used to monitor the presence of PEs in various *Jatropha* derived products such as oil, biodiesel, kernel meal, cake, and latex. It could also be used for detecting the presence of PEs in soil and other matrices in the ecosystem interacting with *Jatropha* production and use chain. However, it must be noted that the presence of trace amounts PEs in test samples should be confirmed by HPLC. In addition, the study has also demonstrated that the PEs exhibit moluscicidal, antifungal and antibacterial activities. Thus, extracted PEEF could also serve as a natural molluscicide, fungicide, and bactericide against snails, fungal phytopathogens and bacteria, which in turn could be used for agricultural and pharmaceutical applications.

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References

Achten, W.M.J., Verchot, L., Franken, Y.J., Mathijs, E., Singh, V.P., Aerts, R., 2008. *Jatropha curcas* bio-diesel production and use. Biomass Bioenerg. 32, 1063–1084.

Adam, S.E., Magzoub, M., 1975. Toxicity of *Jatropha curcas* for goats. Toxicol. 4, 347–354.

Ahmed, O.M., Adam, S.E., 1979. Effects of *Jatropha curcas* on calves. Vet. Pathol. 16, 476–482.

Aiyelaagbe, O.O., Adesogan, E.K., Ekundayo, O., Adeniyi, B.A., 2000. The antimicrobial activity of roots of *Jatropha podagrica* (Hook). Phytother. Res. 14, 60–62.

Becker, K., Makkar, H.P.S., 1998. Effects of phorbol esters in carp (*cyprinus carpio L.*). Vet. Hum. Toxicol. 40, 82–86.

Commission of the European Communities, 1996. Technical guidance document in support of commission directive 93/67/EEC on risk assessment for new notified substances and commission regulation (EC) no. 1488/94 on risk assessment for existing substances. Office for Oficial Publications of the European Communities, Luxembourg.

Devappa, R.K., Makkar, H.P.S, Becker, K., 2010b. Nutritional, biochemical, and pharmaceutical potential of proteins and peptides from *Jatropha*: review. J. Agric. Food Chem. 58, 6543–6555.

Devappa, R.K., Makkar, H.P.S., Becker, K., 2010a. Quality of biodiesel prepared from phorbol ester extracted *Jatropha curcas* oil. J. Am. Oil Chem. Soc. 87, 697–704.

Devappa, R.K., Makkar, H.P.S., Becker, K., 2010c. *Jatropha* toxicity—a review. J. Toxicol. Environ. Health B Crit. Rev. 13, 476–507.

Devappa, R.K., Makkar, H.P.S., Becker, K., 2010d. Optimization of conditions for the extraction of phorbol esters from *Jatropha* oil. Biomass Bioenerg. 34, 1125–1133.

Devappa, R.K., Makkar, H.P.S., Becker, K., 2011. *Jatropha* diterpenes—a Review. J. Am. Oil Chem. Soc. 88, 301–322.

Devappa, R.K., Rajesh, S.K., Swamylingappa, B., 2009. Antioxidant and antibacterial Properties of *Jatropha (Jatropha curcas)* meal Extracts. J. Food Sci. Technol. Nep. 5, 73–81.

El-Badwi, S.M., Adam, S.E., 1992. Toxic effects of low levels of dietary *Jatropha curcas* seed on brown Hisex chicks. Vet. Hum. Toxicol. 34 (2), 112–115.

Gandhi, V.M., Cherian, K.M., Mulky, M.J., 1995. Toxicological studies on Ratanjyot oil. Food Chem. Toxicol. 33, 39–42.

GEXSI, 2008. Available from 〈http://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf〉.

Gutleb, A.C., Ropstad, E., Brandt, I., Murk, A.J., 2004. *In vitro* bioassays-valuable tools contributing to the conservation of endangered species. IUCN Otter Spec. Group Bull. 21A.

Haas, W., Strerk, H., Mittelbach, M., 2002. Novel 12-deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J. Nat. Prod. 65, 1434–1440.

ISO-6341, 1996. Water quality—Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea*)—Acute toxicity test.

Kinghorn, A.D., Harjes, K.K., Doorenbos, N.J., 1977. Screening procedure for phorbol esters using brine shrimp (*Artemia salina*) larvae. J. Pharm. Sci. 66, 1362–1363.

Kumar, V.P., Chauhan, N.S., Padh, H., Rajani, M., 2006. Search for antibacterial and antifungal agents from selected Indian medicinal plants. J. Ethnopharmacol. 107, 182–188.

Li, C.Y., Devappa, R.K., Liu, J.X., Makkar, H.P.S., Becker, K., 2010. Toxicity of *Jatropha curcas* phorbol esters in mice. Food Chem. Toxicol. 48, 620–625.

Liu, S.Y., Sporer, F., Wink, M., Jourdane, J., Henning, R., Li, Y.L., Ruppel, A., 1997. Anthraquinones in *Rheum palmatum* and *Rumex dentatus* (Polygonaceae), and phorbol esters in *Jatropha curcas* (Euphorbiaceae) with molluscicidal activity against the schistosome vector snails *Oncomelania, Biomphalaria*, and *Bulinus*. Trop. Med. Int. Health 2, 179–188.

Makkar, H.P.S., Becker, K., 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur. J. lipid Sci. Technol. 111, 773–787.

Makkar, H.P.S., Becker, K., Sporer, F., Wink, M., 1997. Studies on Nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J. Agric. Food Chem. 45, 3152–3157.

Makkar, H.P.S., Siddhuraju, P., Becker, K., 2007. A laboratory manual on quantification of plant secondary metabolites. Humana Press, New Jersey, p. 130.

Makkar, H.P.S., Maes, J., De Greyt, W., Becker, K., 2009. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J. Am. Oil Chem. Soc 86, 173–181.

Makkar, H.P.S., Martinez Herrera, J., Becker, K., 2008. Variations in seed number per fruit, seed physical parameters and contents of oil, protein and phorbol ester in toxic and nontoxic genotypes of *Jatropha curcas*. J. Plant Sci. 3, 260–265.

Moreno, L., Bello, R., Primo-Yufera, E., Esplugues, 1997. *In vitro* studies of methanol and dichloromethanol extracts of *Juniperus oxycedrus* L. Phytother. Res. 11, 309–311.

OECD, 2004. *Daphnia* sp. Acute immobilization test method 202, Organization for Economic Cooperation and Development Guidelines for the Testing of Chemicals 202. OECD, Paris.

Ruebhart, D.R., Cock, I.E., Shaw, G.R., 2008. Brine shrimp bioassay: importance of correct taxonomic identification of *Artemia* (Anostraca) species. Environ. Toxicol. 23, 555–560.

Rug, M., Ruppel, A., 2000. Toxic activities of the plant *Jatropha curcas* against intermediate snails and larvae of schistosomes. Trop. Med. Int. Health 5, 423–430.

Saetae, D., Suntornsuk, W., 2010. Antifungal activities of ethanolic extract from *Jatropha curcas* seed cake. J. Microbiol. Biotechnol. 20, 319–324.

Terasaki, M., Makino, M., Tatarazako, N., 2009. Acute toxicity of parabens and their chlorinated byproducts with *Daphnia magna* and *Vibrio fisheri* bioassays. J. Appl. Toxicol. 29, 242–247.

Vairappan, S.C., 2003. Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (*Rhodomelaceae, Ceramiales*). Biomol. Eng. 20, 255–259.

Van den berghe, D.A., Vlietinck, A.J., 1991. Screening for antibacterial and antiviral agents. In: Hostettmann, K. (Ed.), Methods in Plant Biochemistry (Vol. 6 - Assays for Bioactivity). Academic Press, London, pp. 47–69.

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CHAPTER - 6

Shelf-life of isolated phorbol esters from *Jatropha curcas* oil

Rakshit K. Devappa, Harinder P.S. Makkar, Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart, Germany

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Abstract

The application of bio-control agents in agriculture is gaining importance. Phorbol esters (PEs) from *Jatropha curcas* provide a means to control pests. Long-term stability of methanol extracted phorbol esters enriched fraction (PEEF) was studied under dark at room temperature (RT, 22−23 °C), 4 °C and -80 °C. Highest degradation of PEs (50%) was at RT after 132 days; while it was 8% and 4% at 4 °C and -80 °C respectively. The snail bioassay conducted at regular intervals revealed that the PEEF lost biological activity more rapidly at RT, becoming ineffective after 260 days; while at 4 °C and -80 °C, the degradation was slow and only 27.5% and 32.5% of bioactivity was remaining after 870 days. The degradation of PEs is due to auto-oxidation. The auto-oxidation is reflected from the changes in fatty acid composition, increase in peroxide value and decrease in free radical scavenging activity of the PEEF. Inclusion of antioxidants as additives increased the protection of PEs against degradation. In conclusion, the PEEF is susceptible to oxidation and addition of antioxidant is required to increase shelf life of PEs.

1. **Introduction**

In recent years, the utilization of bio-control agents in agriculture is gaining importance in both developed and developing countries. This has enrouted interest in the utilization of novel natural compounds from plants, animals and microorganisms. *Jatropha curcas* seeds are one of the potential and promising feedstocks for biodiesel production and are also an abundant source of phytochemicals that exhibit a wide range of biological activities on species from microorganisms to higher animals (Devappa et al., 2010a: Devappa et al., 2010b; Devappa et al., 2011). For example, organic solvent extracts from seeds, oil and vegetative parts have moluscicidal activity against *Biomphalaria glabrata* (which cause schistosomiasis), insecticidal activity against mosquitoes e.g. *Aedes aegypti* L. (which cause dengue fever) and *Culex quinquefasciatus* (lymphatic filariasis vector) and anti-birth activity against houseflies (*Musca domestica*) (Solsoloy and Solsoloy, 1997; Goel et al., 2007; Devappa et al., 2010b). The extracts also exhibit toxicity in higher animals (mice, rat, chicken, goat, sheep etc.) and possess antibacterial, fungicidal and rodenticidal properties (Amin et al., 1972; Rahuman et al., 2008; Karmegam et al., 1996; Adam, 1974; Chivandi et al., 2000; Becker and Makkar, 1998; Devappa et al., 2008). The phorbol esters (PEs) are considered to be the most active principle present in various organic extracts prepared from the seeds. *J. curcas* seeds and oil contain at least 6 different PEs (Haas et al., 2002). These PEs are diterpenes containing tigliane as a backbone and hydroxylation of its tigliane skeleton in various positions and ester bonding to various acid moieties characterize large number of compounds termed as PEs (Goel et al., 2007). PEs also promote uncontrolled cell growth following exposure to a solitary chemical initiator (e.g. dimethylbenz(a)anthracene), through activation of protein kinase C (PKC). The predicted structural features of PEs responsible for biological activities are (a) polar functional groups near to O-3, O-4, O-9, O-20 of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (b) free C-20 hydroxyl group (c) no sterric hindrance near the five membered ring, and (d) hydrophobic moiety near C-20 (Goel et al., 2007; Jeffry and Liskamp, 1986).

By 2015, the global projected Jatropha oil production is 26 million tonnes and majority of its total production will be concentrated in Asia, with India, Myanmar and China together playing a major role (GEXSI survey 2009). This indicates the future potential to generate a large amount of feedstock oil for the production of biodiesel. In addition, Jatropha oil is also a rich source of PEs (3−6 mg/g), indicating the future potential to produce ~0.08–0.15 million tonnes of PEs. During oil extraction from *J. curcas* seeds, 70−75% PEs are extracted along

with the oil and 25−30% of PEs still remains strongly bound to the matrix of the kernel meal (Makkar et al., 2009a; Makkar et al., 2009b). Generally, oil is further subjected to processing treatments such as degumming, stripping and esterification to produce biodiesel. In all these stages, the PEs undergoes partial or complete destruction depending on the deodorisation conditions (Makkar et al., 2009a). However, these PEs could be extracted from Jatropha oil with minimum resources and in a short time (< 5 min) before subjecting it to the biodiesel production. The extracted PEs could be concentrated upto 14 fold and the biodiesel produced from the residual oil after PEs extraction meet the European and American biodiesel standards (Devappa et al., 20010c; Devappa et al., 2010d). The extracted PEs has a potential to be used in various agricultural and pharmaceutical applications. In addition, the extraction of PEs could be advantageous in (a) providing a value added coproduct which enhances the economic viability and sustainability of biodiesel based production chain and, (b) preventing health risk and creating sustainable working environment for workers involved in the biodiesel industries.

The information on environmental degradation of a biochemical/phytochemicals used as a pesticide is another important parameter. PEs present in Jatropha oil and Jatropha seed cake are completely biodegradable in soil and the degraded products appear to be innocuous (Devappa et al., 2010e). Despite its potency and applicability, the stability of these compounds (PEs) is an important trait for their use in various applications. It is well documented that the biological activity of PEs differs widely from one source to another. Jatropha PEs are reported to be unstable and difficult to isolate in the pure form (Hass et al., 2002). However, it is anticipated that the stability of PEs in the form of an enriched fraction would be higher than that of the purified PEs. Our long term aim is to use the extracted PEs in agricultural applications. In this study we examined: (1) stability of the PEs present in an extract kept under different conditions, (2) bioactivity of PEs present in the extract at regular storage intervals, (3) possible mode of PEs degradation products formation on storage, and (4) effect of antioxidants on stability of the PEs.

2. Materials and methods

2.1. Materials

J. curcas seeds (toxic Indian variety) were from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E),

Rajasthan, India. Phorbol 12-myristate-13-acetate (PMA) and 2, 2′-Azobis-(2-methyl-butyronitril) (Vazo−67) was obtained from Sigma (St. Louis, USA) and all other chemicals and solvents used were of analytical grade.

2.2. Preparation of Jatropha oil

J. curcas seeds were mechanically pressed using a screw press to obtain oil. The oil was centrifuged at 3150 × *g* for 20 min to remove residues and the supernatant was collected and stored in a refrigerator (4 °C) until further use.

2.3. Extraction of Phorbol ester rich fraction

J. curcas oil was mixed with methanol (1:2, w/v) in a capped container and the contents were stirred at 55 °C for 15 min using a magnetic stirrer (300 rpm). Thereafter, the mixture was centrifuged at 3150 x *g* for 5 min to get upper methanolic and lower oily layers. The methanol layer was rotaevaporated at 65 °C under vacuum (300 mbar) to get oily PEs enriched fraction (PEEF) (Devappa et al., 2010c). The oily PEEF used in the experiment was golden yellow in colour and free of methanol, liquid at room temperature, partially solid (~15−25%) at 4 °C and completely frozen at -80 ^{o}C.

2.4. Phorbol ester analysis

Phorbol esters were determined at least in duplicate according to Makkar et al. (2007), based on the method of Makkar et al. (1997). Briefly, 0.5 g of oil sample or PEEF was extracted four times with 2% tetra hydrofuran in methanol. A suitable aliquot was loaded into a high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 x 4 mm id, from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at RT (23°C) and the flow rate was 1.3 ml/min using a gradient elution as described by Makkar et al. (2007). The four phorbol ester peaks (containing 6 PEs) appeared between 25.5 and 30.5 min were detected at 280 nm. The spectra of each peak were taken using Merck-Hitachi L-7450 photodiode array detector. Phorbol-12-myristate 13-acetate (PMA) was used as an external standard which appeared between 31 and 32 min. The area of the four phorbol ester peaks was summed and the concentration was expressed equivalent to PMA. The PEs detection limit in the HPLC was 3 to 4 µg. The PEs in PEEF was analyzed in triplicates at regular interval of shelf life studies.

2.5. Long term or real time stability studies without additives

The PEEF was sub-sampled into a plastic eppendorf vials in triplicate and kept at three different temperatures (RT, 23−25 °C, 4 °C and -80 °C) in dark for > 2 year. At regular intervals Eppendorf tubes were taken out and aliquots were taken for analysis of PEs. The PEEF was sub-sampled in such a way that for each time interval a fresh eppendorf vial was available for the analysis.

2.6. Bioassay for toxicity in snails

Among the various bioassays (Daphnia, *Artemia salina* and snails (*Physa fontinalis*) tested in our laboratory, snails were found to be highly susceptible to PEs (our unpublished results; also see Results and Discussion section). Tests with snails were performed according to the method of Rug and Ruppel (2000). All tests were carried out in deionized water. Stock solutions of extracts were prepared in methanol and further diluted in water. Groups of 10 snails were placed in glass containers with 400 ml of water containing the test substance (methanol extract diluted in water). The glass containers were placed in a water bath adjusted at 26 °C. Snails were prevented from crawling out of the containers by a fine stainless steel mesh suspended just above the water surface. After 24 h of incubation the snails were transferred to de-ionized water, fed and maintained for another 48 h. Mortality of the snails was determined by lack of reaction to irritation of the foot with a needle. Control experiments were performed with the same quantity of methanol in water as used for the test preparations and no mortality was recorded in the control containers. All tests were independently repeated three times. Toxicity is expressed as percent mortality referring to concentrations killing 100% of the snails.

2.7. Oxidation by chromic acid

Degradation of PEs was observed in our study (see results). We presumed that PEs are susceptible to oxidative degradation. To test this hypothesis, we carried out chemical oxidation reaction, wherein the PEs rich fraction (0.4 g) was mixed with freshly prepared chromic acid (0.16 ml), used as an oxidising agent, at room temperature and kept in dark for 30 min. The reaction mixture was then centrifuged at 3000 × *g* for 5 min. The known weight of oily supernatant layer was collected and re-dissolved in methanol for further analysis of PEs.

2.8. Peroxide value (PV) analysis

The PV was analyzed by the modified standard International Dairy Federation method. In brief, Fe(II) is oxidized to Fe(III) by hydroperoxides, leading to the formation of reddish Fe(III)-thiocyanate complex, the colour of which is measured using a spectrophotometer. The procedure followed was that of Hornero-Mendez et al. (2001).

2.9. Antioxidant Assay

The assay was carried out according to Sharma and Bhat (2009). In brief, the stock solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (200 μM) was prepared in methanol and kept in cold and dark place until use. Different volumes of the aliquots of the test samples, kept at different temperature were taken and the volume was adjusted to 3 ml with methanol, to obtain different concentrations. The reaction was started by adding 1 ml of 200 μM DPPH stock solutions (final DPPH concentration in the assay 50 μM). After shaking for 30 min at 30 °C in the dark the absorbance was measured at 517 nm against methanol using a spectrophotometer.

2.10. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared by the boron trifluoride method (AOAC, 1990; Schlectreim 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.

2.11. Screening of antioxidants

As the degradation mechanism of chemical compounds vary from one compound to another, choosing a suitable assay to monitor the degradation as well as effect of stabilizers on degradation of compounds is critical. Our preliminary studies indicated that PEs are

sensitive to increase in temperature (as low as 60 °C; see section 3.7) when present as a component in PEEF. Therefore, a milder degradation assay was optimized to study the effect of stabilizers (antioxidants) on the PEs present in PEEF.

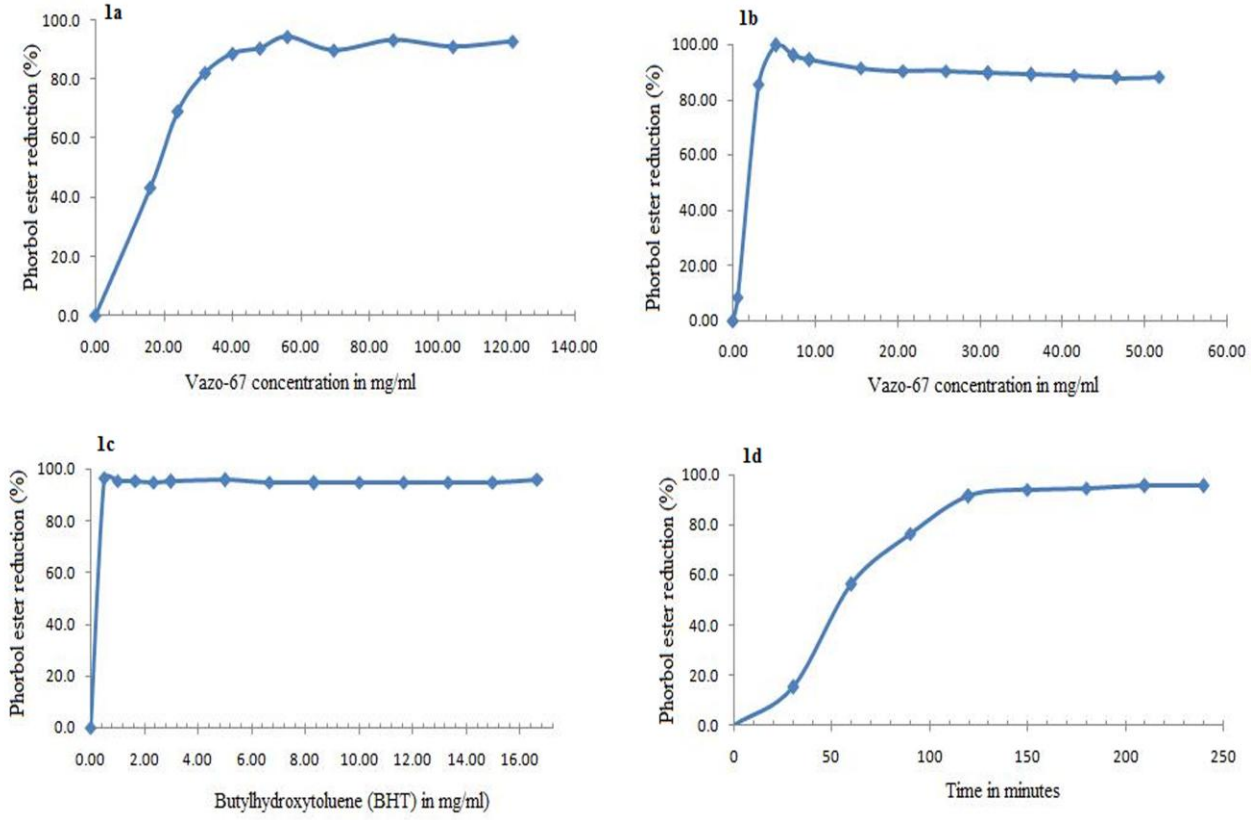


Figure 1. Optimization of chemical oxidation assay using Vazo-67 for screening of antioxidants.

2.11.1 Development of chemical oxidation assay

Initially, assay was carried out by taking a fixed weight of the PEEF containing PEs (0.2 g PEEF/ml of acetone) and concentration of diazo initiator was optimized. The diazo initiator used in the experiment was 2, 2′-Azobis-(2-methyl-butyronitril) (synonym: Vazo-67). An aliquot (250 µl) of PEEF was mixed with 100 µl of varying concentrations of Vazo-67 dissolved in acetone, followed by addition of 100 µl of acetone. It was incubated at 37 °C and 55 °C for 4 h. The reaction was stopped by adding 50 µl of BHT (butylhydroxytoluene, 25 mg/ml). The assay mixture was extracted with methanol and PEs were measured (section 2.4), to determine their decrease. The minimum concentration of Vazo-67 at which PEs were reduced by 90% was recorded. Using this assay, PEs were reduced by 90% at 48 mg and 6

mg of Vazo-67 at 37 °C and 55 °C respectively, and further increase in the concentration of Vazo-67 did not have any major effect in reducing the PEs (Figure 1a and 1b). This study demonstrated that increase in temperature increases the rate of the reaction with lower amount of Vazo-67.

Further, studies were conducted to optimize the effective concentration of the antioxidant (BHT). It was chosen because it is the most commonly used and effective antioxidant. The PEEF (250 µl) was mixed with Vazo-67 at the optimized concentration (100 µl, 60 mg/ml acetone) and incubated for at 55 °C for 4 h. The reaction was stopped by adding varying concentrations of BHT in acetone (100 µl). The results showed that 0.5 mg of BHT in the assay mixture was effective in arresting the reaction (Figure 1c). Further, studies were conducted to optimize the time for the assay reaction. The assay mixture (250 µl of PEEF mixed with Vazo-67 (100 µl, 60 mg/ml acetone)) was incubated for varying time intervals at 55°C and 100 µl of BHT (5 mg/ml in acetone) was added to arrest the reaction. The result indicated that PEs reduced by 90% at 3 h (Figure 1d).

2.11.2. Screening of antioxidants using the chemical oxidation assay

Screening of antioxidants was carried out by using the optimized chemical oxidation assay (section 2.11.1). An aliquot (250 µl) of the PEEF dissolved in methanol (0.8−0.9 g/ml; PEs concentration was 86 mg/g PEEF) was mixed with 100 µl of Vazo-67 dissolved in acetone (60 mg/ml) and 100 µl of acetone containing varying concentrations of antioxidants. The assay mixture was incubated for 3 h at 55 ºC and the reaction stopped by adding 100 µl BHT (5 mg/ml). The assay mixture was extracted with methanol to extract PEs, which were analysed by the method given in section 2.4. The antioxidants quercetin, pyrogallol, TBHQ (Tertiary butylhydroquinone), BHA (butylated hydroxy anisole), BHT, baynox and α-tocopherol up to 10,000 ppm were evaluated against chemical oxidation in PEEF. The antioxidant (mg) required to completely protect PEs (mg) under the assay conditions was noted.

2.12. Shelf life studies in presence of antioxidant

Shelf life studies of the PEEF were conducted at RT using the potent antioxidants as screened by using chemical oxidant (Vazo-67) as in section 2.11.2. The studies were conducted up to 132 days, at which 50% of PEs reduction was observed during the shelf life studies conducted without antioxidants (Figue 1, Section 2.5).

2.13. Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of 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.

3. Results and discussion

The activity of bio-chemicals either in pure form or in formulations tends to decrease gradually with time. Therefore, it is essential to monitor and improve the storage stability of any phytochemicals for its effective use. Generally, stability is determined for a wide variety of pure substances or their end user products. In the present study, PEEF was extracted from *J. curcas* oil with the aim to utilize it in suitable agricultural applications as a bio-control agent. Consequently, the stability and efficacy of the extracted PEEF were evaluated over a long period of storage time under defined laboratory conditions; at RT, 4 °C and -80 °C for 870 days. The physical and chemical changes occurring were monitored at regular intervals.

3.1. Stability studies without antioxidant

The PEs concentration in methanol soluble PEEF at day-0 was 12.43 mg/g and when stored at RT the concentration decreased slightly (2%) in the initial days (9 days), reaching 50% ($P<0.05$) after 132 days (Fig. 2). The rate of reduction was calculated up to 254 days, and the equation derived was $y = 0.322x + 3.34$ (where x = 'number of days' and y = % reduction) with R^2 of 0.97. After 178 days, the PEEF kept at RT was not completely soluble in methanol, resulting in un-dissolved oily layer at the bottom. But, the methanol dissolved PEEF still contained 30% PEs. Further increase in the storage time increased methanol insoluble layer (8 to 12% w/w of PEEF) and 76.7% of PEs reduction was observed after 254 days. After 557 days >99% reduction was observed. At day-254, although the peaks appeared at the same retention time as of PEs, their absorption spectrum did not match the absorption spectrum of PEs peaks analyzed at zero day (absorption spectra not given). Concurrently, additional peaks started to appear in the HPLC chromatogram indicating the appearance of degradation products in PEEF after day-254. After day-870, the area of the additional peaks (non PEs) increased rapidly. These observations clearly show the degradation of PEs in the

PEEF (Figure 3). The formation of additional peaks might be auto-oxidized products. The PEs in the PEEF when kept a RT are unstable.

At 4 °C and -80 °C, the reduction of PEs was very low up to 870 days (Figure 2). Nevertheless, small amount of methanol insoluble layer started to form after 260 days (1.4% w/w, insoluble layer : PEEF) which thereafter increased slowly up to 870 days (4.5% w/w, insoluble layer : PEEF). Overall, marginal reductions in PEs (7.9% and 5.4 % at 4 °C and –80 °C respectively) were observed after 870 days. At 4 °C, additional PEs degraded peaks started to appear after 590 days and were prominent at day-870 (Figure 3c). However, PEs degraded peaks were not observed up to day 870 when stored at -80 °C (Figure 3d). These results demonstrate that PEs in the PEEF is stable up to 29 months when stored at 4 °C and -80 °C.

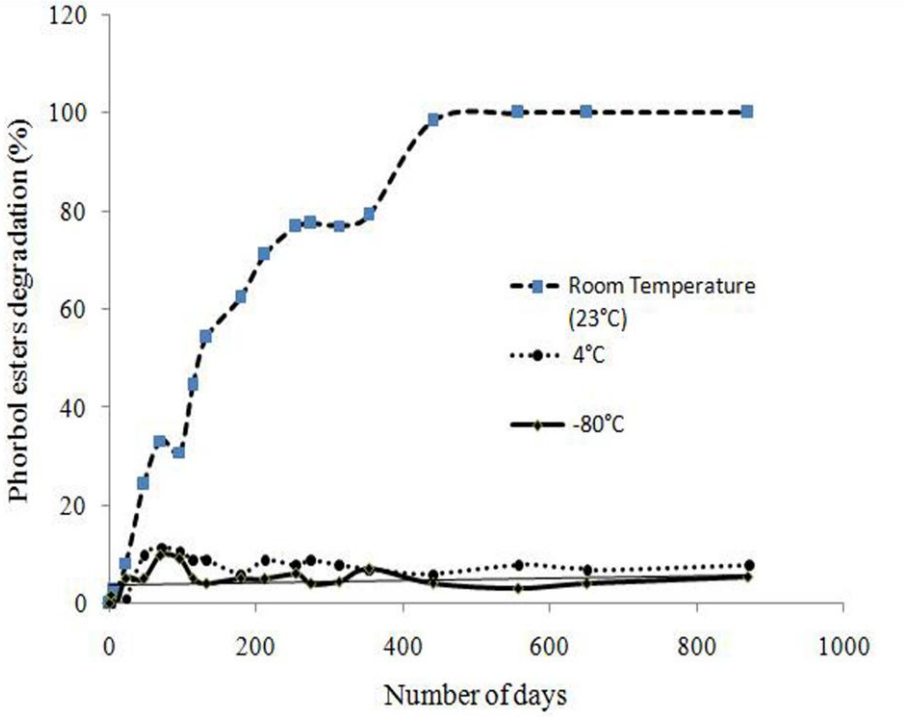


Figure 2. Shelf life studies of phorbol esters enriched fraction (PEEF) at room temperature (RT), 4 °C and -80 °C.

3.2. Bioactivity of PEEF during storage

At day-0, methanol extract of the PEEF containing PEs was effective against snails, exhibiting 10%, 60% and 100% mortalities at 0.01 ppm, 0.1 ppm and 1 ppm concentrations respectively. The bioactivity of PEs in the PEEF at regular intervals of storage was evaluated at 1 ppm equivalent concentration at day-0. The storage under RT reduced PEEF bioactivity by 40% and 85% after day 45 and 125 respectively (Figure 4) and the methanol extract from

the PEEF after day-260 was nontoxic to snails. The decrease in biological activity was due to decrease in the PEs content of the PEEF. At 4°C and -80°C bioactivity decreased with increase in number of storage days (Figure 3) albeit to a lower extent than at room temperature.

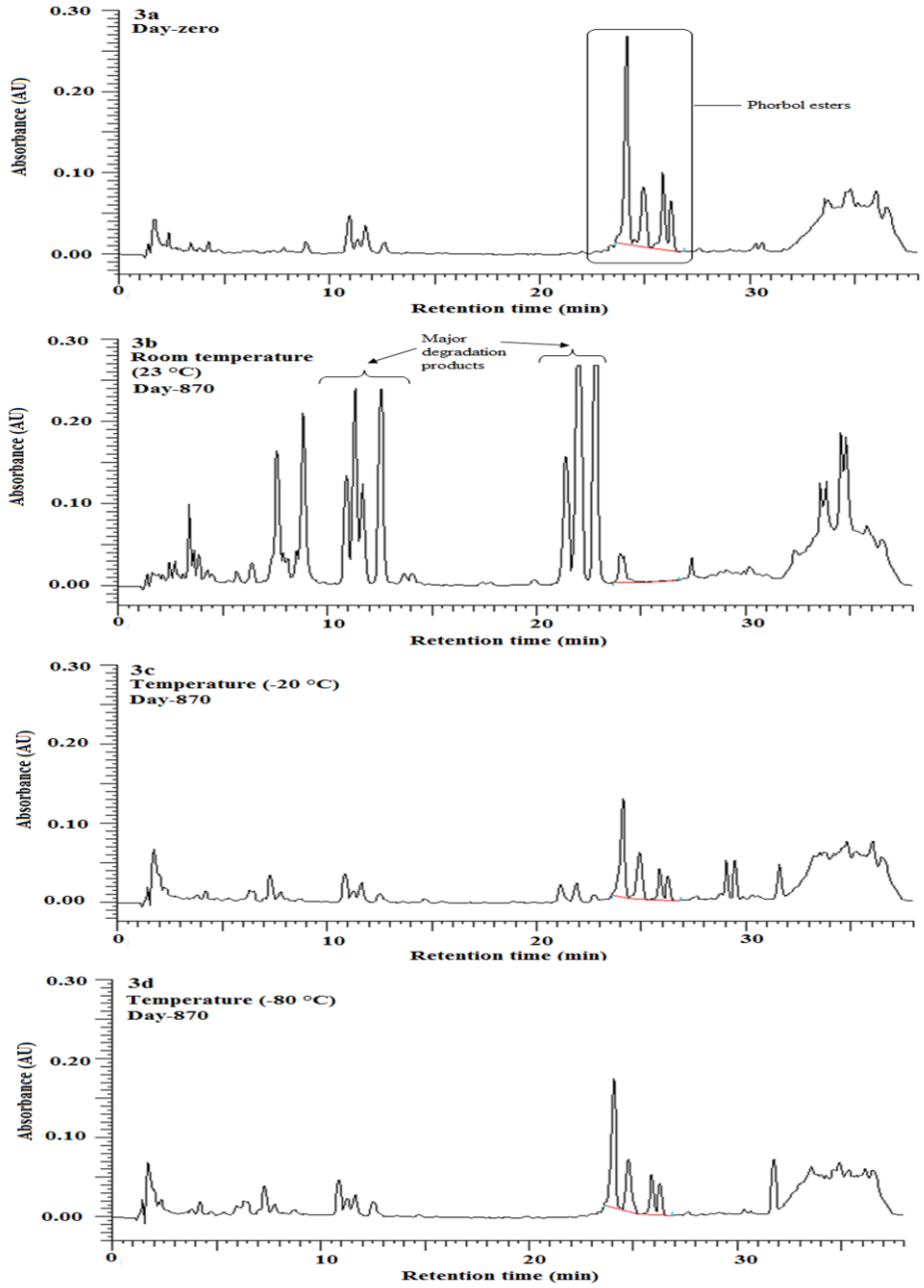


Figure 3. Comparison of HPLC chromatograms of phorbol esters enriched fraction (PEEF) during shelf life studies.

At day-870, the bioactivity was 27.5% and 32.5% at 4 °C and -80 °C respectively. However, the biological activity values do not correlate with the PEs content in the PEEF. The results

indicated that even though PEs are present in the PEEF (as quantified by the HPLC), there was a gradual reduction in the bioactivity of the PEEF. The reduction in the efficacy or biological activity of the PEEF may be due to change in the chemical nature or structural features responsible for biological activities of the PEs. The presence of degradation products in the chromatograms at 4 °C and -80 °C (Figure 3) supports the structural changes in PEs present in PEEF.

The stability of the compound depends upon physical and chemical properties of the end product in a test material. PEs are large and complex molecules having number of reactive functional groups which render the compound unstable under different storage conditions. In our study, we hypothesize that the degradation of PEs in the PEEF is due to oxidation. The comparison of spectra and retention time of oxidation products induced by a chemical reaction with those obtained on real time storage of the PEEF; and evaluation of the peroxide value, inherent free radical scavenging activity and fatty acid composition could provide information on the possible mode of degradation of PEs during storage.

3.3. Chemical oxidation using chromic acid

Chemical oxidation of PEs using chromic acid (CA) was carried out to ascertain whether the degradation of PEs during storage is due to oxidation. The CA-oxidized PE peak pattern of the PEEF in the HPLC chromatogram was compared with the chromatogram of PEs in the PEEF stored at different temperatures. As the appearance of PEs degradation products (peaks) was prominent at RT, the chromatogram of RT (day-260) was used to compare with that of the CA-oxidized PEs. Both chromatograms showed degradation peaks (I and II) at similar retention times indicating susceptible nature of PEs in the PEEF towards auto-oxidation during storage (Fig. 5a and 5b). In another study on degradation of PEs in soil, Devappa et al. (2010e) have reported that the HPLC chromatogram of day-9 of the PE amended soil also showed similar peak pattern (Fig. 5c). This suggests that PEs are susceptible to oxidation reaction in the environment. It has also been reported that purified PEs decompose in the presence of acids and bases. Schmidt and Hecker (1975) reported that purified TPA in acetone was stable when stored in the dark at -20 °C, but it decomposed slowly in the dark at 4 °C in 3 months and the degradation was extensive at 25 °C in diffused daylight in 3 months. During decomposition 7-hydroperoxide was formed as a major product. Similar decomposed products may be formed during the PEEF storage. In addition, TPA stored (3 months) as a powder at 25 °C (under diffused light) formed hydroperoxides, and at

4 °C (under dark) the hydroperoxides were formed to a very limited extent. Similarly, Tremp and Hecker (1985) also observed significant reduction of 12-*O*-retinoylphorbol-13-acetate solution in acetone when stored at room temperature (20 °C) for a period of 5 weeks; while slight decomposition of PEs in solutions stored in amber glassware wrapped with black foil, with and without argon at 20 °C was observed; and at -20 °C and -70 °C no decomposition was observed. They suggested that solutions stored in the refrigerator be wrapped in black foil.

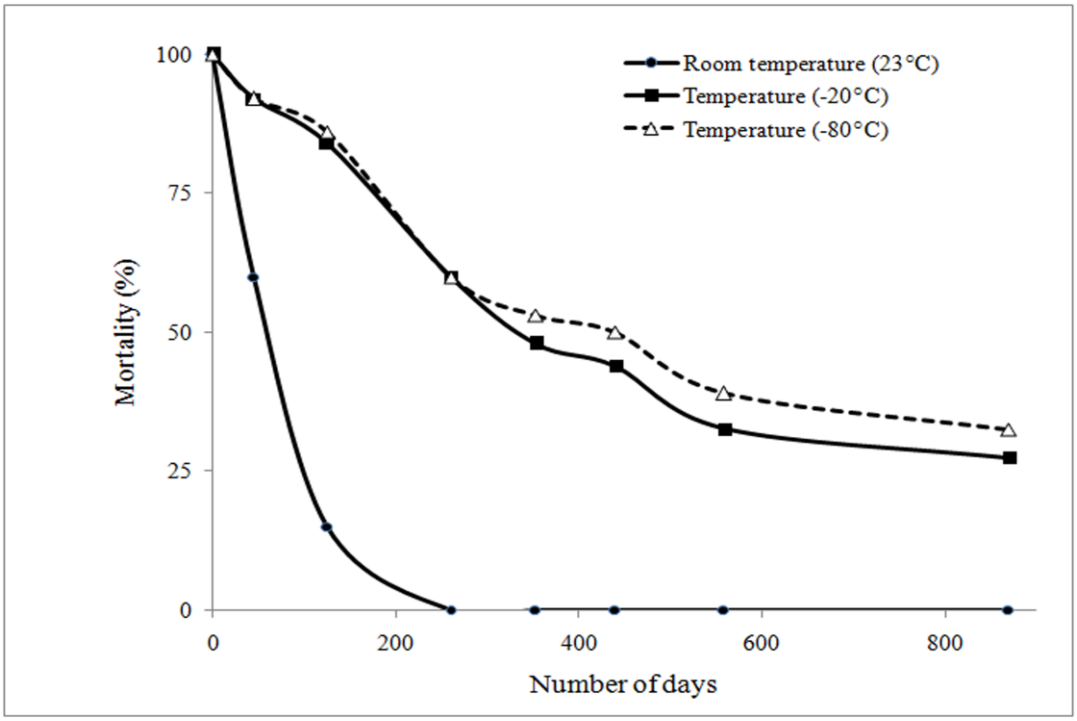
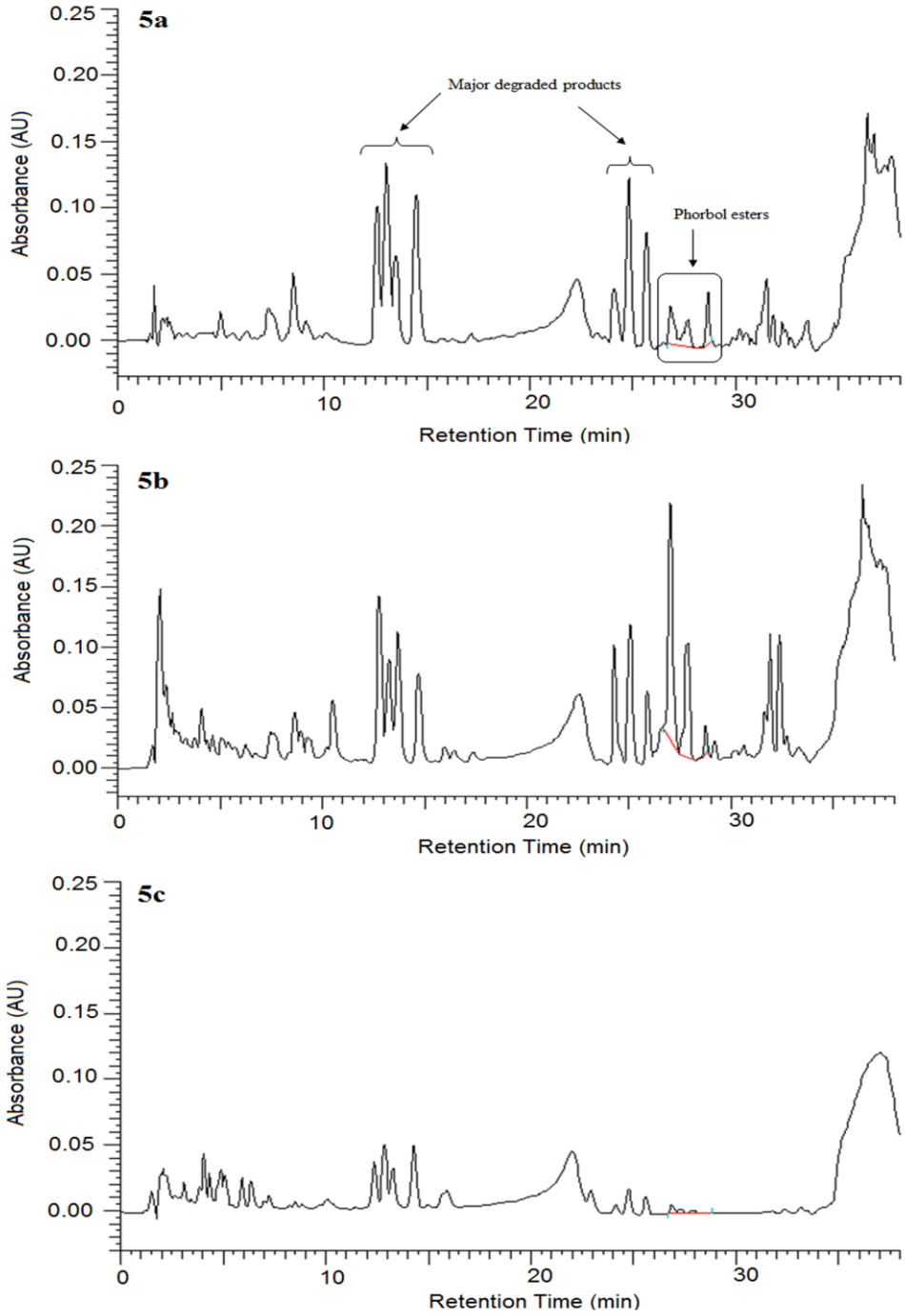


Figure 4. Bioactivity of phorbol esters enriched fraction (PEEF) during storage at room temperature (RT), 4 °C and -80 °C.

3.4. Peroxide value

Peroxide value (PV) as an indicator of current status of oxidation was evaluated. In general, as the fatty oils or the alkyl monoesters of fatty oils are oxidized, hydroperoxide ROOH level increases. In our study, at day-0 PEEF had PV of 13.80 (Table 1). The PV values of PEEF after day-980 kept at 4 °C and -80 °C were 16.35 and 17.86 respectively; an increase of 1.2 and 1.3 fold in PV. Whereas, PV of the RT stored sample increased by 3.6

fold, indicating higher oxidation of products formed when the PEEF is stored at RT. The results suggest that majority of destruction of PEs in the PEEF is due to oxidation.



Fiure 5. Comparison of chromatograms among Chromic acid (CA)-oxidized phorbol esters enriched fraction (PEEF; 5a), room temperature stored 260-day PEEF (RT-260 day PEEF; 5b) and phorbol esters (PEs) degraded in soil at 9th day (chromatogram for 9th day PEs degradation in soil taken from Devappa et al. (2010e); 5c).

3.5. Free radical scavenging activity

In general, vegetable oils contain natural antioxidants which impart certain amount of protection against oxidation. The most common are tocopherols, which are phenolic chain breaking antioxidants having high reactivity towards free radicals, forming stable compounds deprived of initiating oxidation reaction. For example, soy oil along with other antioxidants such as sterols contains approximately 500−3000 ppm of α-tocopherols (Tarandjiska et al., 1996). However, naturally occurring antioxidants are often lost during processing or storage, necessitating the addition of exogenous antioxidants. In our study, we estimated the inherent/native antioxidant activity of the PEEF as an indicator to evaluate its potential for protection against oxidation. As the oxidation increases, inherent antioxidant activity to scavenge free radical decreases. At day-0, the PEEF had 12.8% of free radical scavenging activity. After 250 days, the PEEF stored at RT, 4 °C and -80 °C had 2%, 11.7% and 12.2% free radical scavenging activity respectively (Table 1). The results indicated that storage at room RT had a strong effect on inherent radical scavenging activity with 84% reduction from day-0. The majority of the reduction may be due to consumption of free radicals produced by oxidation reaction in the PEEF. Whereas, storage at cold temperatures (-4 °C and -80 °C) had slow oxidation reaction which was reflected by a marginal reduction of free radical scavenging activity with a reduction of only 9% and 5% respectively.

3.6. Fatty acid composition

Fatty acid composition (FAC) is another characteristic for evaluation of changes in the PEEF during storage. The prime fatty acids in Jatropha oil consist of monounsaturated (40.8%), followed by polyunsaturated (37.9%) and saturated (21.3%) fatty acids. The fatty acid composition of the *J. curcas* oil observed in this study was similar to that observed by Devappa et al., 2010d).The PEEF extracted from *J. curcas* oil also had similar fatty acid composition with monounsaturated (39.3%), polyunsaturated (41%) and saturated (19.7%) fatty acids. In general, oil containing higher linoleic acid content is susceptible to oxidation. At day-0, the PEEF contained oleic (40%), linoleic (37.8%), palmitic (15.3%) and stearic (5.8%) as major fatty acids (Table 1). Unsaturated fatty acids such as linoleic (18:2) and linolenic (18:3) acids content in fatty oils or esters are generally more susceptible to oxidation or autoxidation than saturated compounds (Neff et al., 1993; Dunn, 2008).

Table 1 - Fatty acid composition of Jatropha oil and phorbol esters enriched fraction (PEEF) stored at room temperature (RT), 4 °C and -80 °C.

					Storage for 870 days		
	Name		**Jatropha oil at day-0**	**PEEF at day-0**	**Room temperature (22−23°C)**	**4 °C**	**-80 °C**
C 16:0	Palmitic	Saturated	15.3	15.5	14.98	11.1	11.7
C 16:1	Palmitoleic	monounsaturated	0.8	1.0	1.25	1.1	1.0
C 18:0	Stearic	Saturated	5.8	4.2	4.39	3.3	3.5
C 18:1	Oleic	Mono unsaturated	40.0	38.3	49.39	43.2	43.0
C 18:2	Linoleic	Poly unsaturated	37.8	40.8	23.13	41.0	40.4
C 18:3	Alpha-linolenic	Poly unsaturated	0.1	0.2		0.2	0.2
C 24:0	lignoceric	saturated	0.2	-	0.35	0.1	0.1
(C 20:1)	Cis-11-Eicosenoic	monounsaturated			0.31		
		Unknown			0.23		
					0.716		
(C 20:2)	Cis-11, 14-Eicosadienoic	polyunsaturated			0.18		
		Unknown			0.96		
(C 22:0)	Behenic	Saturated			0.95		
(C 22:1)	Erucic	monounsaturated			1.31		
		Unknown			1.13		
	Total saturated		21.3	19.7	20.7	14.5	15.3
	Total monounsaturated		40.8	39.3	52.2	44.3	44.0
	Total polyunsaturated		37.9	41.0	23.3	41.2	40.6
Peroxide value	-	-	5.66	13.8	50.16	16.35	17.86
% DPPH scavenging activity	-	-	Not determined	12.8	2	11.7	12.2

In our study the PEEF at day-650 had a slight change in the FAC with 11−13% increase in oleic acid content and 24.5−28% decrease in palmitic acid content for both the PEEF stored at 4 °C and -80 °C. Overall, there was decrease in total saturated fatty acid by 26.3% and 22.0% and increase in total monounsaturated fatty acids by 113% and 112% at 4 °C and -80 °C respectively. On the other hand, at room temperature linoleic (18:2) acid decreased (43%) and oleic (18:1) acid increased (23%), indicating the occurrence of oxidation. In addition, several other new fatty acid products such as cis-11-eicosenoic acid, cis-11, 14-eicosadienoic acid, behenic acid and erucic acid were formed in lesser amounts. The results suggest that the PEEF when stored is susceptible to oxidation which is reflected by decrease in linoleic acid content compared to that at zero day.

3.7. Degradation of phorbol esters at 60 °C

Further we wanted to evaluate the sensitivity of PEs present in PEEF towards the temperature. There was 50% degradation of PEs after 5 days and >90% degradation after 15 days when stored at 60 ℃ in the dark (Figure 6). The HPLC chromatogram shows that the degradation of PEs increased with increase in number of storage days and the degradation peaks that appeared were similar to those of the CA-oxidized PEs and of the PEEF stored for 260 days at RT (Figure 7). The PEs in PEEF was more susceptible to oxidation when stored at high temperatures. This indicated that care should be taken in choosing *in vitro* oxidation assays to screen the antioxidants for stabilizing PEs present in PEEF.

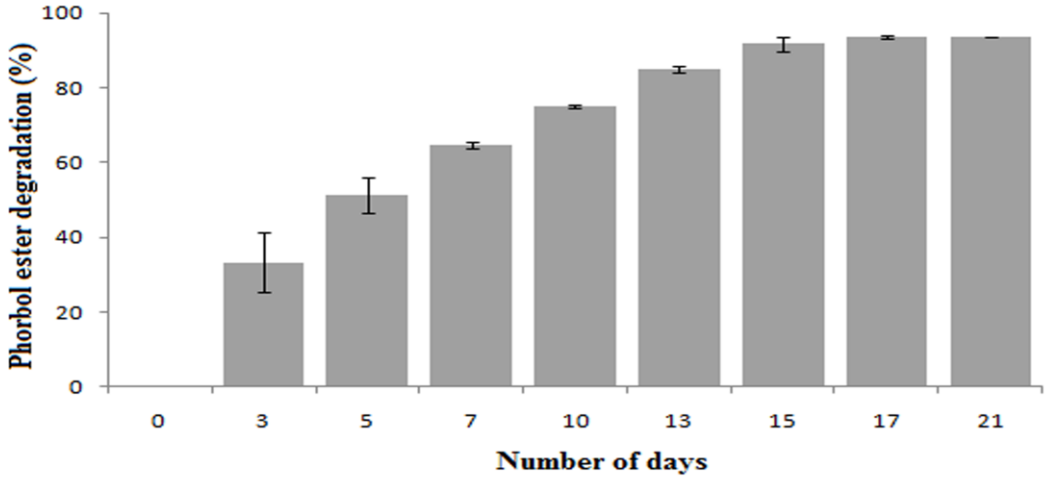


Figure 6. Degradation of phorbol esters (PEs) in the phorbol esters enriched fraction (PEEF) at 60 ℃.

3.8. Stability studies with antioxidants

PEs in the PEEF has short shelf life, especially at room temperature, which could be a serious impediment towards their use as a bio-control agent. Among various factors, lipid auto-oxidation contributes significantly towards deterioration and reduction of the shelf life of many products. However, stability can be increased in several ways, for example by using appropriate storage and packaging conditions, suitable solvents or by adding stabilizers. Our results show that PEs undergoes auto-oxidation reaction and this could be inhibited by adding antioxidants as stabilizers.

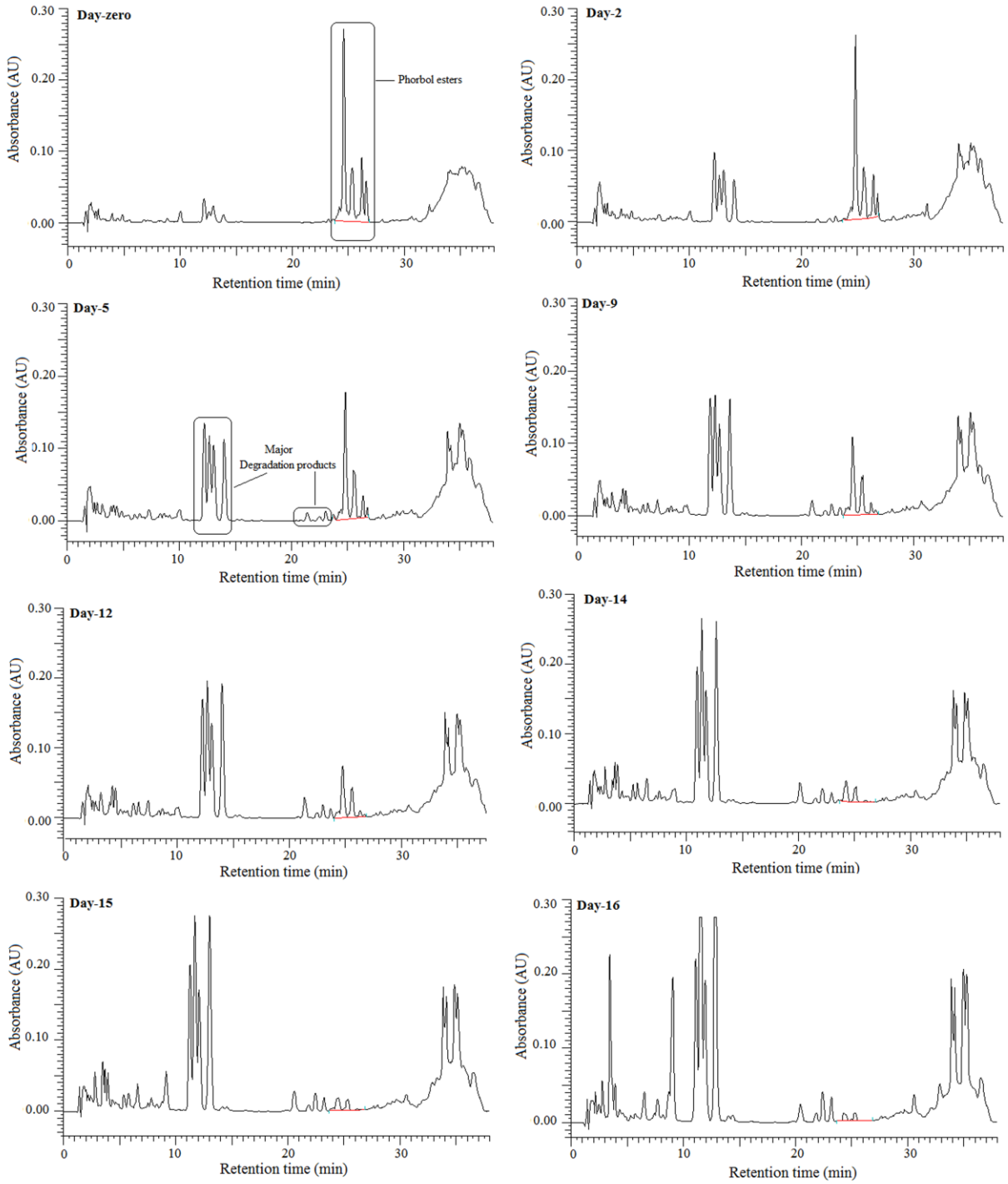


Figure 7. HPLC chromatogram of phorbol esters enriched fraction (PEEF) at different days of storage at 60 °C.

It is known that various antioxidants, inhibitors and scavengers can stabilize compounds against spontaneous decomposition/auto-oxidation. However, different chemical compounds require different stabilizers as the mechanisms of their decomposition vary from one active compound class to the other. Therefore, selection of a stabilizer is an empirical process. Valid comparison of antioxidant activity depends on condition of oxidation and on

the analytical method used to determine the extent and endpoint of oxidation (Frankel et al., 1994). Generally, two commonly used methods are (a) Rancimat or (b) Oxidative Stability Instrument, which determine the oxidative stability index (OSI) of a material at elevated temperatures (100 to 130ºC) while exposing the sample to a stream of air. However, these are not applicable to our situations due to sensitivity of the PEs towards high temperatures (Section 3.7). Hence, we developed and optimized a new system for screening the antioxidants for their potency to protect PEs, so that potent ones could be used in the stability studies.

3.8.1. Screening of antioxidants using chemical oxidation assay (Vazo-67)

The assay was aimed at ranking the antioxidants in preventing the PEs degradation in the PEEF. In the assay, diazo-type free radical sources were used as initiators and accelerators for oxidation reactions. Upon gentle heating, these diazo-compounds decompose and form two free radicals which are highly reactive towards both mono- and polyunsaturated fatty acid moieties in the substrates to accelerate the generation of lipid radicals. Consequently, the lipid oxidation process is initiated and accelerated at low temperatures, against those that use high temperature for example Rancimat and OSI methods. By initiating and accelerating the lipid oxidation processes at lower temperatures, the efficacy of antioxidants can be evaluated on a substrate of interest in a shorter time and under conditions which are closer to the actual storage conditions. In the present study, the rate of PEs degradation was very low after it reached 90% (Figure 2). Thus, the optimization of chemical oxidation assay (Figure 1) was carried out with a cut off point of 90% reduction in the PEs in the PEEF. In addition, a higher cut off point of say 100% reduction could lead to misleading ranking of the test substances due to their possible use at concentrations beyond the concentration that gives just 100% reduction.

The potency of antioxidants as effective concentration (in mg) to protect mg of PEs against oxidation are in the following order: BHA (0.50) > baynox (0.54) > α-tocopherol (0.79) > BHT (0.99) > TBHQ (1.29) > pyrogallol (2.01) > quercetin (2.48). Although the chemical oxidation assay was rapid and the results show the requirement of higher concentrations of antioxidants to protect PEs against oxidation; the assay was used as a tool to eliminate ineffective antioxidants. The best five antioxidants were chosen as additives and real time shelf life studies were conducted for 132 days at RT. The duration of the experiment was

chosen solely because at this time 50% PEs were degraded in the real time shelf life studies (Figure 1).

3.8.2. Stability studies with antioxidant at room temperature

Generally, the concentrations of antioxidants are kept low (500–1000 ppm) to minimize the cost (Dunn RO, 2008). In the present study, the concentrations in the range from 100−7000 ppm were chosen. The antioxidant-mediated protection for PEs against oxidative degradation is shown in Figure 8. In the present study, effectiveness of antioxidants in real time stability studies reflected the chemical oxidation assay indicating the requirement of higher concentrations of antioxidants to prevent oxidation in the PEEF. The potency of antioxidants as effective concentration (in mg) to protect one mg of PEs against oxidation are in the order: BHA (0.09) > baynox (0.11) > tocopherol (0.13) > TBHQ (0.18) > BHT (0.25). The order of first three potent antioxidants is similar to the order observed during the chemical oxidation using Vazo-67. At higher concentration the antioxidants tested were effective in reducing the oxidation (Figure 8). However, the effective concentrations of antioxidants used to protect PEs against oxidation in the PEEF are well above the usual concentrations (500−1000 ppm) used. The studies are also conducted for cold temperatures exhibiting only 3−4% decrease in PEs with or without addition antioxidants (Data not shown). Further studies should be carried out with additive formulations containing combinatorial antioxidants.

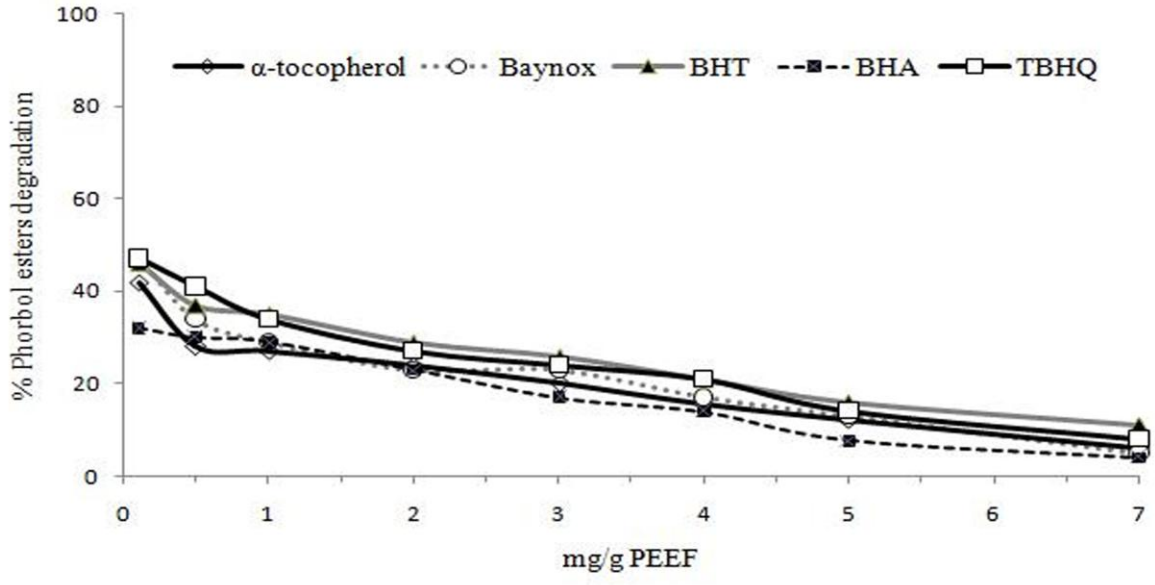


Figure 8. Shelf life of PEs in phorbol ester enriched fraction (PEEF) (with additives) carried out at room temperature (RT) for 132 days.

4. Conclusions

The PEEF has shown earlier to possess insecticidal and moluscicidal activities and it could be used as a bio-control agent. The PEs in the PEEF are biodegradable; however they do not have good stability at room temperature. The degradation of PEs especially at room temperature is due to auto-oxidation, which is reflected by change in FAC, PV, free radical scavenging activity. Storage of the PEEF at 4 °C and -80 °C reduces auto-oxidation. The bioactivity of PEs in the PEEF decreases with time even when stored at these cold conditions, albeit to a lower extent than at room temperature. During storage, stability of PEs in the PEEF could be increased by addition of commercially used antioxidants such as BHT and baynox.

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References

1. Adam SEI. 1974. Toxic effects of *Jatropha curcas* in mice. Toxicol 2:67−76.
2. Amin MA, Daffalla AA, el-Moneim OA. 1972. Preliminary report on the molluscicidal properties of Habat El Mulluk, *Jatropha* Sp. T Roy Soc Trop Med H 66:805−806.
3. AOAC. 1990. Official methods of analysis (963.33), 15th ed. Vol II. AOAC, Arlington: VA.
4. Becker K., Makkar HPS. 1998. Effects of phorbol esters in carp (*cyprinus carpio* L.). Vet Hum Toxicol 40:82−86.
5. Chivandi E., Erlwanger KH, Makuza, S M, Read J S, Mtimuni JP. 2006. Effects of dietary *Jatropha curcas* meal on percent packed cell volume, serum glucose, cholesterol and triglyceride concentration and alpha-amylase activity of weaned fattening pigs. Res J Anim Vet Sci 1:18–24.
6. Devappa RK, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya, S. 2008. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. Food Chem Toxicol 46:3621−3625.
7. Devappa RK, Maes J, Makkar HPS, De Greyt W, Becker K. 2010d. Quality of biodiesel prepared from phorbol ester extracted *Jatropha curcas* Oil. J Am Oil Chem Soc 87:697−704.

8. Devappa RK, Makkar HPS, Becker K. 2010a. Nutritional, biochemical, and pharmaceutical potential of proteins and peptides from Jatropha: review. J Agric Food Chem 58:6543−6555.
9. Devappa RK, Makkar HPS, Becker K. 2010b. Jatropha toxicity – A review. J Toxicol Environ Health B Crit Rev 13:476−507.
10. Devappa RK, Makkar HPS, Becker K. 2010c. Optimization of conditions for the extraction of phorbol esters from Jatropha oil. Biomass Bioenerg 34:1125−1133.
11. Devappa RK, Makkar HPS, Becker K. 2010e. Biodegradation of *Jatropha curcas* phorbol esters in soil. 90:2090–2097.
12. Devappa RK, Makkar HPS, Becker K. 2011. Jatropha Diterpenes: a Review. J. Am oil chem 88:301-322.
13. Dunn RO. 2008. Biofuels, Bioprod Bioref 2:304–318.
14. Frankel EN, Huang SW, Kanner J , Bruce German J. 1994. Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions J Agric Food Chem 42:1054−1059.
15. GEXSI. 2008. http://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf
16. Goel G, Makkar HPS, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 26:279–288.
17. Haas W, Strerk H, Mittelbach M. 2002. Novel 12 deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J Nat Prod 65:1434−1440.
18. Hornero-Mendez D, Perez-Galvez A, Minguez-Mosquera MIA. 2001. rapid spectrophotometric method for the determination of peroxide value in food lipids with high carotenoid content. J Am Oil Chem Soc 78:1151−1155.
19. Jeffrey AM, Liskamp RM. 1986. Computer-assisted molecular modelling of tumor promoters: rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin. Proc Natl Acad Sci USA 83:241–245.
20. Karmegam J, Sakthivadivel M, Daniel T. 1996. Indigenous plant extracts as larvicidal agents against *Culex quinquefasciatus* say. Bioresour Technol 59:137−140.
21. Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 45:3152−3157.
22. Makkar HPS, Becker K. 2009b. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J lipid sci Technol 111:773–787.
23. Makkar HPS, Maes J, Becker K. 2009a. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J Am Oil Chem Soc 86:173−181.
24. Makkar HPS, Siddhuraju P, Becker K. 2007. A laboratory manual on quantification of plant secondary metabolites. New Jersey: Humana Press; 2007, p. 130.
25. Neff WE, Mounts TL, Rinsch WM, Konishi H. 1993. Photooxidation of soybean oils as affected by triacylglycerol composition and structure, Ibid 70:163−168.

26. Rahuman AA, Gopalakrishnan G, Venkatesan P, Geetha K. 2008. Larvicidal activity of some *Euphorbiaceae* plant extracts against *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Cluicidae). Parasitiol Res 102:867−873.
27. Rug M, Ruppel A. 2000. Toxic activities of the plant *Jatropha curcas* against intermediate snails and larvae of schistosomes. Trop Med Int Health 5:423−430.
28. Schlechtriem C, Ricci M, Focken U, Becker K. 2004. The suitability of the free-living nematode *Panagrellus redivivus* as live food for first-feeding fish larvae. J Appl Ichthyol 20:61−68.
29. Schmidt R, Hecker E. 1975. Autoxidation of phorbol esters under normal storage conditions. Cancer Res 35:1375−1377.
30. Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem 113:1202−1205.
31. Solsoloy AD. 1995. Pesticidal efficacy of the formulated physic nut, *Jatropha curcas* L. oil on pests of selected field crops. Philippine J Sci 124:59–74.
32. Tarandjiska RB, Marekov IN, Niklilova-Damyanova BM, Amidzhin BS. 1996. Determination of triacylglycerol classes and molecular species in seed oils with high content of linoleic and linolenic acids. J Sci Food Agr 72:403−410.
33. Tremp GL, Hecker E. 1985. Stability of the "second stage" promoter 12-O -retinoylphorbol-13-acetate. Cancer Res 45:2390-2391.

CHAPTER - 7

Biodegradation of *Jatropha curcas* phorbol esters in soil

Rakshit K. Devappa, Harinder P.S. Makkar*, Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart, Germany

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Biodegradation of *Jatropha curcas* phorbol esters in soil

Rakshit K Devappa, Harinder PS Makkar* and Klaus Becker

Abstract

BACKGROUND: *Jatropha curcas* seed cake is generated as a by-product during biodiesel production. Seed cake containing toxic phorbol esters (PEs) is currently used as a fertiliser and thus it is of eco-toxicological concern. In the present study the fate of PEs in soil was studied.

RESULTS: Two approaches for the incorporation of PEs in soil were used. In the first, silica was bound to PEs, and in the second, seedcake was used. At day 0, the concentration of PEs in soil was 2.6 and 0.37 mg g^{-1} for approach 1 and 2 respectively. PEs from silica bound PEs were completely degraded after 19, 12, 12 days (at 130 g kg^{-1} moisture) and after 17, 9, 9 days (at 230 g kg^{-1} moisture) at room temperature, 32 °C and 42 °C respectively. Similarly at these temperatures PEs from seed cake were degraded after 21, 17 and 17 days (at 130 g kg^{-1} moisture) and after 23, 17, and 15 days (at 230 g kg^{-1} moisture). Increase in temperature and moisture increased rate of PEs degradation. Using the snail (*Physa fontinalis*) bioassay, mortality by PE-amended soil extracts decreased with the decrease in PE concentration in soil.

CONCLUSION: Jatropha PEs are biodegradable. The degraded products are innocuous.

Keywords: *Jatropha curcas*; phorbol esters; biodegradation; soil; toxicity

INTRODUCTION

Jatropha curcas (Euphorbiaceae) is an oil seed plant distributed widely in tropical and subtropical regions of Central America, Asia and Africa. Traditionally, Jatropha plants are grown as hedge plants and different plant parts (seed, leaves, root and oil) are used in ethno medicines. The seeds are generally mechanically pressed to obtain 240–300 g kg^{-1} of oil and, in the process, 700–760 g kg^{-1} of seed cake is obtained.[1] The non-edible oil is used for biodiesel production upon esterification. The cake left after oil extraction is rich in nitrogen, but it has a limited use due to the presence of anti-nutritional (trypsin inhibitors, curcin, tannins, saponins, phytates) and toxic factors (phorbol esters, PEs).[2] PEs are the most active compounds responsible for toxicity in Jatropha seeds and their concentration ranges from 2 to 3 mg g^{-1} in defatted kernel meal and 2 to 8 mg g^{-1} in the oil.[1] Animals do not feed on Jatropha plant, leaves or seeds. However, force-feeding studies using Jatropha cake, oil or kernel meal in higher animals (rats, chicken, mice, fish, goat pigs etc.,) resulted in acute toxicity.[3–6] Similarly, various aqueous and organic solvent extracts from the seeds of toxic genotypes were also toxic to insects, microorganisms and higher animals.[6] The PEs are considered to be the most biologically active compound present in these extracts.[4,6]

The kernels from *J. curcas* contain at least six different PEs as shown in Fig. 1.[7] PEs are diterpenes containing tigliane as a fundamental backbone. Hydroxylation of the tigliane skeleton in various positions and ester bonding to various acid moieties characterise the large number of compounds termed as PEs. PEs have purgative, skin irritant and tumour promoting properties. These themselves do not elicit tumours but promote uncontrolled growth following exposure to a sub-carcinogenic dose of a solitary chemical initiator (e.g. dimethylbenz(*a*)anthracene), through activation of protein kinase C (PKC) which regulates many cellular processes including signal transduction.[8] In addition to tumour promotion, PEs exhibit a wide range of other biochemical and cellular effects; for example, they alter cell morphology, serve as a lymphocyte mitogen, induce platelet aggregation, elevate cyclic GMP levels, stimulate ornithine decarboxylase, exhibit antileukaemic activity and prostaglandin production.[8] However, not all PEs are toxic and their activity is strictly structure dependent with the α form of phorbol being inactive and the β form being active.[8]

In recent years, the Jatropha plant has been extensively cultivated on wastelands as an energy crop to meet future fuel demands in many tropical and subtropical countries. Siang[9] reported 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, with India and China together playing a major role. This indicates the potential to generate large amount of Jatropha seed cake in future (>80 million tons) by biodiesel industries. PEs are hydrophobic in nature, oil soluble and heat stable in seed cake/meal. During extraction of oil from Jatropha seeds, 70–75% of PEs are extracted along with the oil and 25–30% of PEs still remains strongly bound

* *Correspondence to: Harinder PS Makkar, Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany. E-mail: makkar@uni-hohenheim.de*

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, 70593 Stuttgart, Germany

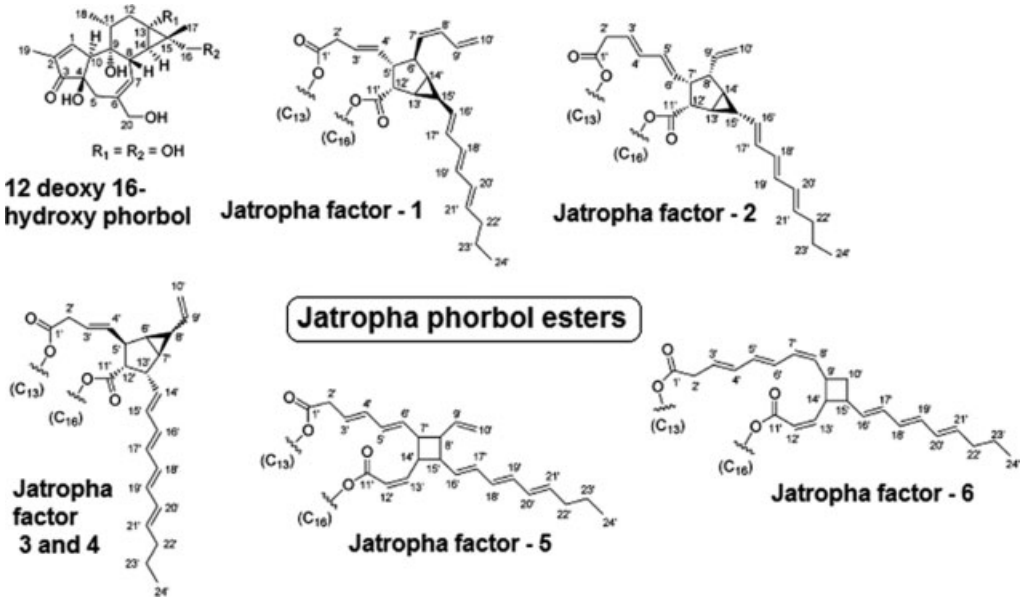

Figure 1. Structure of phorbol esters present in *Jatropha curcas* (Source: Haas *et al.*[6]).

to the matrix of seed meal.[10,11] Due to the toxicity of PEs, seed cake cannot be used in animal feeds without detoxification.[1] On the other hand, nutrient-rich seed cake is suitable as a fertiliser.[12,13] Ghosh *et al.*[14] have reported that the application of Jatropha cake as a fertiliser (0.75–3 t ha^{-1}) significantly increased the seed yield (13–120%) of *J. curcas* plantation over the control plantation (zero input). Similar results were observed when the seed cake was applied to edible crops such as pearl millet (5 t ha^{-1}), cabbage (2.5 t ha^{-1}), rice (10 t ha^{-1}), resulting in 46%, 40–113% and 11% increases in yield.[12] However, users and experts are concerned over the application of seed cake as fertilisers due to its potential toxicity, safe handling/disposal and its impact on the beneficial microbial communities, insects, invertebrates and plant/animal communities.[6,12,15] Rug and Ruppel[16] quoted a report by Koschmieder (personal communication) that PEs of *J. curcas* decompose completely within 6 days; however, no quantitative information was provided nor were the experimental conditions reported. Hitherto, there has been no authentic study describing the fate of Jatropha PEs in the environment. In the present study we have attempted to evaluate the biodegradability of PEs from seed cake and of the PE-enriched fraction extracted from Jatropha oil in the soil. To achieve this, we examined (1) the degradation of PEs in soil incorporated with (a) PEs adsorbed to silica, and (b) seed cake containing PEs; and (2) the bioactivity of PEs as their degradation progressed in the soil using bioassay with snails (*Physa fontinalis*).

Table 1. Chemical evaluation of soil, Jatropha seed cake, Jatropha oil and PEs rich fraction isolated from Jatropha oil ($n = 4$)

Parameter	
Soil	
Nitrogen (g kg^{-1})	1
Carbon (g kg^{-1})	12
PH	6.6
Moisture (g kg^{-1})	30
Soil type	Silty loam
Phorbol esters (mg g^{-1}, on a dry weight basis)	
Jatropha seed cake (sieved)	1.2
Oil	3.5
PEs rich fraction	48
Jatropha seed cake (sieved)	
Nitrogen (g kg^{-1})	53
Carbon (g kg^{-1})	455
Fat (g kg^{-1})	120
Moisture (g kg^{-1})	73
PEs rich fraction	
Carbon (g kg^{-1})	762
Nitrogen (g kg^{-1})	2.5

MATERIALS AND METHODS

Materials

J. curcas seeds used in the experiment were obtained from Jaipur, Rajasthan, India. All chemicals and solvents used were of analytical grade.

Preparation of Jatropha cake

J. curcas seeds were mechanically pressed using a screw press to obtain oil and seed cake. The oil was centrifuged at 3150 × *g* for 20 min to remove residues and the clear oil was collected and stored in a refrigerator (4 °C) until further use. The expellar pressed seed cake was powdered and sieved (mesh size 85) to void majority of the hull fractions. The resulting sieved cake was homogeneously mixed and was analysed for major chemical constituents (Table 1). The moisture content was analysed by the AOAC method.[17]

Preparation of soil sample

A soil sample was collected (top 10–15 cm) from the local agricultural field (University of Hohenheim, Stuttgart, Germany; 400 m above mean sea level; silty loam soil texture; mean annual temperature 8.8 °C; mean annual precipitation 697 mm), which has never been exposed to prior application of Jatropha cake or involved in growing Jatropha plants. The soil was air dried (at 28 ± 1 °C), powdered and sieved (mesh size 85). The resulting

sample was stored at room temperature until further analysis. The soil used in the experiment had carbon content 12 g kg^{-1}, nitrogen 1 g kg^{-1}, pH 6.67 and moisture 30 g kg^{-1} (Table 1).

Chemical evaluation

The soil, Jatropha cake and PE-rich fraction were analysed for carbon and nitrogen by using an automated CN analyser. The results were expressed in g kg^{-1} material. Soil pH was measured by using a glass electrode in a 1 : 2.5 soil : distilled water (w/v) suspension. The moisture content was analysed according to the procedure of the AOAC.[17]

Preparation of the PE-rich fraction

Jatropha oil was mixed with methanol (1 : 2, w/v) in a capped container at room temperature (60 °C) for 15 min using a magnetic stirrer (300 rpm). Thereafter, the mixture was centrifuged at 3150 × *g* for 5 min to obtain the upper methanolic and lower oily layers. Both the layers were separated. The oily layer was re-extracted three more times with fresh solvent in a ratio of 1 : 1.5, 1 : 1 and 1 : 1 (w/v), respectively. The methanolic layers were pooled and rota-evaporated (65 °C, 300 mbar) to obtain the oily PE-rich fraction.[18]

Quantification of phorbol esters

Phorbol esters were determined at least in duplicate according to the method of Makkar *et al*.[19] Briefly, 0.5 g of PE-containing samples (oil, seed cake, PE-rich fraction and PE-amended soil) were extracted four times with methanol. A suitable aliquot of the extracted material was loaded in a high-performance liquid chromatography (HPLC) reverse-phase C18 LiChrospher 100, 5 µm (250 × 4 mm i.d.) from Merck (Darmstadt, Germany). 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^{-1} using a gradient elution.[1] The PE peaks were detected at 280 nm and appeared between 26 and 30.5 min. The results are expressed as equivalent to phorbol-12-myristate 13-acetate (Sigma, St Louis, MO, USA), which appeared between 31 and 32 min.

Incorporation of phorbol esters in soil

Two approaches were used for the incorporation of PEs in soil. In the first approach silica was bound to PEs (SPE) and in the second approach seed cake (CPE) containing phorbol esters was used. The ratios of soil to SPE and CPE were chosen such that the PE concentration in the methanol extracts of SPE and CPE mixtures in soil both at day 0 and initial days of incubation are consistently above the detection limit of HPLC (5 µg).

Adsorption of PEs to silica and its incorporation into soil (approach 1)

Considering the difficulty in mixing Jatropha oil with the soil for degradation studies, an adsorbent (silica) was chosen to incorporate the PEs from the PE-rich fraction homogenously with the soil. The stock solution of the oily PE-rich fraction diluted in methanol (11.1 mg PEs mL^{-1}) was mixed with silica gel G-60 (Merck, Darmstadt, Germany; particle size 0.04–0.063 mm; 230–400 mesh) in a ratio of 1.5 : 1 (v/w). The resulting mixture was vortexed for 30 min and subjected to rota-evaporation (65 °C, 300 mbar) until 75% of the methanol had been recovered. The resulting wet silica-bound PE sample (SPE) was dried at 40 °C and mixed well. The dried SPE was stored in 4 °C until further analysis. The SPE had a PE concentration of 16.6 mg g^{-1}. The SPE was mixed with soil in a ratio 1 : 6 (w/w) to obtain S-SPE, with a PE concentration of 2.6 mg g^{-1} (moisture <6 g kg^{-1}). Further, recovery of PEs from S-SPE was determined by extracting with methanol. A high recovery of 99.9% of PEs was obtained, indicating that the S-SPE mixture is homogenously mixed and methanol could be used as a suitable solvent for extraction of PEs. Further, the protocol for quantification of PEs was carried out as mentioned in the previous section.

Incorporation of Jatropha seed cake into soil (approach 2)

As the hulls contain a very low level of PEs; sieving of the seed cake was done to remove majority of hulls. The removal of hulls increased PE content in the sieved seed cake (1.2 mg g^{-1} on a dry matter basis). This sieved seed cake was mixed homogenously with soil in a ratio of 2 : 5 (w/w) to obtain S-CPE with a PE concentration of 0.37 mg g^{-1} (on a dry matter basis). As in approach 1, methanol was used as a suitable solvent for extracting PEs in the S-CPE samples at regular intervals of the experiment.

Experimental analysis

The test was carried out in triplicates using a complete randomised block design. The S-SPE and S-CPE degradation studies of phorbol esters were conducted in a incubator chamber at three different temperatures (room temperature (RT (23 °C), 32 °C and 42 °C). The experimental soil samples (15 g) from both approach 1 and approach 2 were taken in a glass vial and the final moisture level was adjusted to 130 and 230 g kg^{-1}. The container was covered with an aluminium foil on which five to six needle holes were made for aeration. Thereafter, it was placed in an incubator. The incubator chamber had a controlled inflow and outflow of air and the experiment was carried out in the dark to minimise the effect of light. The moisture content was maintained by weight basis. The difference in initial weight in grams (for both 13% and 23% moisture) and the weight in grams on the next day of incubation gives the loss of moisture in grams. Distilled water in millilitres equivalent to loss in moisture in grams was added to keep the moisture the same. At regular intervals, glass vials containing soil samples from approach 1 and approach 2 were taken out and the soil samples (0.5 g) were analysed for PEs and the percentage degradation was expressed on a moisture-free basis. The moisture content was analysed using the AOAC method.[17]

In order to ascertain the possibility of residual degradation of PEs by soil microbes, SPE and CPE were mixed with the soil in separate capped glass containers. The mixtures, containing approximately 230 g kg^{-1} moisture, were then autoclaved (121 °C for 15 min) twice, with a gap of 4 h in between. The autoclaving was done to be absolutely sure that the PE-amended soil was free of microbes and other biological contaminants. The PE content of autoclaved and un-autoclaved soil-amended SPE and CPE were similar. These mixtures were incubated at 42 °C for up to 14 days. The reason for taking 42 °C and 23 g kg^{-1} moisture was that the rate and extent of PE degradation was higher for the un-autoclaved mixtures under these conditions (see below).

Toxicity in snails

Snails (*P. fontinalis*) are highly susceptible to PEs (our unpublished data). Tests with snails were performed according to the method of Rug and Ruppel.[16] All tests were carried out in deionised water. Stock solutions of PE-containing extracts were prepared in methanol and further diluted in water. Groups of 10 snails were

placed in glass containers with 400 mL of water containing the test substance. The experiment was conducted under controlled RT (23 °C). Snails were prevented from crawling out of the containers by a fine stainless steel mesh suspended just above the water surface. After 24 h of incubation the snails were transferred to deionised water and maintained for another 48 h. Death of the snails was determined by lack of movement and lack of reaction to irritation of the foot with a needle.

All toxicity tests were independently carried out in triplicates (three groups with 10 snails each). The concentration at which 100% snail mortality observed was calculated. The methanol extracts containing PEs from PE-rich fractions and seed cake produced 100% mortality at a concentration of 1 ppm. In our another study, PEs extracted from Jatropha oil also showed 100% mortality at 1 ppm of PEs.[16] In the present study, methanol extracts from both approach 1 and approach 2 containing PEs equivalent to 1 ppm produced 100% mortality at day 0. Further, the toxicity was observed at regular intervals of incubation up to a day where the methanol extracts from both approaches 1 and 2 became non-toxic (no mortality observed). At day 0, a methanol extract containing 1 ppm of PEs (0.2–0.4 mL) was taken for assessing the toxicity of approaches 1 and 2 at regular intervals (in days). Control experiments were performed with the same quantity of methanol (as taken for the test sample; generally 0.2–0.4 mL) in water and no mortality was recorded in the control containers.

Oxidation of phorbol esters by chromic acid

From the results of above studies we presume that PEs are susceptible to oxidative degradation mediated by microbial enzymes) in soil. To test this hypothesis, we carried out chemical oxidation reaction, wherein the PEs rich fraction (0.4 g) was mixed with freshly prepared chromic acid (0.16 mL) at room temperature and kept in dark for 30 min. The reaction mixture was then centrifuged at 3000 × *g* for 5 min. The known weight of oily supernatant layer was collected and re-dissolved in methanol for further analysis of PEs.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of 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

In Jatropha, curcin and PEs are considered to be the main toxic compounds. Curcin is unlikely to exhibit significant toxicity at *in vivo* conditions.[12,15,20–22] In addition, curcin, being a protein, could easily be degraded in soil. Therefore, focus should be on PEs. It has been well documented that PEs are toxic to a wide range of organisms including insects and bacteria/fungi.[6,8] Since the toxicity is not specific, it is likely that non-target as well as target organisms could be affected. If the products containing PEs are to be used as fertilisers, it is critical to investigate the environmental fate and effects of these compounds in *in vitro* and *in vivo* models. The result of present study would provide important information on the likely toxicity of Jatropha cake when applied as a fertiliser and of the accidental exposure of oil in the soil environment.

Degradation of phorbol esters

Degradation of phytochemicals in soil depends on (1) temperature, moisture, pH, adsorption capacity, clay mineralogy and organic matter content; (2) chemical and physical properties of the compound; and (3) microbial populations. In addition to the above parameters, degradation of phytochemicals also depends on particle size of the soil; smaller soil particles have larger relative surface areas and lower porosities, which favour microorganism settlement, thus promoting microbial growth and activity.[23,24] The sieved seed cake used in the experiment contained 53 g kg^{-1} of nitrogen, 455 g kg^{-1} of carbon, 120 g kg^{-1} of fat and 73 g kg^{-1} of moisture. The seed cake, oil and PE-rich fraction had PE contents of 1.2 mg g^{-1}, 3.5 mg g^{-1} and 48 mg g^{-1}, respectively (Table 1).

The initial PEs content in approach 2 (at day 0) taken for soil biodegradation studies was rather low to fully elucidate the soil biodegradation of PEs. Therefore, an additional approach was chosen with soil mixed with silica-bound PEs, thus allowing higher concentrations of PEs (approach 1) to be tested. The PE-rich fraction considered in this study (approach 1) would also enable extrapolation of the results to the degradation of Jatropha oil PEs in the soil. At day 0, the concentration of PEs in soil was 2.60 and 0.37 mg g^{-1} in approaches 1 and 2, respectively.

Approach 1: soil mixed with silica-bound phorbol esters

At 130 g kg^{-1} moisture

The initial degradation of PEs at RT was low up to 6 days (26.7%) and the % degradation increased to 89.2% and 95.3% in 9 and 12 days. Further degradation at RT was low and reached 100% degradation at day 19. Whereas at both 32 °C and 42 °C the initial rate of PE degradation was higher up to 4 days (>85% reduction) and further reduction in PEs was achieved at a slower rate, reaching 100% degradation at day 12 (Fig. 2A). The calculated initial rate (% day^{-1}) of degradation (up to 4 days) was 2.9, 21.8 and 22.4 at RT, 32 °C, and 42 °C, respectively.

At 230 g kg^{-1} moisture

The results observed were similar to those obtained at 130 g kg^{-1} moisture level. At RT, the degradation of PEs was low up to 6 days (28%) and reached 90% at day 9. Further degradation of PEs was low, reaching 100% degradation at day 17. However, at both 32 °C and 42 °C, the extent of PE degradation was higher (>91%) at day 4 when compared do degradation at 130 g kg^{-1} moisture levels and reached 100% degradation at day 9 (Fig. 2B). The calculated initial rate (% day^{-1}) of degradation (up to 4 days) was 4.1, 23.0, and 23.3 at RT, 32 °C and 42 °C, respectively.

Approach 2: soil mixed with seed cake

At 130 g kg^{-1} moisture

The initial degradation of PEs at RT was low up to 9 days (17.3%) and the degradation increased slowly with an increase in the number of days, reaching 100% at day 21. The initial degradation of PEs was higher at 32 °C and 42 °C with 41% and 38%, respectively, compared to the concentration at RT (9.6%) at day 3. Further, 100% PE degradation was observed at day 17 both at 32 °C and 42 °C (Fig. 2C). The calculated initial rate of degradation, % day^{-1} (up to 6 days) was 2.6, 7.9 and 8.7 at RT, 32 °C, and 42 °C, respectively.

At 230 g kg^{-1} moisture

At RT, the initial degradation at day 9 was higher (68%) when compared to degradation at RT at 130 g kg^{-1} moisture

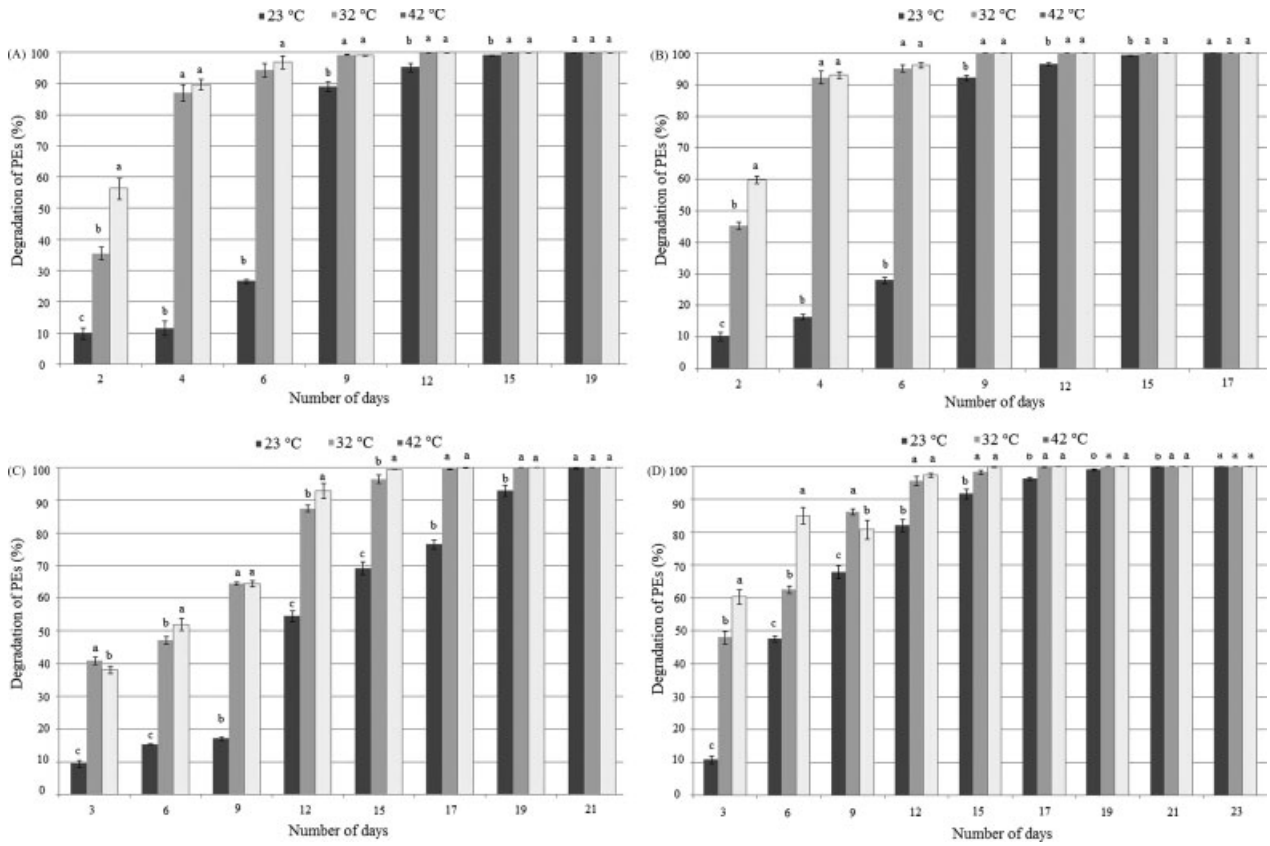

Figure 2. Degradation of PEs: (A) approach 1 at 130 g kg^{-1} moisture; (B) approach 1 at 230 g kg^{-1} moisture; (C) approach 2 at 130 g kg^{-1} moisture; (D) approach 2 at 230 g kg^{-1} moisture. Error bars represent the standard deviation from the mean ($n = 3$). Bars with the different letters at respective days differ significantly ($P < 0.05$).

levels. However, further degradation was low, reaching 100% degradation at day 23. At 32 °C and 42 °C, initial degradation was higher (48% and 60%) at day 3 and reached 100% degradation at day 17 and day 15, respectively (Fig. 2D). The calculated initial rate of degradation, % day^{-1} (up to 6 days) was 7.9, 10.4 and 14.2 at RT, 32 °C, 42 °C, respectively.

Most of the phytochemicals and other biochemicals are degraded in soil by microbial, chemical or photodegradation. The degradation reaction can be influenced by acidic or alkaline hydrolysis or photodegradation by sunlight. The present experiment was carried out completely in the dark to minimise the effect of light on degradation. Overall, from the results of both approach 1 and approach 2, it was evident that PEs present either in Jatropha oil or Jatropha seed cake are degradable and the degradation of PEs increased with an increase in temperature and moisture content in the soil.

Toxicity studies in snails

PEs are toxic even at low concentrations depending on the sensitivity of different organisms. For example, carp (*Cyprinus capio*) were sensitive up to 15 ppm and mice had an LD_{50} of 27.34 mg kg^{-1} body mass.[4,5] Other toxicity tests for PEs are based on snails (*P. fontinalis*) and *Artemia salina* showed that snails (*P. fontinalis*) were more sensitive than *A. salina* with 100% mortality at 1 ppm and 4 ppm, respectively (unpublished data), and hence a snail-based bioassay was preferred in this study. We carried out the snail toxicity studies with the assumption that after PE degradation in the soil (1) although the PE content is reduced to undetectable levels as quantified by HPLC (sensitivity 5 µg), the remaining PEs might cause residual toxicity; and (2) PEs may be converted into unknown secondary products which are still toxic.

Approach 1: soil mixed with phorbol esters bound to silica

At 130 g kg^{-1} moisture, the extracts from S-SPE at RT, 32 and 42 °C caused 47%, 43% and 27% mortality at day 3. However, the extracts from S-SPE at RT, 32 and 42 °C were non-toxic after 12, 9 and 6 days, respectively (Fig. 3A). At 230 g kg^{-1} moisture, similar results were observed, with 43%, 23% and 7% mortality at day 3, at RT, 32 and 42 °C, respectively. The extracts from both RT, 32 and 42 °C became non-toxic to snails after 9, 6 and 6 days, respectively (Fig. 3B).

Approach 2: soil mixed with seed cake

At 130 g kg^{-1} moisture level, the extracts from RT were toxic up to 4 days with 100% mortality, and 40% mortality was observed at day 9 (Fig. 3C). Although, the extract at day 15 had detectable concentrations of PEs, it was non-toxic to snails. At 32 °C, the mortality was reduced to 30% at day 2 and, gradually, the extract became non-toxic after 12 days. Similarly at 42 °C, the mortality was reduced to 10% at day 2 and the extract was non-toxic at day 6 of the experiment. At 230 g kg^{-1} moisture levels, similar trend was observed. No mortality was observed after 15, 9 and 6 days of the experiment (Fig. 3D).

The results of toxicity studies are in agreement with the degradation of PEs (Fig. 2). Compared to approach 2, soil from

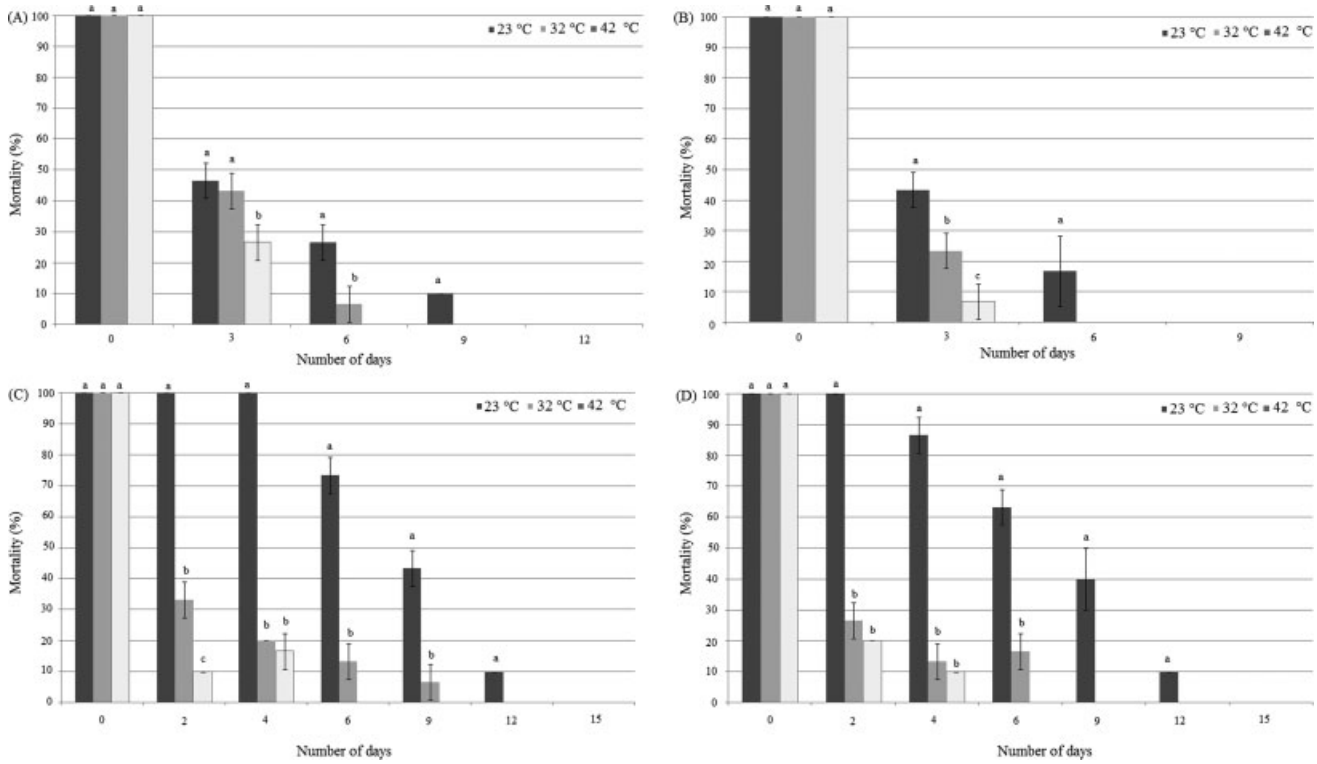

Figure 3. Toxicity evaluation from approach 1 and approach 2 in snails: (A) approach 1 at 130 g kg^{-1} moisture; (B) approach 1 at 230 g kg^{-1} moisture; (C) approach 2 at 130 g kg^{-1} moisture; (D) approach 2 at 230 g kg^{-1} moisture. Error bars represent the standard deviation from the mean ($n = 3$). Bars with the different letters at respective days differ significantly ($P < 0.05$).

approach 1 had higher PE content and the degradation could be easily monitored both quantitatively and qualitatively. The degradation of PEs increased with increased days of incubation as shown in Fig. 4. As the PE content decreased in both approaches 1 and 2, the toxicity of the methanol extracts from the soils also decreased. At 100% degradation, there was no prominent peak representing PEs in the HPLC chromatogram (Fig. 4e). The absorption spectra of non-prominent peaks did not match with the spectra of PEs. No significant reduction in PE content was observed when the autoclaved soil samples were used in both approaches 1 and 2 (data not shown), suggesting that the degradation is microbial in nature.

The results demonstrate that PEs in soil are completely degraded by microbes and the degraded products are non-toxic to snails. If the cake is applied as a fertiliser on a repeated basis, rapid microbial degradation is more likely due to build-up of the PE-degrading microbes. As the population of these organisms increases, degradation would further accelerate and the amount and exposure of the toxic moieties available for causing toxicity to living organisms will be limited.

The comparison of degradation between temperate and tropical soils is difficult due to different soil characteristics (e.g. moisture, temperature and mineralogy).[25] Consequently, the comparison if made could form an avenue for advancing our understanding of PE degradation process in different types of soil. However, from the results we expect that the degradation of PEs would be greater in tropical humid soils.

Phorbol ester degradation products in soil

The exposure of any toxic chemical in the soil environment leads to different chemical reactions which would further increase or decrease contaminant toxicity. These reactions include adsorption/desorption, precipitation, polymerisation, dissolution, complexation and oxidation/reduction. As the concentration of PEs decreased during degradation, additional peaks (degradation products) appeared in the HPLC chromatogram prior to the retention time of the PE peaks (Fig. 4); as evident from the new peaks shown in rectangular blocks I and II and the decrease of PEs in the chromatogram at day 2 (Fig. 4a at day 0 and Fig. 4b at day 2). An increase in the incubation period (day 4) decreased the concentration of both the set of degraded products (shown in rectangular blocks I and II) as well as the PEs present in the soil (Fig. 4c).

Complete degradation of PEs was observed at day 9 (Fig. 4e), while the degraded products (shown in rectangular blocks I and II) were observed until day 12 (Fig. 4f) and a further increase in incubation days (day 15) resulted in complete degradation of PE-degraded products. The chromatogram at day 15 was devoid of any peaks. It was interesting to note that the treatment of the PE-rich fraction by chromic acid, a strong oxidising agent, also produced a set of peaks with similar retention times (Fig. 4g) as observed on incubation of the PE-rich fraction in soil (Fig. 4b). In addition, spectra of the prominant peaks from the chemically oxidised (chromic acid) and enzymatically oxidised (in soil) were similar. These observations suggest that the PEs in soil are degraded by microbial enzymes involving oxidation as one of the intermediate steps. In the soil, these intermediate oxidised products are further degraded with increase in incubation time to non-toxic products.

Further studies should be conducted to evaluate the eco-toxicity of PEs (particularly the extent of leaching of PEs from different soils), as well as the effects of PEs and their degraded products on water channels and water bodies.

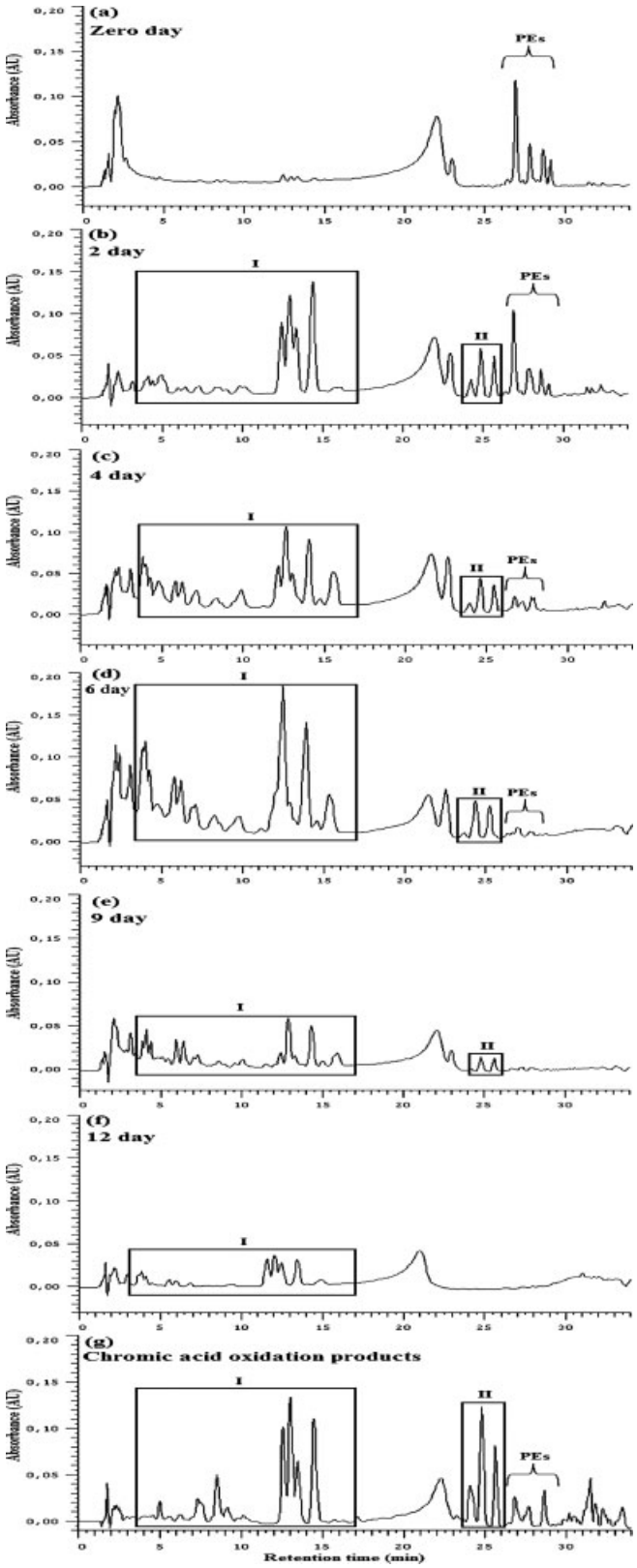

Figure 4. HPLC chromatograms from biodegradation studies (from day 0 to day 12; a–f) showing degradation of phorbol esters (PEs) when silica-bound phorbol esters were mixed with soil (32 °C, 230 g kg^{-1} moisture); and the chromatogram of the chromic acid oxidised products (g).

CONCLUSION

Jatropha cake is a nutrient-rich by-product from the biodiesel industry. The application of seed cake as an organic fertiliser has been found to increase the yield of Jatropha seeds and edible crops. The present study demonstrates that the PEs, the main toxic compounds present in the cake, are completely biodegradable in soil and their degraded products appear to be innocuous. The degradation of phorbol esters in the soil depends on the temperature and the moisture levels. The highest rate of degradation was observed at 42 °C (230 g kg^{-1} moisture level). The snail-based bioactivity determination also followed the same pattern as the levels of PEs in soil.

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REFERENCES

1 Makkar HPS, Becker K, Sporer F and Wink M, Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. *J Agric Food Chem* **45**:3152–3157 (1997).
2 Makkar HPS, Aderibigbe AO and Becker K, Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. *Food Chem* **62**:207–215 (1998).
3 Devappa RK, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P and Bhagya S, Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. *Food Chem Toxicol* **46**:3621–3625 (2008).
4 Li CY, Devappa RK, Liu JX, Makkar HPS and Becker K, Toxicity of *Jatropha curcas* phorbol esters in mice. *Food Chem Toxicol* **48**:620–625 (2010).
5 Becker K and Makkar HPS, Effects of phorbol esters in carp (*Cyprinus carpio* L). *Vet Hum Toxicol* **40**:82–86 (1998).
6 Devappa RK, Makkar HPS and Becker K, Jatropha toxicity – a review. *J Toxicol Environ Health* (In press).
7 Haas W, Sterk H and Mittelbach M, Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. *J Nat Prod* **65**:1434–1440 (2002).
8 Goel G, Makkar HPS, Francis G and Becker K, Phorbol esters: Structure, biological activity and toxicity in animals. *Int J Toxicol* **26**:279–288 (2007).
9 Siang CC, *Jatropha curcas* L.: *Development of a new oil crop for biofuel*. [Online]. The Institute of Energy Economics, Japan (2009). Available: http://eneken.ieej.or.jp/en/data/pdf/467.pdf [22 June 2010].
10 Makkar HPS, Martinez Herrera J and Becker K, Variations in seed number per fruit, seed physical parameters and contents of oil, protein and phorbol ester in toxic and nontoxic genotypes of *Jatropha curcas*. *J Plant Sci* **3**:260–265 (2008).
11 Makkar HPS, Maes J, De Greyt W and Becker K, Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. *J Am Oil Chem Soc* **86**:173–181 (2009).
12 Achten WMJ, Verchot L, Franken YJ, Mathijs E, Singh VP, Aerts R, *et al*, *Jatropha curcas* bio-diesel production and use. *Biomass Bioenergy* **32**:1063–1084 (2008).
13 Francis G, Edinger R and Becker K, A concept for simultaneous wasteland reclamation, fuel production, and socio-economic development in degraded areas in India: need, potential and perspectives of Jatropha plantations. *Nat Res For* **29**:12–24 (2005).
14 Ghosh A, Patolia JS, Chaudhary DR, Chikara J, Rao SN and Kumar D, Response of *Jatropha curcas* under different spacing to Jatropha de-oiled cake, in *FACT seminar on Jatropha curcas L. agronomy and genetics, 26–28 March 2007, Wageningen*. FACT Foundation, Wageningen, The Netherlands, Article no. 8 (2007).
15 Gressel J, Transgenics are imperative for biofuel crops. *Plant Sci* **174**:246–263 (2008).

16 Rug M and Ruppel A, Toxic activities of the plant *Jatropha curcas* against intermediate snails and larvae of schistosomes. *Trop Med Int Health* **5**:423–430 (2000).
17 Association of Official Analytical Chemists, *Official Methods of Analysis of the AOAC International, Methods 934.01*. AOAC International, Arlington, VA (2000).
18 Devappa RK, Makkar HPS and Becker K, Optimization of conditions for the extraction of phorbol esters from *Jatropha curcas oil*. *Biomass Bioenergy* **32**:1125–1133 (2010).
19 Makkar HPS, Siddhuraju P and Becker K, *A Laboratory Manual on Quantification of Plant Secondary Metabolites*. Humana Press, New Jersey (2007).
20 Lin J, Yan F, Tang L and Chen F, Antitumor effects of curcin from seeds of *Jatropha curcas*. *Acta Pharmacol Sin* **24**:241–246 (2003).
21 Barbieri L, Battelli M and Stirpe F, Ribosome-inactivating protein from plants. *Biochim Biophys Acta* **1154**:237–282 (1993).
22 King JA, He W, Cuevas JA, Freudenberger M, Ramiaramanana D and Graham IA, Potential of *Jatropha curcas* as a source of renewable oil and animal feed. *J Exp Bot* **60**:2897–2905 (2009).
23 Amellal N, Portal JM, Vogel T and Berthelin J, Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates. *Biodeg* **12**:49–57 (2001).
24 Delhomenie MC, Bibeau L and Heitz M, A study of the impact of particle size and adsorption phenomena in a compost-based biological filter. *Chem Eng Sci* **57**:4999–5010 (2002).
25 Six J, Feller C, Denef K, Ogle SM, Joao Carlos de Moraes Sa and Albrecht A, Soil organic matter, biota and aggregation in temperate and tropical soils – Effects of no-tillage. *Agronom* **22**:755–775 (2002).

CHAPTER - 8

Potential of using phorbol esters as an insecticide against *Spodoptera frugiperda*

Rakshit K. Devappa[a], Miguel A. Angulo-Escalante[b], Harinder P.S. Makkar[a] and Klaus Becker[a]

[a]*Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart-70599, Germany*

[b]*Centro de Investigación en Alimentación y Desarrollo (CIAD), Hermosillo, Sonora, México*

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Abstract

Jatropha curcas oil is a promising candidate for biodiesel production. The oil is also rich in bioactive diterpenoids (phorbol esters, PEs). In the present study, the extracted PEs (PEs enriched fraction, PEEF) from Jatropha oil was evaluated for insecticidal activity against *Spodoptera frugiperda* (third instar larvae), which is a common pest in corn field across the tropical and subtropical areas. The PEEF exhibited contact toxicity with an LC_{50} of 0.83 mg ml^{-1} (w/v). The corn leaves treated with PEEF also declined the food consumption (33%), relative growth (42%) and food conversion efficientcy (38%) at a concentration of 0.25 mg ml^{-1} (w/v). Higher reduction (39 and 45%) in the relative consumption rate was observed at 0.625 and 0.125 mg PEs ml^{-1} (w/v) of PEEF. Considering the rapid growth in Jatropha biodiesel industry, large amount of PEs can be harnessed, which can be further utilized as a biocontrol agent.

Keywords: Jatropha, phorbol esters, *Spodoptera frugiperda*, biocontrol, byproduct

1. Introduction

Terpenoids are the largest class of plant metabolites comprising more than 40,000 structures (Bohlmann and Keeling, 2008). In plants, they may have different physiological, metabolic and structural roles or may have more discrete, specialized interactions with other organisms. The specialized interactions may include interaction with other organisms in context of reproduction, defence or symbiosis (Gershenzon and Dudareva, 2007). The role of specialized terpenoids may include, among others, as repellents, anti-feedentants, attractants, toxins or antibiotics. Due to their array of biological activities in nature, they have been widely exploited by humans as industrially relevant compounds in crude or purified forms for a long time, for example as a flavours, fragrances, pharmaceuticals, food supplements (vitamins), sweetners or biocontrol agents (pesticides) (Bohlmann and Keeling, 2008). The chemical diversity of terpenoids often originates from complex biosynthetic pathways and they may serve as a large volume feedstock resource for the production of industrial biomaterials. This resource can be utilized both in their naturally occurring forms or metabolically engineered forms in agriculture, forestry and horticulture (Bohlman and Keeling, 2008). Harnessing the bioactive and economically valuable terpenoids requires interdisciplinary research which involves chemistry, biology and pharmacology, among others, giving ample opportunity and new means for the exploitation of terpenoids in agricultural and pharmaceutical applications.

In the present study, the diterpene from the *Jatropha curcas* plant is studied. Jatropha (Euphorbiaceae) seeds are an attractive feedstock resource of seed oil for biodiesel production. The rapidly increasing commercial cultivation gives ample opportunity to harness highly bioactive compounds as coproducts (Kumar and Sharma, 2008). Among the secondary metabolites, the Jatropha plant contains variety of terpenoids. So far, more than 65 types of diterpenes have been identified (Devappa et al., 2011). Among them, phorbol esters (PEs, a group of tigliane diterpenes) are the most investigated and these compounds possess high potency and bioactivity. There are 6 types of PEs present in the Jatropha oil (Haas et al., 2002). Previous reports suggest that the aqueous/organic solvent extracts from Jatropha oil/seed are effective as insecticidal and antimicrobial agent in vitro; and in majority of the studies, the activities are attributed to the presence of PEs (Devappa et al., 2010b). In the present study, preliminary investigation has been carried out to evaluate the potential of phorbol ester enriched fraction (PEEF), isolated from Jatropha oil, as an insecticide or insect deterrent in the insect, *Spodoptera frugiperda.* The *S.*

frugiperda (Lepidoptera: Noctuidae) is a polyphagous species that commonly attacks economically important crops in several countries. This insect damages following crops: corn, sorghum, rice, wheat, alfalfa, beans, peanuts, tomato, cotton, potatoes, cabbage, spinach, pumpkin and cabbage (Cruz et al., 1999; Praca et al., 2006).

2. Material and methods

2.1. Materials

J. curcas seeds (toxic Indian variety) were collected from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. Phorbol 12-myristate-13-acetate (PMA), other chemicals was obtained from Sigma (St. Louis, USA) and all other chemicals/solvents used were of analytical grade.

2.2. Extraction of phorbol ester enriched fraction

J. curcas seeds were mechanically pressed using a screw press to obtain oil. The oil was centrifuged at 3150 × *g* for 20 min to remove residues. The extraction of phorbol esters enriched fraction (PEEF) was carried as reported by Devappa et al. (2010a). The oil was mixed with methanol (1:2, w/v) and the mixture was mixed at 55 °C for 15 min using a magnetic stirrer (300 rpm). Further, the mixture was centrifuged (3150 x *g* for 5 min) to get upper methanolic and lower oily layers. The methanol layer was rotaevaporated (Buchi, Germany) to get oily PEEF. The oily PEEF was stored at 4 °C for 4 months until further analysis.

2.3. Preparation purified phorbol ester rich extract (PEs-rich extract)

In brief, the PEEF was subjected to flash chromatography on 50 g of silica gel (40-63 μm, Merck), which had been preconditioned with dichloromethane (DCM). The column was eluted successively with DCM (500 ml), 1% DCM in MeOH (500 ml) then 5% DCM in MeOH (250 ml, collected in 50 ml fractions). Fractions containing PEs eluted with the 5% DCM in MeOH mixture and were concentrated *in vacuo* to yield a yellow PEs-rich extract. The PEs- rich extract was stored at 4 °C for 4 months until further analysis.

2.4. Phorbol ester analysis

The PEs were determined at least in duplicate according to Makkar et al. (2007a), based on the method of Makkar et al. (1997). Briefly, 0.5 g of PEEF or PEs-rich extract was dissolved in 2% tetra hydrofuran containing methanol. A suitable aliquot was loaded into a high-Performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 x 4 mm id, from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at RT (23 °C) and the flow rate was 1.3 ml min^{-1} using a gradient elution Makkar et al. (2007a). The four phorbol ester peaks (containing 6 PEs) appeared between 25.5 and 30.5 min were detected at 280 nm. Phorbol-12-myristate 13-acetate was used as an external standard which appeared between 31 and 32 min. The area of the four phorbol ester peaks was summed and the concentration was expressed equivalent to PMA. The PEs detection limit in the HPLC was 3 to 4 µg.

2.5. Contact toxicity

Stock solutions of PEEF and PEs-rich extract was dissolved in acetone and applied topically (10 µL total volume) to the dorsal region of the thorax of third instar larvae of *S. Frugiperda* using a Hamilton microsyringe. The test included different concentrations (0.0313, 0.0625, 0.125, 0.25, 0.5, 1 and 20 mg ml^{-1}) of PEEF and PFE; and a control (only acetone). Treated groups consisted of 10 larvae at each dose, replicated two times. The mortality of larvae was recorded after 24 h (25 ± 2 ºC). Larvae that were unable to make coordinated movements within 10 seconds of prodding were assigned as dead. Results were corrected for mortality in the untreated larvae group and analyzed using the probit method.

2.6. Ingestion toxicity assay

The bioassay was carried out on *S. frugiperda* using leaf disc by no choice method to analyze the effects of PEEF on insect development by survival and for antifeedant activity of the insect. One *S. frugiperda* larvae (third instar) was placed in the centre of Petri dish and fed on corn leaves (1.5 cm in diameter) previously dipped into one of the treatment solutions of either the pure compounds in acetone (0.0625, 0.125 and 0.25 mg ml^{-1}) or solvent (acetone). Ten larvae were used for each dose with two replications. The ingestion toxicity was recorded after 24 h for 10 days (25 ± 2 ºC). Data collected were: % mortality, % food consumption (visual), larvae weight (mg) and corn leaf disc weight (mg). The

nutritional indexes calculated were: Relative growth rate (RGR) = (fw - iw)/(gw × T) [where fw = final weight; iw = initial weight; gw = geometric weight calculated as (iw × fw)$^{1/2}$ and T = time (ten days)]; Relative consumption rate (RCR) = I/(gw × T) [where I = ingested food calculated as (initial weight of food × % food consumption); gw = geometric weight and T= time (ten days)]; and Food conversion efficiency (FCE) = (fw - iw) / I × 100 [where, fw = final weight; iw = initial weight; I = ingested food calculated as (initial weight of food × % food consumption)].

3.0. Results and discussion

The pests infesting economically important crops inflict marked losses in the agrarian production. The insects have always been recognised as one of the most serious agricultural problems. When compared to synthetic insecticides, usage of biological control agents offer the advantage of being compatible with the environment, often with high specificity, and represent a long-term solution for controlling insects that are particularly resistant to organic chemical based controlling agents. Therefore, many efforts have been made for controlling insects using natural biocontrol agents, such as plant phytochemicals. In the present study, the insect (*S. frugiperda*) chosen is a pest commonly present in corn fields in the tropical/subtropical countries such as Mexico and Brazil.

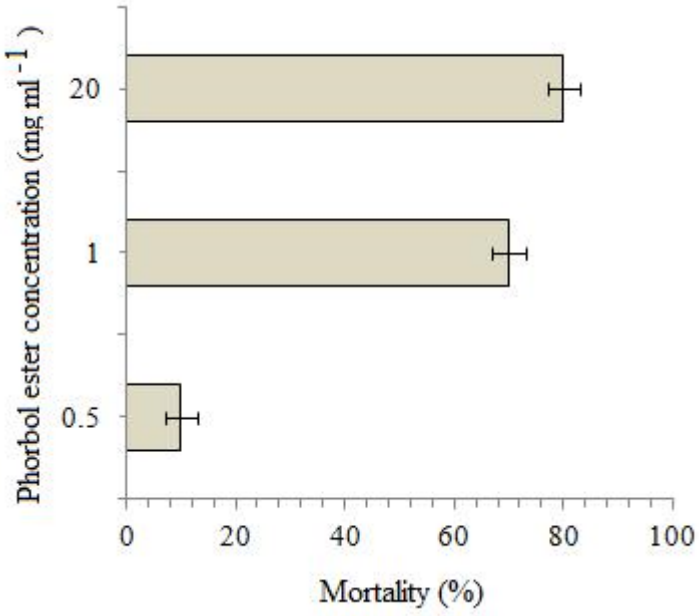

Fig. 1. Insecticidal activity of phorbol esters enriched fraction (PEEF)

The concentration of PEs in PEEF and PEs-rich extract was 62.82 mg g^{-1} and 1.8 g g^{-1} respectively. In the present study, the test concentration of PEs present in PEEF and PEs-rich extract was expressed equivalent of PMA. The contact toxicity of PEs present in PEEF and PEs-rich extract on *S. frugiperda* is shown in Fig. 1. The PEEF exhibited dose dependent increase in mortality of larvae, exhibiting insecticidal activity. The LC_{50} of PEEF was 0.83 mg of PEs ml^{-1} (w/v). However, no effect was observed for the PEs-rich extract. This may be due to degradation of PEs during storage (4 months at 4 °C) prior to conducting the experiment. The PEs content in the PEs-rich extract (in the dried form) were reduced by 50% in 3 months when stored at 4 °C (Devappa et al., 2009). In another study, purified TPA decomposed slowly within 3 months when kept under dark at 4 °C and the degradation was extensive in 3 months at 25 °C in the diffused daylight. During decomposition 7-hydroperoxide was formed as a major product (Schmidt and Hecker, 1975). Similar decomposed products may have been formed during the PEs-rich extract

Table 1. Ingestion toxicity and nutritional indeces of Spodoptera frugiperda (average of 10 days) on phorbol ester enriched fraction (PEEF) treated corn leaves (no-choice method)

Phorbol esters in PEEF (mg/mL)	Mortality (%) *	Food consumption (%) (visual) *	Relative growth rate *	Relative consumption rate *	Food conversion efficiency *
0 (acetone blank)	20	67.04	0.11	7.29	1.45
0.0625	30	64.12	0.06	4.45	1.31
0.125	10	76.13	0.07	4.04	1.64
0.25	40	44.73	0.06	6.85	0.91

* average of ten larvae

storage in our study and thereby losing its bioactivity. In addition, TPA stored as a powder for 3 months at 25 °C (under diffused light) formed hydroperoxides, and at 4 °C (under dark) the hydroperoxides were formed but to a very limited extent. Whereas, the PEs present in the PEEF were found to be stable even after 2 years at 4 °C (Devappa et al., 2009).

Since PEs-rich extract was ineffective, only the PEEF was taken to test the antifeedant activity against *S. frugiperda*. Application of higher test concentration would cause mortality due to contact toxicity rather than exhibiting antifeedant activity. Therefore, concentrations at which lowest or no contact toxicity observed were further chosen for the antifeedant/ingestion toxicity studies. The effects of the PEEF at different concentrations on food consumption, relative growth rate, relative consumption rate and food conversion efficiency are shown in Table 1. When compared with control, dietary intake by *S. frugiperda* was severely affected when fed on corn leaves treated with the PEEF (0.0625 to 0.25 mg ml^{-1}). The adverse effect of PEEF on the feeding and growth of *S. frugiperda* was evident from the nutritional index (Table 1). At highest concentration of the PEEF (0.25 mg ml^{-1}, w/v), food consumption and relative growth rate of 3rd instar *S. frugiperda* were reduced by 33% and 42% respectively (Table 1). The food conversion efficiency (FCE) value also declined by 38% compared with the control counterparts, while the relative consumption rate (RCR) was decreased by only 6% at 0.25 mg ml^{-1} of PEEF. But higher decline in RCR was observed at 0.625 mg ml^{-1} and 0.125 mg PE ml^{-1} of PEEF (39 and 45% respectively) (Table 1). The toxicity was attributed to be the outcome of direct toxic effect from phorbol esters present in PEEF instead of starvation effect because a substantial amount of the leaves embedded with PEEF were consumed. Overall, the results indicate dose dependent effects of phorbol esters present in PEEF on the observed parameters, except at one concentration (0.125 mg/ml). The reason for low toxicity at this concentration is not clear.

In another study, methanol extract (similar to PEEF) from *J. curcas* oil exhibited potent insecticidal effects against *Busseola fusca, Sesamia calamistis* larvae, *Helicoverpa armigera* and *Manduca sexta* larvae (Makkar et al., 2007b; Sauerwein et al. 1993; Mengual, 1997; Ratnadass et al., 2009). There are several studies reported wherein the potential of crude and aqueous/solvent extracts from Jatropha (leaves, root, bark and oil) have been investigated as biocontrol agents (Devappa et al. 2010b). For example, ethanolic (95%) and ethyl acetate extracts from leaves of *Jatropha gossypifolia* exhibited weak insecticidal activity against second instar of *Spodoptera exigua* (dipping method) with an LC_{50} (24 h) of 35,000 ppm and 6182 ppm respectively (Khumrungsee et al., 2009; Khumrungsee et al., 2010). In addition, *J. curcas* oil or its extract containing PEs were also found to be effective against *Callosobruchus maculatus, Sitophilus zeamais, Callosobruchus chinensis, Lipaphis*

erysimi, *Pieris rapae*, *Sarocladium oryzae*, *Phthorimaea operculella*, *Myzus persicae*, *Tetranychus urtica*e, *Periplaneta Americana*, *Blattella germanica*, *Oncopeltus fasciatus* and *Coptotermes vastator* (Wink et al., 1997; Adebowale and Adedire, 2006; Solsoloy, 1995; Jing et al., 2005; Shelke et al., 1985; Shelke et al., 1987; Devappa et al. 2010b). From these studies it is evident that the Jatropha extracts have a wide spectrum of insecticidal activity, and extracts especially from plant parts containing PEs could be potential candidates for use as an insecticidal agent. In the present study, although the insecticidal potential of PEEF was carried out in *S. Frugiperda* (found in tropical and subtropical regions of American continent), similar insecticidal activities could be expected in other *Spodoptera* species found across the world. Further studies are required in this direction.

In our previous studies, we found that the PEEF extraction procedure did not hamper the quality of residual oil, and the residual oil can be further processed (transesterification) into high quality biodiesel which meets European (EN 14214:2008) and American biodiesel standards (ASTM D6751-09) (Devappa et al. 2009). The PEs have poor shelf life in purified form when compared with PEs present in its oily phase, Therefore, it is realistic to use PEs as enriched fraction (PEEF) in agricultural applications to control biological pests/insects. Apart from obtaining the PEEF as a value added co-product, the extraction process will also make the process of biodiesel preparation friendly for both the workers and environment.

4. Conclusions

It is evident from our study that PEs rich PEEF exhibits contact toxicity and antifeedant activity against *S. frugiperda,* indicating the potential of the PEEF as a bio-control agent. Further in depth field experiments on the effects of the PEEF on *S. frugiperda* will pave the way for its use under field conditions.

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References

1. Acda MN. 2009. Toxicity, tunneling and feeding behavior of the termite, *Coptotermes vastator*, in sand treated with oil of the Physic nut, *Jatropha curcas*. J. Insect Sci. 6, 1 – 8.
2. Adebowale KO, Adedire CO. 2006. Chemical composition and insecticidal properties of underutilized *Jatropha* curcas seed oil. Afr. J. Biotechnol. 5, 901 – 906.
3. Bohlmann J, Keeling CI. 2008. Terpenoid biomaterials. The Plant J. 54, 656 – 669.
4. Cruz I, Figueiredo M, De LC, Matoso MJ. 1999. Controle Biológico de *Spodoptera frugi-perda* utilizando o parasitóide de ovos *Trichogramm*a. Sete Lagoas. In Circular Técnica 30. Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Embrapa Milho e Sorgo.
5. Devappa RK, Makkar HPS, Becker K. 2010a. Optimization of conditions for the extraction of phorbol esters from Jatropha oil. Biomass Bioenerg. 34, 1125 − 1133.
6. Devappa RK, Makkar HPS, Becker K. 2010b. Jatropha toxicity – A review. J. Toxicol. Environ. Health B Crit. Rev. 13, 476 − 507.
7. Devappa RK, Makkar HPS, Becker K. 2011. Jatropha Diterpenes: a Review. J. Am. Oil Chem. 88, 301 − 22.
8. Devappa RK, Maes J, Makkar HPS, Greyt WD, Becker K. 2009. Isolation of phorbol esters from *Jatropha curcas* oil and quality of produced biodiesel. 2nd International Congress on Biodiesel: The Science and the Technologies, Munich, Germany.
9. Gershenzon J, Dudareva N. 2007. The function of terpene natural products in the natural world. Nat. Chem. Biol. 3, 408 – 414.
10. Haas W, Sterk H, Mittelbach M. 2002. Novel 12-deoxy-16- hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J. Nat. Prod. 65:1434 − 1440.
11. Jing L, Fang Y, Ying X, Wenxing H, Meng X, Syed MN, Fang C. 2005. Toxic impact of ingested Jjatropherol-I on selected enzymatic activities and the ultrastructure of midgut cells in silkworm, *Bomboxy mori* L. J. Appl. Entomol. 129, 98 − 104.
12. Khumrungsee N, Pluempanupat W, Kainoh Y, Saguanpong U, Bullangpotin V. 2010. Toxicity of ethyl acetate extract from *Jatropha gossypifolia* senescent leaves against *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) and *Meteorus pulchricornis* (Hymenoptera: Braconidae). Commun. Agric. Appl. Biol. Sci. 75, 405−410.
13. Khumrungsee N, Bullangpoti V, Pluempanupat W. 2009. Efficiency of *Jatropha gossypifolia* L. (Euphorbiaceae) against *Spodoptera exigua* HÜbner (Lepidoptera:Noctuidae): Toxicity and its detoxifying enzyme activities. KKU Sci. J. 37, 50−55.
14. Kumar A, Sharma S. 2008. An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. Ind. Crop. Prod. 28, 1-10.

15. Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J. Agric. Food Chem. 45, 3152 − 3157.
16. Makkar HPS, Francis G, Becker K. 2007b. Bioactivity of phytochemicals in some lesser known plants and their effects and potential applications in livestock and aquaculture production systems. Animal 1, 1371 – 1391.
17. Makkar HPS, Siddhuraju P, Becker K. 2007a. A laboratory manual on quantification of plant secondary metabolites. New Jersey, Humana Press, p. 130.
18. Mengual L. 1997. Extraction of bioactive substances from J. curcas L. and bioassays on *Zonocerus variegatus*, *Sesamia calamistis* and *Busseola fusca* for characterisation of insecticidal properties, in: Gübitz, G.M., Mittelbach, M., Trabi, M. (Eds.), Biofuel and industrial products from *Jatropha curcas*. Graz: Dbv-Verlag University, pp 211–215.
19. Praça L.B., Silva, Neto, S.B., Monnerat, R.G., 2006**.** *Spodoptera frugiperda* J. Smith 1797 **(**Lepidoptera: Noctuidae) Biologia, amostragem e métodos de controle**.** In *Documentos 199***.** Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Embrapa Recursos Genéticos e Biotecnologia.
20. Ratnadass A, Togola M, Cissé B, Vassal JM. 2009. Potential of sorghum and physic nut (*Jatropha curcas*) for management of plant bugs (Hemiptera: Miridae) and cotton bollworm (*Helicoverpa armigera*) on cotton in an assisted trap-cropping strategy. http://ejournal.icrisat.org/Volume7/Sorghum_Millets/SG703.pdf (Accessed on 13th December 2011)
21. Sauerwein M, Sporer F, Win M, 1993. Insect-toxicity of phorbol esters from *Jatropha curcas* seed oil. Planta Med. 59, A686.
22. Schmidt R, Hecker E. 1975. Autoxidation of phorbol esters under normal storage conditions. Cancer Res. 35, 1375 − 1377.
23. Shelke SS, Jadhav LD, Salunkhe GN. 1985. Ovipositional and adult repellent action of some vegetable oils/extracts against potato tuber moth. J Maharashtra Agric. Univ. 10, 284 – 286.
24. Shelke SS, Jadhav LD, Salunkhe GN. 1987. Ovicidal action of some vegetable oils and extracts in the storage pest of potato, *Phthorimaea operculella* Zell. Biovigyanam 13, 40 – 41.
25. Solsoloy AD. 1995. Pesticidal efficacy of the formulated physic nut, *Jatropha curcas* L. oil on pests of selected field crops. Philippine J. Sci. 124, 59 – 74.
26. Wink M, Koschmieder C, Sauerweien M, Sporer F. 1997. Phorbol esters of *J. curcas*— Biological activities and potential applications, in; Gubitz, G. M., Mittelbach, M., Trabi (Eds.), Biofuel and industrial products from *Jatropha curcas*, Graz:DBV, pp. 160 – 166.

CHAPTER - 9

Isolation, characterization, stability and bioactivity of *Jatropha curcas* phorbol esters

Joy S. Roach[a], **Rakshit K. Devappa[a]**, Harinder P.S. Makkar[a], Uwe Beifuss[b], Klaus Becker[a]

[a]*Institute for Animal Production in the Tropics and Subtropics, (480b), University of Hohenheim, Stuttgart, Germany.*
[b]*Institut fur Chemie, Universit3t Hohenheim, Stuttgart, Germany.*

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Joy S. Roach, Rakshit K. Devappa, Harinder P.S. Makkar *, Klaus Becker

Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart-70599, Germany

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ABSTRACT

Jatropha curcas seed oil, which can be utilized for biodiesel production upon transesterification, is also rich in phorbol esters (PEs). In this study, PEs from *J. curcas* oil (*Jatropha* factors C_1 and C_2 (purified to homogeneity), *Jatropha* factors C_3 and ($C_4 + C_5$) (obtained as mixtures) and PE-rich extract (containing all the above stated *Jatropha* factors) were investigated. The concentrations of *Jatropha* PEs were expressed equivalent to *Jatropha* factor C_1. In the snail (*Physa fontinalis*) bioassay, the order of potency (EC_{50}, μg/L) was: PE-rich extract < factor C_3 mixture < factor C_2 < factor C_1 < factor ($C_4 + C_5$). In the *Artemia salina* bioassay, the order of potency (EC_{50}, mg/L) was: factor C_2 < factor C_3 mixture < factor C_1 < factor ($C_4 + C_5$) mixture. In addition, *Jatropha* PEs exhibited platelet aggregation (ED_{50}, μM, factor C_2 < factor C_3 mixture < factor C_1 < factor ($C_4 + C_5$) mixture. The stability of a PE-rich extract was evaluated and found to be low at room temperature but favourable in ethanol over a range of temperatures. By integrating the isolation methodology developed in this study in the *Jatropha* biodiesel industry, PEs could be obtained as value-added co-products.

1. Introduction

Jatropha curcas L. is a promising feedstock of oil for biodiesel production. *J. curcas* is one of approximately 175 species in its genus and a member of the Euphorbiaceae family [1]. It is a perennial shrub or small tree native to South and Central America but cultivated in many tropical regions, including Africa and Asia. It thrives in poor, stony soils and under adverse climatic conditions. *J. curcas* has a variety of uses. In many parts of the world it is used as a live fence and for erosion control. The seed kernel of the plant contains up to 60% oil [2], which can be transesterified to biodiesel. The oil is also incorporated in cosmetics and soap production. The seed kernel meal remaining after oil extraction is rich in nutrients and is used as an organic fertilizer [3,4]. Although high in protein the use of the defatted kernel meal as livestock feed is precluded by the presence of phorbol esters (PEs), which are the major toxic principles in *J. curcas* seeds [3].

Jatropha oil cannot be used for edible purposes without detoxification, making it attractive for biodiesel production. During mechanical extraction, the majority of PEs (~70%) present in the seed is extracted with the oil fraction [5]. During biodiesel production, the oil is subjected to many treatments (stripping, degumming and esterification), which leads to partial or complete destruction of the PEs [5]. Instead of losing the PEs, if a suitable method can be adopted to extract and isolate these esters before biodiesel production, the PEs could be valued co-products, which would contribute to enhancing economic viability and sustainability of the *Jatropha* oil-based biodiesel production chain. This is subject to the condition that the extraction of PEs from the oil does not adversely affect the quality of biodiesel produced.

Haas et al. [6] identified six *J. curcas* PEs, namely *Jatropha* factors C_1–C_6 (Fig. 1). The reason for choosing this group of compounds (PEs) for this study was that they are highly bioactive both *in vitro* and *in vivo*, but they are currently considered to be merely toxic, unwanted biomaterial in the *Jatropha* biodiesel production chain [7]. The recent increase in the cultivation of *Jatropha* means that there are potentially huge quantities of PEs that could be used for various applications. In this paper, we provide an optimized method for obtaining a *J. curcas* PE-rich extract and purified *Jatropha* PEs. In

* Corresponding author at: Institute for Animal Production in the Tropics and Subtropics (480b), University of Hohenheim, Stuttgart-70599, Germany. Tel.: +49 711 459 23640; fax: +49 711 459 23702.
E-mail address: Harinder.Makkar@fao.org (H.P.S. Makkar).

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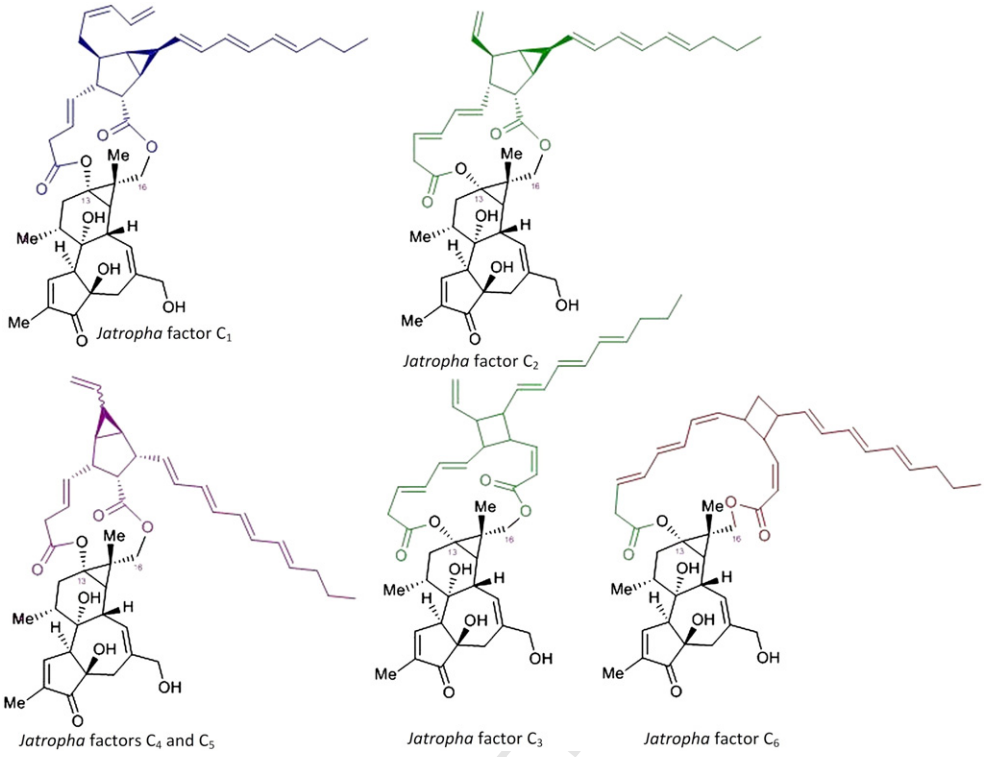

Fig. 1. Phorbol esters present in *Jatropha curcas* oil (Haas et al. [6]).

addition, the storage stability of the PE-rich extract and the biological activity of purified PEs are also presented.

2. Materials and methods

2.1. Processing of J. curcas oil

J. curcas seeds (Jaipur, India; Jaipur; geographical coordinates: 26°55′0″ N, 75°49′0″ E) were mechanically pressed and the resulting oil was centrifuged at 3150×*g* for 20 min to remove solid material. Clear oil was collected and stored at room temperature (23 °C) for further studies.

2.2. Phorbol ester-rich extract preparation and Jatropha factors C_1–C_5 isolation

Approximately 1 L of *J. curcas* seed oil (904.1 g) was extracted with methanol (MeOH, 750 ml) by stirring (300 rpm) in a 60 °C water bath for 15 min. The layers were allowed to separate then the MeOH layer was collected. The extraction process was repeated three times on the remaining oil. Combined MeOH extracts were concentrated *in vacuo* (60 °C) to yield a yellow oily residue (41.8 g). The crude MeOH extract was subjected to flash chromatography on 50 g of silica gel (40–63 μm, Merck), which had been preconditioned with dichloromethane (DCM). The column was eluted successively with DCM (500 ml), 1% DCM in MeOH (500 ml) then 5% DCM in MeOH (250 ml, collected in 50 ml fractions). Fractions containing PEs eluted with the 5% DCM in MeOH mixture and were concentrated *in vacuo* to yield a yellow PE-rich residue (0.26 g). The PEs were identified by a gradient elution high performance liquid chromatography (HPLC) method described by Makkar et al. [3,8]. Briefly, samples were injected on a reverse phase C_{18} Licrospher 100, 5 μm (250×4 mm) from Merck (Darmstadt, Germany), protected by a guard column of the same material. Starting with 60% solvent A (1.75 ml of 85% *o*-phosphoric acid in 1 L water and 40% solvent B (acetonitrile), B was increased to 50% over 10 min before increasing again to 75% over the next 30 min. From 30 to 45 min, B was increased to 100% before washing the column with solvent C (2% tetrahydrofuran in MeOH). Monitoring at 280 nm, four PEs peaks appeared between 25.5 and 30.5 min.

Additional oil was extracted, 1 L at a time, to yield a combined 2.0 g of the PE-rich product. This was subjected to semi-preparative HPLC on a reverse-phase C_{18}, 5 μm (250×10 mm) column from Phenomenex (California, USA). The column was eluted with 75% acetonitrile–water (0.01% trifluoroacetic acid) at a flow rate of 4 ml/min, monitored at 280 nm. Four prominent peaks were observed, which corresponded to *Jatropha* factors C_1 (18.3 min), C_2 (20.5 min), C_3 (23.4 min) and a C_4+C_5 mixture (25.2 min). Each of the four samples was subjected to open column chromatography on Sephadex LH-20 for purification. *Jatropha* factors C_1 and C_2 obtained were confirmed by 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, TOCSY, HMBC) NMR and comparison with data published by Haas et al. [6]. *Jatropha* factors C_3 and the factor (C_4+C_5) mixture needed further purification.

2.3. Bioassay for toxicity in Artemia salina

The assay was carried out by a slight modification of the method described by Kinghorn et al. [9]. The *A. salina* cysts

were incubated in a hatching chamber containing artificial seawater under a light source (60 W bulb) and constant aeration. After 48 h, the hatched active nauplii, free from egg-shells, were collected from the brighter portion of the hatching chamber and used for the assay. For toxicity tests, PEs were dissolved in dimethyl sulfoxide (DMSO) and serially diluted in a micro well plate to a final volume of 100 μl. Further, *A. salina* nauplii were diluted to a concentration of 100 organisms/ml and 100 μl aliquots were transferred to a 96-well polystyrene micro well plate. The plates were incubated at room temperature under illumination. DMSO was used as an assay blank. Deaths were recorded after 24 h of PE exposure and the surviving animals were killed by the addition of 100 μl of 5% (v/v) phenol to each well and counted for total number of nauplii. Results were expressed as percentage of mortality ($n=3$) and LC_{50} was calculated using the statistic software SPSS (Probit analysis).

2.4. Bioassay for toxicity in Physa fontinalis

Snail tests were performed as described by Devappa et al. [10]. All tests were carried out in deionized water. Stock solutions of *Jatropha* PE-rich extracts and purified *Jatropha* PEs were prepared in methanol and further diluted in water. Groups of 10 snails were placed in glass containers with 400 ml of water containing the test substance. The glass containers were placed in a water bath adjusted to 26 °C. Snails were prevented from crawling out of the containers by a fine stainless steel mesh suspended just above the water's surface. After 24 h of incubation, the snails were transferred to deionized water, fed and maintained for another 48 h. Snail death was determined by lack of reaction to irritation of the foot with a needle. Control experiments were performed with the same quantity of methanol in water as used for the test preparations and no mortality was recorded in the control containers. All tests were repeated three times. Toxicity is expressed as percent mortality, referring to concentrations killing 100% of the snails and LC_{50} was calculated using the statistic software SPSS (Probit analysis).

2.5. Platelet aggregation assay

The whole blood from a healthy volunteer was collected and citrated (0.13 M). The assay was carried out as described by Akarasereenont et al. [11]. The platelet rich plasma (PRP) was obtained by centrifuging the citrated blood at 250×*g* for 10 min at 20 °C. The PRP was further centrifuged at 1500 x *g* for 15 min at 20 °C and the supernatant was collected as platelet-poor plasma (PPP). Platelets were adjusted to a concentration of approximately 3×10^8 platelets/ml. Aggregation was initiated by adding 5 μl of agonists to the PRP at the following concentrations: 0.05 μM to 1 μM (PMA) and 0.05 to 50 μM (*J. curcas* PEs). Saline was used as a control. After addition of the agonists, kinetic reading of the plate was started immediately and absorbance (Abs) was noted at 625 nm for 20 min, with readings at 2 min intervals. During the run time, the plate was incubated at 37 °C and was agitated using the automix function of the reader. The experiment was carried out at least in triplicate for each sample. Percentage aggregation was calculated from the following formula:

$$\%\text{Aggregation} = ((A-B)/(A-C))x100$$

where A = Abs PRP plus normal saline; B = Abs PRP plus agonist; C = Abs PPP plus normal saline.

ED_{50} values were calculated as the concentrations of test compound producing 50% of the maximal aggregation rate.

2.6. Phorbol ester-rich extract stability studies

Dried PE-rich extract samples were stored in sealed glass vials at room temperature (23 °C), 4 °C and −20 °C. Over a period of one year, at predetermined intervals, samples were analyzed by the HPLC gradient elution method described above. The peak area for total PEs was recorded for each sample and expressed equivalent to *Jatropha* factor C_1.

3. Results and discussion

3.1. Purification of phorbol esters

Jatropha factors C_1 and C_2 were purified to homogeneity while, factor C_3 and factors (C_4+C_5) were obtained as mixtures, as indicated by shoulder peaks in their HPLC chromatograms (Fig. 2). Since the shoulder peaks were small, the impurities in the factor C_3 and factors (C_4+C_5) mixtures were considered to be minute. Generally, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (synonym: phorbol 12-myristate 13-acetate, PMA) is used as a reference compound for the quantitative expression of *J. curcas* using HPLC [3,10,12]. However, the absorption peak area (monitored at 280 nm on HPLC) of *Jatropha* PEs was higher (w/w) when compared to TPA. According to the *Jatropha* factor C_1 calibration curve (data not shown), where absorption peak area was plotted against *Jatropha* factor C_1 weight, absorption peak area for 1 μg of factor C_1 was 770636. Similarly, 1 μg of TPA corresponded to an absorption peak area of 18679, equivalent to 0.0242 μg of factor C_1. Therefore, the ratio of TPA to factor C_1 was calculated to be 41.3:1.

Jatropha oil (5.45 mg/g of PEs, TPA equivalent; average of 6 values) was analyzed by HPLC and the proportion of individual peaks to the total area of all PE peaks was determined. Only peaks representing factor C_1 and factor C_2 were clearly separated while shoulders appeared in the peaks of factors C_3 to C_6. The peaks representing factors C_1 and C_2 constituted 51% and 21%, respectively, of the total PE peak area obtained for *Jatropha* oil and the rest of the combined peak area (28%) was represented by factors C_3 to C_6 (data not shown). As a percentage of total area contributed by PEs, *Jatropha* factor C_1 is the predominant PE (51%), and hence it is suggested that the concentration of *Jatropha* PEs be expressed equivalent to factor C_1. Consequently, data in Table 1, Figs. 3 and 4 are expressed in *Jatropha* factor C_1 equivalents.

3.2. Biological activity of purified PE-rich extract

Devappa et al. [10] reported that a methanol extract containing PEs was most sensitive in the snail and *Artemia* bioassays with EC50 (48 h, TPA equivalent) of 330 μg/L and 26.5 mg/L, respectively (or 8 μg/L and 0.64 mg/L equivalent to *Jatropha* factor C_1, respectively). As a result, these organisms were selected for the current study.

The bioactivity of the *Jatropha* PEs and PE-rich extract obtained are presented in Figs. 3 and 4. In the snail bioassay,

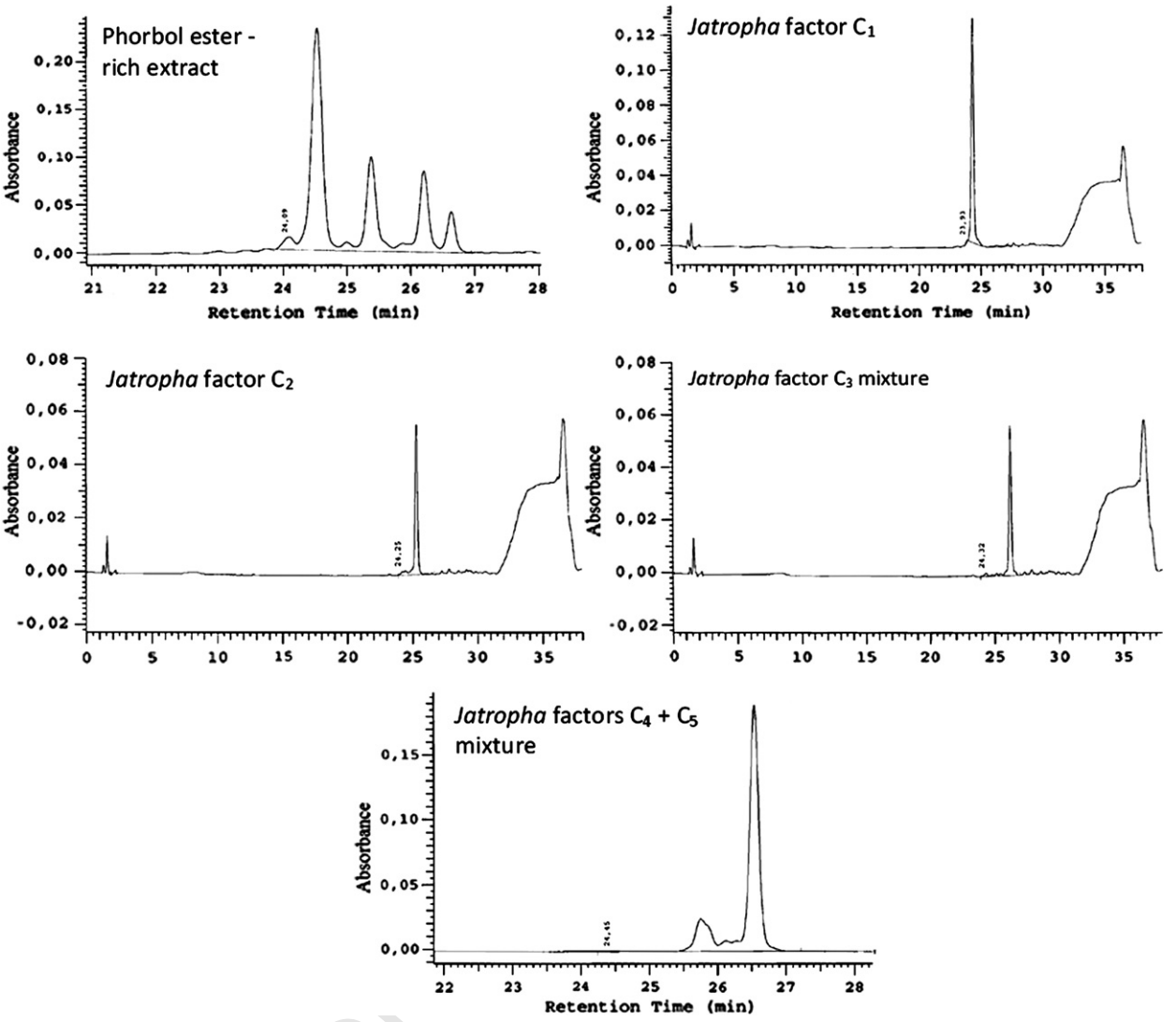

Fig. 2. HPLC chromatograms of purified phorbol esters from *Jatropha curcas* oil.

the order of potency based on EC_{50} (μg/L, equivalent to *Jatropha* factor C_1) was: PE-rich fraction (11.33)<factor C_3 mixture (6.78)<factor C_2 (6.54)<factor C_1 (4.12)<factors (C_4+C_5) mixture (2.18). In the *Artemia* bioassay, the order of potency based on EC_{50} (ppm, equivalent to *Jatropha* factor C_1) was: factor C_2 (11.8)<factor C_3 mixture (1.08)<factor C_1 (0.43)<factors (C_4+C_5) mixture (0.043). From these results, it is evident that all the *Jatropha* PEs were active and showed increased bioactivity when compared to the PE-rich extract.

Rug and Ruppel [13] reported that crude *Jatropha* oil and a methanol extract from *Jatropha* oil exhibited toxicity against snails (*Biomphalaria glabrata*) with an LC_{50} value of 50,000 μg/L and 5,000 μg/L; whereas, in the genus *Bulinus*, the methanol extract from *Jatropha* oil was potent with an LC_{50} of 200 μg/L [13]. Liu et al. [14] reported that a methanol extract from *Jatropha* kernels produced 50% mortality at a concentration of 10,000 μg/L against *Oncomelania hupensis*; while, 4β-phorbol-13-decanoate was toxic against both snails (*Biomphalaria glabrata and O. hupensis*) at a concentration of 10,000 μg/L. However, 4α-phorbol, 4β-phorbol and 'α' forms of PEs were inactive [14]. The toxicity of the methanol fraction from *Jatropha* oil was attributed to the presence of PEs (Rug and Ruppel, 2000; Liu et al., 1997). Kinghorn et al. [9] reported that in an *Artemia* bioassay, phorbol 12-tetradeconoate 13-acetate, phorbol 12, 13-didecanoate and phorbol 12, 13-dibenzoate elicited toxicity with an effective mortality dose

Table 1
Platelet Aggregation by *Jatropha curcas* phorbol esters.

Phorbol ester	ED_{50} (μM)[a]	ED_{50} (μM)[b]
Jatropha factor C_1	0.11	4.4
Jatropha factor C_2	0.19	7.9
Jatropha factor C_3 mixture	0.15	6.1
Jatropha factor (C_4+C_5) mixture	0.04	1.8
Phorbol 12-myristate 13 acetate	0.01	0.5

[a] Equivalent to *Jatropha* factor C_1.
[b] Equivalent to Phorbol 12-myristate 13 acetate (PMA or TPA).

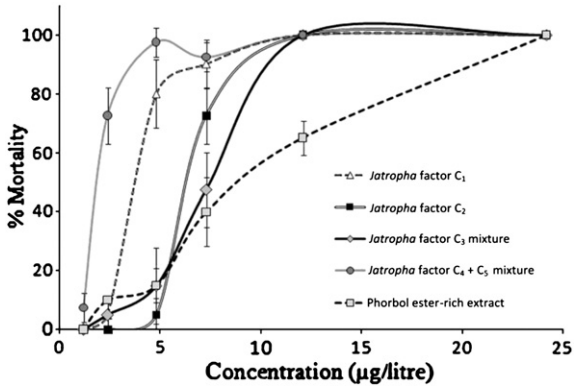

Fig. 3. Bioactivity of *Jatropha curcas* phorbol esters and phorbol ester-rich extract using the snail (*Physa fontinalis*) bioassay (concentrations are expressed equivalent to *Jatropha* factor C_1).

(ED_{50}) of 3.8, 6.8 and 11.8 mg/L, respectively, while phorbol and 4α-phorbol 12, 13-didecanoate were relatively nontoxic.

Comparison of the aforementioned reports [13,14] with our results indicates that the *Jatropha* PE-rich fraction could be used as a molluscicidal agent against schistosomiasis vector snails. In addition, the *Jatropha* PEs tested in our study all have the 4β configuration, similar to the active PEs discussed above [9,13].

3.3. Platelet aggregation by purified Jatropha phorbol esters (PEs)

The effective concentration at which platelet aggregation was observed is presented in the Table 1. Potent tumour promoters PMA and teleocidin are known platelet aggregators [15–17]. In our study, the effect of *Jatropha* PEs on platelet aggregation was compared with PMA. The order of potency for platelet aggregation based on the ED_{50} (μM, factor C_1 equivalent) was: factor C_2 (0.19) < factor C_3 mixture (0.15) < factor C_1 (0.11) < factors ($C_4 + C_5$) mixture (0.04). The PMA induced platelet aggregation at 0.5 μM concentration (ED_{50}; 0.012 μM as factor C_1 equivalent). All the *Jatropha* PEs were less effective than PMA in the platelet aggregation assay.

Many daphnane and tigliane derivatives of PEs were reported to exert platelet inhibition (measured using a platelet aggregometer). The ED_{50} (μM) results were: PMA (0.3), 12-deoxy phorbol 13-phenyl acetate (0.38), 12-deoxy phorbol-13-acetate (8.0), sapintoxin A (1.76), sapintoxin B (4.3), sapintoxin D (7.2) and proresiniferatoxin (0.248), phorbol-12, 13-dideanoate (6.6), phorbol-12, 13 dibenzoate (0.13) and phorbol −12, 13-diacetate (3.1) [16,18]. The difference in the biological activity of the individual phorbol esters was presumed to be due to their pharmacokinetic differences [16]. Although, poor correlation was observed between the rapid platelet aggregation assay and tumour promoting properties, the former assay was suggested for screening suspected tumour promoting compounds [16]. It has also been reported that the stimulation of PKC by tumour promoting phorbol esters (PMA) induces platelet aggregation and secretion [17]. *Jatropha* PE DHPB, which was later re-characterized as *Jatropha* factor C_1 by Haas et al. [6], hyper activated PKC *in vitro* [19] and showed tumour promotion in mice [20]. From our studies, we presume that *Jatropha* PEs also have potential tumour promoting properties.

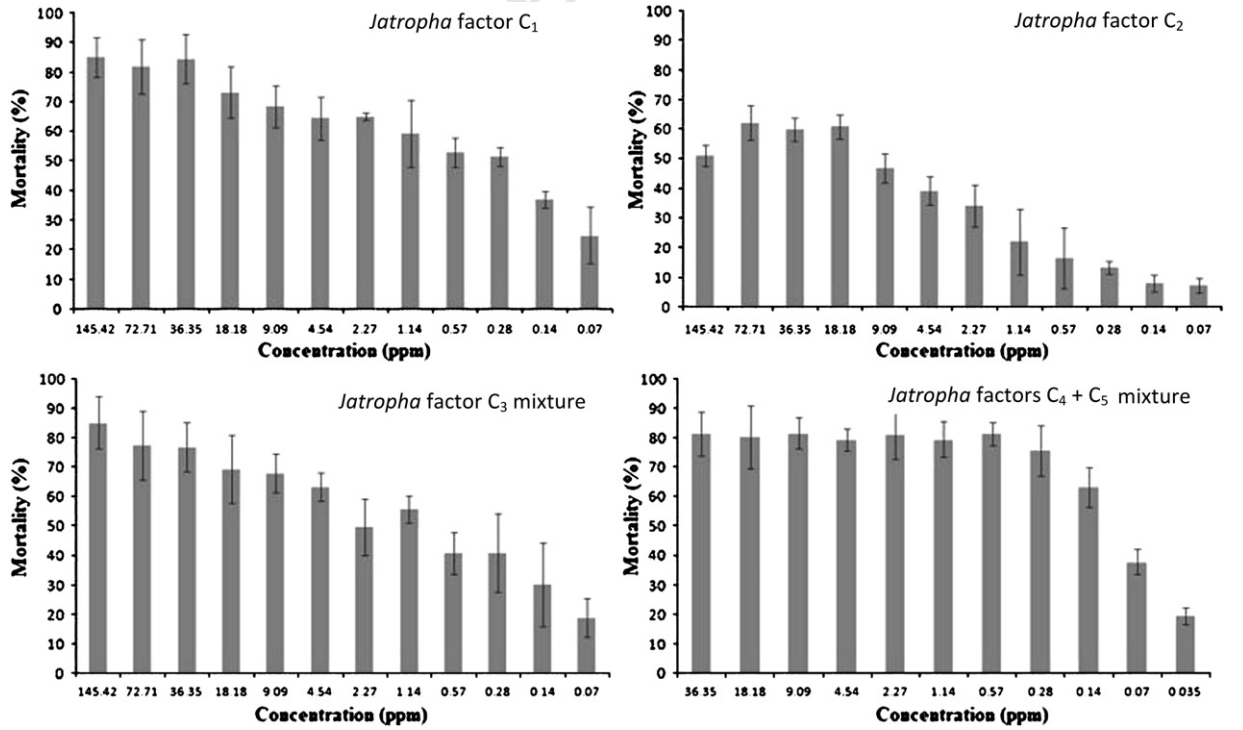

Fig. 4. Bioactivity of *Jatropha curcas* phorbol esters using the *Artemia salina* bioassay (concentrations are expressed equivalent to *Jatropha* factor C_1).

3.4. Stability of phorbol ester-rich extract

The PE-rich extract exhibited high activity in the snail bioassay (Section 3.2). With an aim to using the PE-rich extract as a molluscicide, we evaluated the storage stability of PEs present in the PE-rich extract at room temperature, 4 °C and −20 °C by storing the PE-rich extract in the dried form and in ethanol.

The results of the stability studies on *J. curcas* PE-rich extract are shown in Fig. 5. When stored in dried form, the PEs present in the PE-rich extract were reduced by 49% after 14 days and further reduced by 98% after 42 days. At 4 °C, the PEs were reduced by 48% after 98 days and by >98% after 273 days. At −20 °C, only 22% reduction was observed after 365 days.

Generally, stability of a compound depends upon physical and chemical properties of the compound in a test material. PEs are large, complex molecules, possessing a number of reactive functional groups, including ester, epoxide, and vinyl ether moieties, which render the compound unstable under different storage conditions. In our study, we hypothesised that the degradation of PEs in the PE-rich extract was due to auto-oxidation. Similar results were observed in our earlier study [12], wherein PEs present in the methanol extract of *Jatropha* oil were degraded by 50% after 132 days and >92% after 410 days. In addition, PE reduction of only 7.9% and 5.4% was observed when the same extract was stored at 4 °C and −80 °C, respectively. Furthermore at day 870 of storage of the methanol extract, reduction in the bioactivity (using the snail bioassay) was 27.5% and 32.5% at 4 °C and −80 °C, respectively. In another study, TPA, when stored for 3 months in a dried form at 25 °C under diffused light, produced hydroperoxides; and at 4 °C (in the dark), the hydroperoxides were formed to a very limited extent [21].

In our study, when the PE-rich extract was stored in ethanol, negligible decreases in PEs were observed at all storage temperatures (RT, 4° and −20 °C) up to 90 days (data not shown). Due to ineffective sealing of the test vials, there was slow evaporation of ethanol from the stored vials with increased storage time. Thus, the degradation of PEs could not be determined quantitatively. However, it should be noted that there was no PEs degradation or degradation products observed up to 365 days. In another study, Schmidt and Hecker [21] reported that TPA in acetone was stable when stored in the dark at −20 °C, but it decomposed slowly in the dark at 4 °C in 3 months and during this time the degradation was extensive at 25 °C in diffused daylight. During decomposition, 7-hydroperoxide was formed as a major product. Similarly, Tremp and Hecker [22] reported reduction of 12-*O*-retinoylphorbol-13-acetate in acetone when stored at room temperature (20 °C) for a period of 5 weeks. In the same study, a slight decomposition of PEs in solutions stored in amber glassware wrapped with black foil, with and without argon, at 20 °C was observed while at −20 °C and −70 °C no decomposition was observed. From these results, we recommend storage of the *Jatropha* PE-rich extract and purified *Jatropha* PEs in ethanol, preferably at −20 °C or below. However, detailed screening studies to identify an effective antioxidant formulation to increase the stability of the PE-rich extract at room temperature are needed.

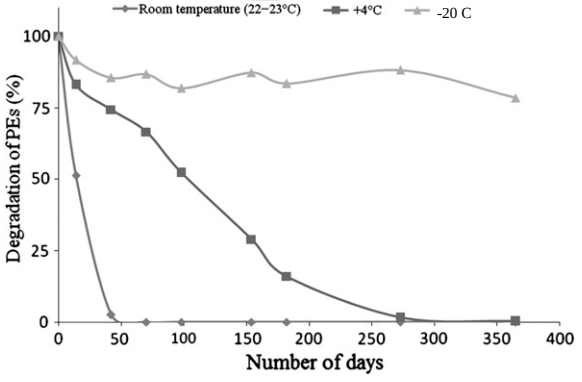

Fig. 5. Stability of *Jatropha curcas* phorbol ester-rich extract (concentrations are expressed equivalent to *Jatropha* factor C_1).

4. Conclusion

From our study, it is evident that *Jatropha* PEs, in the form of the PE-rich extract, could be used in suitable formulations as molluscicidal agents, particularly against schistosomiasis vector snails. The likely tumor-promoting potential of *Jatropha* PEs, and or toxicity to mammals, would necessitate proper handling of such products. Considering the projected oil yield of 26 million tons/annum by 2015 [23], integration of the methodology for the extraction of the PE-rich extract into the *Jatropha* biodiesel production chain could make *Jatropha* a new stock for the pest control industry.

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References

[1] Devappa RK, Makkar HPS, Becker K. *Jatropha* Diterpenes: a Review. J Am Oil Chem Soc 2011;88:301–22.
[2] Makkar HPS, Aderibigbe AO, Becker K. Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem 1998;62:207–15.
[3] Makkar HPS, Becker K, Sporer F, Wink M. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 1997;45:3152–7.
[4] Makkar HPS, Becker K. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J Lipid Sci Technol 2009;111:773–87.
[5] Makkar H, Maes J, De Greyt W, Becker K. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J Am Oil Chem Soc 2009;86:173–81.
[6] Haas W, Sterk H, Mittlebach M. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas*. J Nat Prod 2002;65:1434–40.
[7] Devappa RK, Makkar HPS, Becker K. *Jatropha* toxicity–A review. J Toxicol Environ Health Part B 2010;13:476–507.
[8] Makkar HPS, Siddhuraju P, Becker K. A laboratory manual on quantification of plant secondary metabolites. Totowa, New Jersey: Humana Press; 2007.

[9] Kinghorn AD, Harjes KK, Doorenbos NJ. Screening procedure for phorbol esters using brine shrimp (*Artemia salina*) larvae. J Pharm Sci 1977;66:1362–3.
[10] Devappa RK, Makkar HPS, Becker K. Biodegradation of *Jatropha curcas* phorbol esters in soil. J Sci Food Agric 2010;90:2090–7.
[11] Akarasereenont P, Tripatara P, Chotewuttakorn S, Palo T, Thaworn A. The effects of estrone, estradiol and estriol on platelet aggregation induced by adrenaline and adenosine diphosphate. Platelets 2006;17: 441–7.
[12] Devappa RK, Maes J, Makkar HPS, Greyt WD, Becker K. Isolation of phorbol esters from *Jatropha curcas* oil and quality of produced biodiesel, 2nd International Congress on Biodiesel: The Science and the Technologies, Munich, Germany; 2009.
[13] Rug M, Ruppel A. Toxic activities of the plant *Jatropha curcas* against intermediate snail hosts and larvae of schistosomes. Trop Med Int Health 2000;5:423–30.
[14] Liu SY, Sporer F, Wink M, Jourdane J, Henning R, Li YL, Ruppel A. Anthraquinones in *Rheum palmatum* and *Rumex dentatus* (*Polygonaceae*), and phorbol esters in *Jatropha curcas* (*Euphorbiaceae*) with molluscicidal activity against the schistosome vector snails *Oncomelania, Biomphalaria* and *Bulinus*. Trop Med Int Health 1997;2:179–88.
[15] Kume S, Yamanaka M, Kaneko Y, Kariya T, Hashimoto Y, Tanabe A, Ohashi T, Oda T. Teleocidin, a tumor promoter, is a potent platelet-aggregating agent. Biochem Biophys Res Commun 1981;102:659–66.
[16] Brynes PJ, Schmidt R, Hecker E. Plasminogen activator induction and platelet aggregation by phorbol and some of its derivatives: Correlation with skin irritancy and tumour-promoting activity. J Cancer Res Clin Oncol 1980;97:257–66.
[17] Elzagallaai A, Rosé SD, Trifaró J-MA. Platelet secretion induced by phorbol esters stimulation is mediated through phosphorylation of MARCKS: a MARCKS-derived peptide blocks MARCKS phosphorylation and serotonin release without affecting pleckstrin phosphorylation. Blood 2000;95:894–902.
[18] Ellis CA, Brooks SF, Evans AT, Morrice N, Evans EJ, Aitken A. The effects of phorbol esters with different biological activities on protein kinase C. Phytother Res 1987;1:187–90.
[19] Wink M, Grimm C, Koschmieder C, Sporer F, Bergeot O. Sequestration of phorbol esters by the aposematically coloured bug *Pachycoris klugii* (*Heteroptera: Scutelleridae*) feeding on *Jatropha curcas* (*Euphorbiaceae*). Chemoecology 2000;10:179–84.
[20] Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wonchai V, Hecker E, Fujiki H. A new tumor promoter from the seed oil of *Jatropha curcas* L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. Cancer Res 1988;48:5800–4.
[21] Schmidt R, Hecker E. Autoxidation of phorbol esters under normal storage conditions. Cancer Res 1975;35:1375–7.
[22] Tremp GL, Hecker E. Stability of the "second stage" promoter 12-*O* -retinoylphorbol-13-acetate. Cancer Res 1985;45:2390–1.
[23] GEXSI. Available athttp://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf2008.

CHAPTER - 10

This Chapter contains 2 subsections describing the effect of purified Jatropha phorbol esters upon (1) oral (intragastric) exposure *in vivo* and (2) dermal and ocular exposure *in vitro.*

CHAPTER - 10.1

Toxicity of *Jatropha curcas* phorbol esters in mice

Cai-Yan Li,[a] **K. Rakshit Devappa**,[b] Jian-Xin Liu,[a] Jian-Min Lv,[c] H.P.S. Makkar,[b] K. Becker[b]

[a] *College of Animal Science, Zhejiang University, 310029 Hangzhou, P.R. China.*
[b]*Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, 70593 Stuttgart, Germany.*
[c]*Laboratory Animal Research Center, Zhejiang Chinese Medicine University, 310053 Hangzhou, P.R.China.*

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Toxicity of *Jatropha curcas* phorbol esters in mice

Cai-Yan Li [a], Rakshit K. Devappa [b], Jian-Xin Liu [a,*], Jian-Min Lv [c], H.P.S. Makkar [b,*], K. Becker [b]

[a] College of Animal Science, Zhejiang University, 310029 Hangzhou, PR China
[b] Institute for Animal Production in the Tropics and Subtropics, University of Hohenheim, 70593 Stuttgart, Germany
[c] Laboratory Animal Research Center, Zhejiang Chinese Medicine University, 310053 Hangzhou, PR China

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ABSTRACT

Phorbol esters are the main toxins in *Jatropha curcas* seed and oil. The aim of this study was to assess the acute toxicity of phorbol esters given by intragastric administration and to determine the LD_{50} for Swiss Hauschka mice. The LD_{50} and 95% confidence limits for male mice were 27.34 mg/kg body mass and 24.90–29.89 mg/kg body mass; and the LD_5 and LD_{95} were 18.87 and 39.62 mg/kg body mass, respectively. The regression equations between the probits of mortalities (Y) and the log of doses (D) was $Y = -9.67 + 10.21 \log(D)$. Histopathological studies on the organs from the dead mice showed: (1) no significant abnormal changes in the organs at the lowest dose (21.26 mg/kg body mass) studied, (2) prominent lesions mainly found in lung and kidney, with diffused haemorrhages in lung, and glomerular sclerosis and atrophy in kidney at doses $\geqslant$32.40 mg/kg body mass, and (3) multiple abruption of cardiac muscle fibres and anachromasis of cortical neurons at the highest dose of 36.00 mg/kg body mass. The results obtained would aid in developing safety measures for the Jatropha based biofuel industry and in exploiting the pharmaceutical and agricultural applications of phorbol esters.

1. Introduction

Jatropha curcas is a multipurpose bush or a small tree belonging to the family of *Euphorbiaceae*. It is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics in Africa, Asia and southern America (Gübitz et al., 1999). The plant is well adapted to barren or drought affected areas and even survives in poor stony soils. *J. curcas* seed contains high amount of oil that can be converted into biodiesel of high quality upon transesterification. Apart from the oil, the seed cake or kernel meal leftover has gained tremendous interest for their utilization in feed formulations (Makkar et al., 1997). *J. curcas* kernel meal is rich in protein and the essential amino acid composition of the protein, except lysine, is comparable to that of soybean meal (Makkar et al., 1998; Devappa and Bhagya, 2008). However, the main toxins present in these by-products are phorbol esters, which prevent their utilization as feed ingredients (Makkar et al., 1997).

Phorbol esters are diterpenes having tigliane skeletal structure. Six phorbol esters have been characterized from *J. curcas* (Haas et al., 2002). Phorbol esters are amphiphilic molecules and have tendency to bind phospholipid membrane receptors. During normal signal transduction process, DAG (diacyl glycerol) activates PKC (protein kinase C), which is involved in various other signal transduction pathways. The phorbol esters act as an analogue for DAG and are strong PKC activators. These hyperactivate PKC and trigger cell proliferation, thus amplifying the efficacy of carcinogens. Phorbol esters themselves do not induce tumors but promote tumor growth following exposure to subcarcinogenic dose of carcinogen. They can thus be regarded as co-carcinogens (Goel et al., 2007). The concentration of phorbol esters varies from 2 to 3 mg/g in kernel and 2 to 4 mg/g in oil in different varieties of *J. curcas* (Makkar et al., 1997). Although phorbol esters are lipophilic, they get strongly bound to the matrix of kernel meal. Approximately 30% of the total phorbol esters were present in kernel meal containing <1% lipid (Makkar et al., 2008).

Studies in the last decade showed that *J. curcas* exhibited toxicity to a wide variety of organisms, from microorganisms to higher animals. But, the toxicity effects studied in animals are mainly by force-feeding either *J. curcas* raw or defatted seed meals, leaves or their various organic solvent/aqueous extracts. Oral administration of Jatropha kernel oil obtained by hexane–soxhlet extraction had an LD_{50} of 6 ml/kg body mass in rats. The rats, at 6, 9 and 13.5 ml/kg body mass exhibited diarrhoea and haemorrhagic eyes; and the autopsy showed inflammation of the gastro-intestinal tract (Gandhi et al., 1995). *J. curcas* oil at a dose of 2 g/kg body mass caused significant acute toxicity by inhibiting the birth of pups in rats (Odusote et al., 2002). The concentrated toxic fraction obtained after successive extraction of oil by 90% methanol and ethyl ether, as reported by Gandhi et al. (1995) exhibited toxicity

* Corresponding authors. Tel.: +49 71145923640; fax: +49 71145923702 (H.P.S. Makkar).
E-mail addresses: liujx@zju.edu.cn (J.-X. Liu), makkar@uni-hohenheim.de (H.P.S. Makkar).

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towards rabbit (100 μl), mice and rats (50 μl). The common symptoms of topical application were erythema, oedema, necrosis, diarrhoea, scaling and thickening of the skins (Gandhi et al., 1995). Feeding of *J. curcas* seeds, fruits or leaves caused toxicity depending on the dose and the animal model tested. Raw or defatted seeds when force-fed to fish, chicks, pigs, goat, mice and rats (caused severe toxicity symptoms before death (Liberalino et al., 1988; Chivandi et al., 2000; Gadir et al., 2003; Adam and Magzoub, 1975; Adam, 1974). Various organic and aqueous extracts also exhibited different toxic symptoms depending on dose, mode of administration and sensitivity of the animals being tested (Trebien et al., 1988; Mariz et al., 2006, 2008). For example, acetonitrile extract of *J. curcas* (seed or oil) when given to albino rats at an oral dose of 50 mg/kg body mass (single dose) produced mild toxicological, biochemical and histopathological changes (Abd-Elhamid, 2004). The methanol, petroleum ether and dichloromethane extracts of *J. curcas* fruit caused foetal resorption indicating pregnancy terminating effect in rats (Goonesekera et al., 1995). The irritant methanol fraction from *J. curcas* oil induced tumor promotion upon topical initiation by 7, 12-dimethylbenz(*a*)anthracene (DMBA) in mice, with 36% of the animals having skin tumors in 30 weeks (Horiuchi et al., 1988).

Hitherto, all the LD_{50} studies, toxicity studies or the histopathological studies conducted were using either *J. curcas* oil, seed, defatted seed cake or crude fractions from them. In the present study we purified phorbol esters from *J. curcas* oil, evaluated their acute toxicity when administered intragastrically and determined LD_{50} for laboratory mice.

2. Materials and methods

2.1. Materials

The *J. curcas* oil used in the study was from toxic genotypes from India. Corn oil was obtained from Sigma Chemical Company (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Phorbol esters isolation and quantification

Phorbol esters were determined in quadruplicate according to Makkar et al. (2007), which was based on the method of Makkar et al. (1997). Briefly, 0.5 g of the sample was extracted four times with methanol. A suitable aliquot was loaded on a high-performance liquid chromatography (HPLC) reverse-phase C18 LiChrospher 100, 5 μm (250 × 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-phorbol esters peaks were detected at 280 nm and appeared between 25.5 and 30.5 min. For LD_{50} studies, the phorbol esters were carefully collected at the above retention times. All the collected fractions were in approximately 90% acetonitrile. These fractions were pooled and kept in a freezer (−20 °C). To avoid oxidation by water, the top acetonitrile layer from the frozen sample was separated and the acetonitrile was rotary evaporated at low vacuum to collect the colorless oily fraction. This fraction was redissolved in methanol and subjected to HPLC for checking the purity and concentration. The results were expressed as equivalent to phorbol-12-myristate 13-acetate.

2.3. Test substance

Phorbol esters were isolated from the *J. curcas* oil as above and diluted in high purity corn oil (Acros Organics Co., Geel, Belgium) for oral administration.

2.4. Animals and conditions

All the conditions used for animal housing and handling were approved by Animal Care and Use Committee at Zhejiang Province (China), and the experimental protocols used followed the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Council of People's Republic of China, 1988).

A total of 70 male specific pathogen-free (SPF) grade Swiss Hauschka mice (21 days old) were obtained from Shanghai Laboratory Animal Center of China Academy of Science (Shanghai, China). The number of laboratory-animal-quality certification was SCXK (Hu) 2003-0003. The mice with an initial body mass of 17–18 g were kept in a large clean 27 × 17 × 13 cm polycarbonate cages (5 mice per cage) with bedding and fed on a laboratory diet (radiated by Co^{60}) for 3 days for acclimatization. After acclimatization, the mice were weighed and numbered.

The experiment was conducted in a barrier system with an experimental facility (License No: SYXK (ZHE) 2003-0003). The housing conditions were controlled automatically with a room temperature of 22 ± 1 °C; relative humidity 50–70%; lighting 150–200Lx, the sequence being 12 h dark and light cycle; noise <50 dB; air cleanliness degree 10,000 grade. During the experiment the mice had *ad libitum* access to food and sterile drinking water.

2.5. Test procedure

The test procedures were in accordance with the Regulatory Guide on the Techniques for Drug Research (The State Food and Drug Administration of People's Republic of China, 2005). A pretest was conducted to observe the range of toxicity so that the proper dose levels could be established for LD_{50} determination. Three dose levels (6, 12 and 18 mg/kg body mass) of phorbol esters were used for the pretesting. Based on the pretest results, six dosages (36.00, 32.40, 29.16, 26.24, 23.62 and 21.26 mg/kg body mass) were established with each group comprised of 10 mice using random block design (average body mass 18–20 g). The mice were kept fasting for 12 h before the phorbol esters doses were given by intragastric administration. The dosage of phorbol esters was given to mice as 0.2 ml/10 g body mass. The remaining 10 mice served as control and were given an equal volume (0.2 ml/10 g body mass) of corn oil. After the oil or phorbol ester administration, the mice in each group were fed as normal, and had free access to food and drinking water.

In the following 19 days, the clinical signs, change in body mass, and toxicity symptoms in mice were observed every day.

2.6. Histopathological studies

All deaths were recorded. Died mice and mice killed by cervical dislocation at the end of experiment were examined for gross and microscopical changes. The specimens of tissues from kidneys, liver, heart, spleen, brain and the lungs were taken for histopathological examination. The tissues were immediately rinsed with physiological saline, fixed overnight in 4% paraformaldehyde, and then dehydrated in a graded series of ethanol and embedded in paraffin for later slices and haematoxylin and eosin staining.

2.7. Calculations and statistical analysis

The LD_{50} determination was based on dose levels that increased by a geometrical progression. Six dose levels were ultimately required for establishing the LD_{50}. Calculations for LD_{50}, 95% confidence limits, LD_5 and LD_{95} were based on the Bliss method as explained in Zhou (1988). The Bliss was calculated by using the NDST Software Version 8.0 (Sun, 1998).

Table 1
Mortality of mice after intragastric administration.

Dose (mg/kg)	Dead	After administration (days)														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15–19
0 (Control)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
36.00	9	0	1	0	2	2	3	0	0	0	0	0	0	0	1	0
32.40	7	0	2	2	1	1	0	0	0	0	0	1	0	0	0	0
29.16	7	0	1	2	2	0	0	1	1	0	0	0	0	0	0	0
26.24	4	0	0	1	0	0	2	0	1	0	0	0	0	0	0	0
23.62	3	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
21.26	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

Table 2
Changes in total body mass (g) of mice after administration (Mean ± SD).

Dose (mg/kg)	Body mass (g) after administration (days)					
	1	3	5	7	14	19
36.00	19.0 ± 0.7	17.6 ± 1.8	17.2 ± 3.5	21.1 ± 5.9	28.4	30.60
32.40	19.1 ± 0.8	17.0 ± 1.1	15.9 ± 2.0	17.9 ± 2.6	23.8 ± 1.7	27.6 ± 0.7
29.16	19.8 ± 0.9	17.9 ± 1.2	16.3 ± 2.3	17.2 ± 4.0	23.8 ± 3.9	28.1 ± 2.2
26.24	18.9 ± 0.8	17.2 ± 0.8	16.8 ± 2.2	18.2 ± 3.3	25.0 ± 2.1	28.3 ± 1.8
23.62	19.5 ± 0.7	17.6 ± 0.7	16.1 ± 1.9	17.0 ± 2.2	22.1 ± 4.7	26.6 ± 6.4
21.26	18.8 ± 0.7	17.1 ± 1.4	16.7 ± 2.6	18.4 ± 3.5	24.4 ± 3.0	28.3 ± 2.2
0 (control)	19.2 ± 0.8	22.6 ± 1.1	23.9 ± 1.4	25.9 ± 1.6	27.5 ± 1.7	29.8 ± 1.9

All the experimental analysis were statistically analyzed using the General Linear Model (GLM) Procedure of SAS by Duncan's new multiple range test to determine the significant differences in each concentration doses ($p < 0.05$).

3. Results

3.1. Mortality and live weight changes

The starting material had a phorbol ester concentration of 45.03 mg/g in corn oil. The death of mice occurred in a dose dependent manner (Table 1). Mice began to die on the 2nd day after administering the phorbol esters, and no more mice died during the extended 15–19 observation days. The time to death seems to depend on the administered dose and the individual susceptibility of mice to the phorbol esters. The higher the dose or less the resistance of an individual, the quicker the animal died.

Changes in body mass after administration of phorbol esters are shown in Table 2. For all doses, body mass of the phorbol esters administered mice decreased significantly after 3 days of the administration ($p < 0.05$). However, at the doses of 21.26–32.40 mg/kg, the body mass of mice those survived for 7 days after phorbol esters administration started reverting back to normal. At the highest dose of 36.00 mg/kg, the recovery of body weight occurred after 5 days of phorbol esters administration.

3.2. LD_{50} determinations

Phorbol esters LD_{50} and its 95% confidence limits for male mice were 27.34 mg/kg body mass and 24.90–29.89 mg/kg body mass; and LD_5 and LD_{95} were 18.87 and 39.62 mg/kg body mass, respectively (Table 3). The regression equation between the probits of mortalities (Y) and the log of doses (D) was derived as: $Y(\text{probit}) = -9.67 + 10.21 \log(D)$.

3.3. Clinical signs and histopathological findings

The mice administrated with phorbol esters showed varied degrees of toxicity reactions at different doses. At the highest dose of 36.00 mg/kg, the affected animals showed depression, languishment, loss of body mass, closing of eyes, humidity of anus, arch of the back and behaviors of being easily affrighted and crouched together. For the mice administrated with phorbol esters at all doses, the stool in rectum was dry in the form of beads, and both small and large intestines were filled with black digesta. In contrast, the stool from the control mice was wet and soft. The forming of dry bead like structure in the rectum of affected animals may be related to the intestinal dehydration, most probably caused by phorbol ester mediated electrolyte imbalance. It is supposed that

Table 3
LD_{50} determinations of phorbol esters for mice.

Dose mg/kg	Log Dose	Tested	Dead	Tested/dead (%)	Experimental probability (Y)	Regression probability (Y)
36.00	1.5563	10	9	90	6.2817	6.2203
32.40	1.5105	10	7	70	5.5240	5.7531
29.16	1.4648	10	7	70	5.5240	5.2859
26.24	1.4190	10	4	40	4.7471	4.8187
23.62	1.3733	10	3	30	4.4760	4.3515
21.26	1.3275	10	1	10	3.7183	3.8843

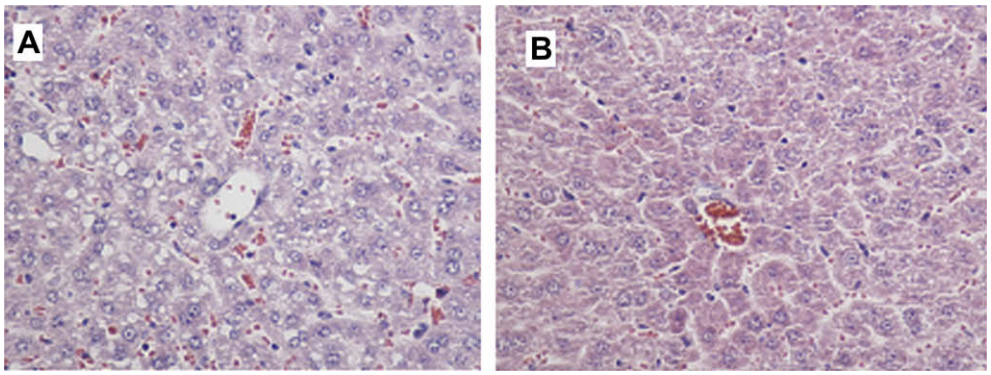

Fig. 1. Pathological changes of liver observed at a dose of 36.00 mg/kg (A) Fatty vacuoles in the live cells (B) Normal liver.

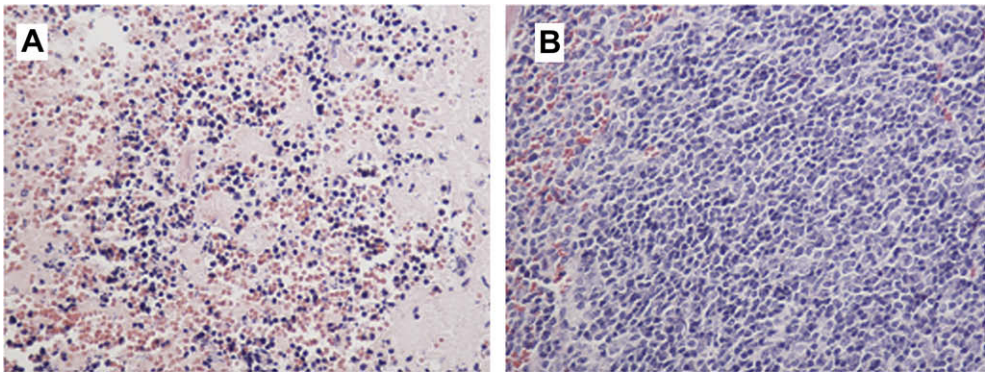

Fig. 2. Pathological changes of spleen observed at a dose of 36.00 mg/kg (A) Widely hyperemia and exudates (B) Normal spleen.

the gastro-intestinal tract haemorrhage may result in the black digesta in intestines. The stool findings showed that the digestive system of mice is sensitive to the presence of phorbol esters.

Histopathological studies of the organs showed normal cellular architecture at the lowest dose (21.26 mg/kg). At a dose of 23.61 mg/kg, no evidence of damage to heart, small intestine, brain and spleen was seen; however, sporadic infiltrated lymphocyte in the liver, seldom haemorrhage in lung and a few of glomerular sclerosis appeared. At a dose of 26.24 mg/kg, congestion of sinus hepaticus, haemorrhage of spleen and a few of glomerular atrophy were seen. At a dose of 29.31 mg/kg, congestion of the pulmonary alveolar capillaries was observed. When the dose increased to 32.40 mg/kg, much more pathological symptoms were seen in lung, with more mice showing congestion of the pulmonary alveolar capillaries and a few of mice exhibiting haemorrhage and burst of alveolus. At the highest dose of 36.00 mg/kg, multiple abruption of cardiac muscle fibres and anachromasis of cortical neurons appeared. Other histopathological changes included the frequent appearance of fatty vacuoles in the liver cells (Fig. 1), widely hyperemia and exudate in spleen (Fig. 2), diffuse haemorrhage and exudate in lung (Fig. 3), and glomerular sclerosis (Fig. 4). Overall, the prominent pathological symptoms were mainly observed in lung and kidney.

4. Discussion

Among the different methods available for LD_{50} determinations, the sensitivity of the Bliss procedure used in the present study is high, despite its complexity (Li et al., 1995).

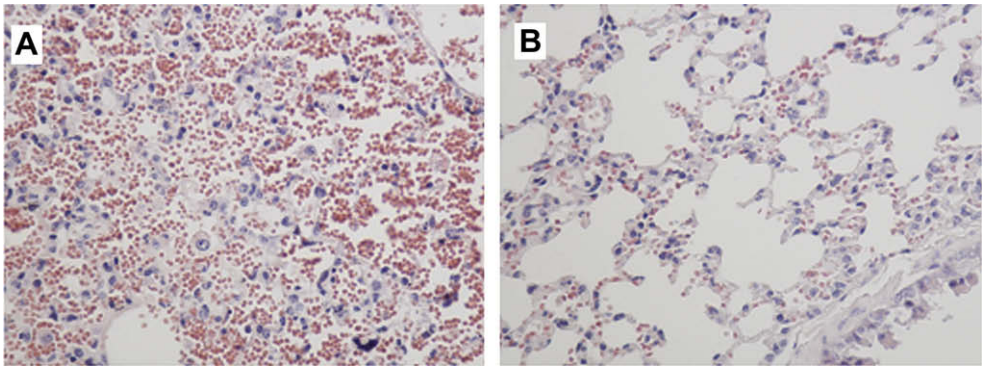

Fig. 3. Pathological changes of lung observed at a dose of 36.00 mg/kg (A) Widely diffuse haemorrhage and exudates (B) Normal lung.

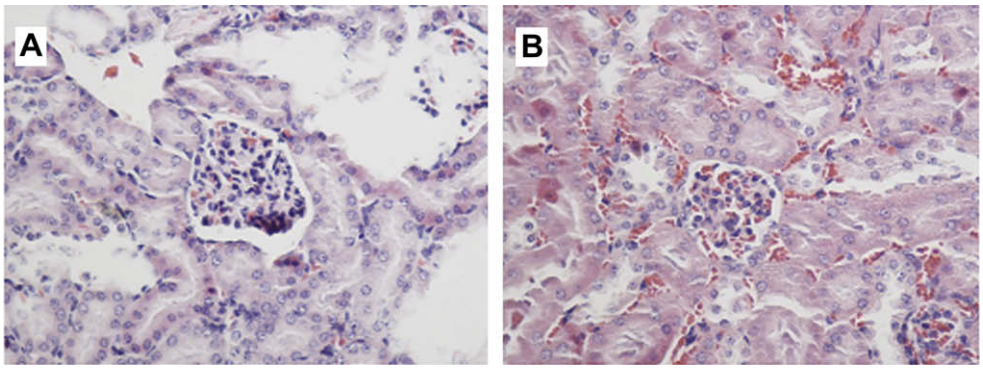

Fig. 4. Pathological changes of kidney observed at a dose of 36.00 mg/kg (A) Glomerular sclerosis (B) Normal kidney.

Table 4
Influence of phorbol ester in ruminants force-fed on powdered *Jatropha curcas* decorticated seeds (kernels).

Animal	Dose (g/kg body mass/day)	100% Mortality (in days)	Calculated value of mg phorbol esters consumed/kg body mass[b]	References
Nubian goat	10	2–3	30–45	Adam and Magzoub (1975)
	10[a]	6	15	
	5	3–4	22.5–30	
	1	7–9	10.5–13.5	
	0.5	11–12	8.25–9.0	
	0.25	18–21	6.75–7.87	
Nubian goat	1	6–7	9–10.5	Ahmed and Adam (1979)
	0.5	16–22	15–20.6	
	0.05	19–25	1.4–1.9	
Desert sheep	1	3–5	4.5–7.5	
	0.5	7–10	5.2–7.5	
Nubian goat kids	1	7–11	10.5–16.5[c]	Gadir et al. (2003)
	0.25	18–21	6.75–7.87[c]	

[a] Single dose.
[b] The average concentration of phorbol esters (Egyptian genotype; 1.5 mg/g, $n = 6$) analyzed in our laboratory was taken for calculation.
[c] The average concentration of phorbol esters (toxic genotype; 2.5 mg/g, $n = 8$) analyzed in our laboratory was taken for calculation.

The LD_{50} study indicates that the purified phorbol esters isolated from the oil are highly toxic to mice and produce severe pathological symptoms. These results support the findings of Makkar et al. (1997, 1998) that phorbol esters are the main toxins in *J. curcas* oil and seeds. Phorbol esters are present in leaves, stems, flowers and roots of *J. curcas* (Makkar and Becker, 2009) and therefore the consumption of *J. curcas* in any form, oil, seeds, seed cake or extracts is toxic to animals, and elicits severe pathological symptoms. In the ruminants, force-feeding (drenching) studies using decorticated *J. curcas* seeds (kernels) caused acute toxicity with 100% mortality depending on the dose administered (Table 4). This highlights the importance of complete removal of phorbol esters from the Jatropha meal before using it in feed formulations. In many parts of the world, *J. curcas* is used as a live fence and the presence of phorbol esters in different parts of the plant and their toxicity is responsible for this use of the plant.

Taking average phorbol esters concentration, determined in our laboratory in the seeds of a number of toxic Indian variety of *J. curcas*, we back calculated the LD_{50} reported for oil by Gandhi et al. (1995) and for methanol extract from oil reported by Oluwole and Bolarinwa (1997). The calculated oral LD_{50} is 19.19 mg/kg body mass (analyzed average value of 3.5 mg/g taken for calculation) in rats and 49.87 mg/kg (intraperitoneal) in mice, respectively (analyzed average value of 1.98 mg/g taken for calculation). Rats fed with diet containing defatted whole seed Jatropha meal (10% protein replacement level) caused severe pathological symptoms and death occurred at a phorbol esters concentration of 47.31 mg/kg body mass (Rakshit et al., 2008).

The symptoms and toxicity of *J. curcas* depend on extract, dose, mode of administration and sensitivity of the animals being tested. For example, topical application of petroleum ether extract of *J. curcas* oil (at a dose of 100 µl) on shaved dorsal skin of rabbit showed erythema and oedema, which later became necrotic and regenerated. In mice, the same extract upon topical application (at a dose of 50 µl) exhibited swelling of the face, haemorrhagic eyes, diarrhoea and skin erythema before death. Whereas, rats (at a dose of 50 µl) showed oedema and erythema at 4 h of topical application on the shaved dorsal skin which subsequently led to severe scaling and thickening of the skin (Gandhi et al., 1995).

All the feeding studies on *J. curcas* showed severe clinical and pathological symptoms. Among the important symptoms observed was transient loss of body mass and mild to severe macroscopic/microscopic changes in the kidney, lungs, heart, liver, and spleen in a dose dependent manner (Adam, 1974; Liberalino et al., 1988; Rakshit et al., 2008). In the present study as well, the major pathological symptoms were observed in lung and kidney, with widely diffuse haemorrhage in lung and glomerular sclerosis in the kidney.

The data obtained in this study would aid in developing: (i) emergency procedures in case of major spills or accidental contacts, (ii) guidelines for the use of appropriate safety clothing and equipment, and (iii) transport regulations. It would also help in establishing occupational exposure limits and in developing material safety data sheets for various by-products obtained during biodiesel production from *J. curcas*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

Abd-Elhamid, H.F., 2004. Investigation of induced biochemical and histopathological parameters of acetonitrile extract of *Jatropha curcas* in albino rats. J. Egypt. Soc. Parasitol. 34 (2), 397–406.
Adam, S.E.I., 1974. Toxic effects of *Jatropha curcas* in mice. Toxicology 2, 67–76.
Adam, S.E.I., Magzoub, M., 1975. Toxicity of *Jatropha curcas* for goats. Toxicology 4 (3), 347–354.
Ahmed, O.M., Adam, S.E., 1979. Toxicity of *Jatropha curcas* in sheep and goats. Res. Vet. Sei. 27 (1), 89–96.
Chivandi, E., Makuza, S.M., Erlanger, K.H., Mtimuni, J.P., Read, J.S., Tivapasi, M., 2000. Effects of dietary *Jatropha curcas* on the haematology of weaned pigs. Zimbabwe Vet. J. 31 (4), 83–91.
Devappa, R.K., Bhagya, S., 2008. Biochemical and nutritional evaluation of Jatropha protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J. Sci. Food Agric. 88, 911–919.
Gadir, A., Onsa, T.O., Ali, W.E.M., El Badwi, S.M.A., Adam, S.E.I., 2003. Comparative toxicity of *Croton macrostachys*, *Jatropha curcas*, *Piper abyssinica* seeds in Nubian goats. Small Ruminant Res. 48 (1), 61–67.
Gandhi, V.M., Cherian, K.M., Mulky, M.J., 1995. Toxicological studies on Ratanjyot oil. Food Chem. Toxicol. 33 (1), 39–42.
Goel, G., Makkar, H.P.S., Francis, G., Becker, K., 2007. Phorbol esters: structure, biological activity and toxicity in animals. Int. J. Toxicol. 26, 279–288.
Goonesekera, M.M., Gunawardana, V.K., Jayasena, K., Mohammed, S.G., Balasubramaniam, S., 1995. Pregnancy terminating effect of *Jatropha Curcas* in rats. J. Ethnopharmacol. 47, 117–123.
Gübitz, G.M., Mittelbach, M., Trabi, M., 1999. Exploitation of the tropical oil seed plant *Jatropha curcas* L. Bioresource Technol. 67, 73–82.
Haas, W., Strerk, H., Mittelbach, M., 2002. Novel 12 deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J. Nat. Prod. 65, 1434–1440.
Horiuchi, T., Suttajit, M., Suguri, H., Endo, Y., Shudo, K., Wongchai, V., Hecker, E., Fujiki, H., 1988. A new tumor promoter from the seed oil of *Jatropha curcas* L., an

intramolecular diesters of 12-deoxy-16-hydroxyphorbol. Cancer Res. 48 (20), 5800–5804.
Li, Q.X., Wang, H., Xiao, Q.Q., Kong, R., 1995. The evaluation and calculation of Median Lethal Dose (LD_{50}) using Bliss method. J. Math. Med. 4, 318–320.
Liberalino, A.A.A., Bambirra, E.A., Moraes-Santos, T., Viera, C.E., 1988. *Jatropha curcas* L. seeds. Chemical analysis and toxicity. Braz. Arch. Biol. Technol. 31, 539–550.
Makkar, H.P.S., Aderibigbe, A.O., Becker, K., 1998. Comparative evaluation of nontoxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem. 62 (2), 207–215.
Makkar, H.P.S., Becker, K., 2009. *Jatropha curcas* an exciting crop for generation of biofuel and value-added products. Eur. J. Lipid Sci. Technol. 11 (8), 773–787.
Makkar, H.P.S., Becker, K., Sporer, F., Wink, M., 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J. Agric. Food Chem. 45, 3152–3157.
Makkar, H.P.S., Martinez-Herrera, J., Becker, K., 2008. Variations in seed number per fruit, seed physical parameters and contents of oil, protein and phorbol ester in toxic and non-toxic genotypes of *Jatropha curcas*. J. Plant Sci. 3 (4), 260–265.
Makkar, H.P.S., Siddhuraju, P., Becker, K., 2007. A Laboratory Manual on Quantification of Plant Secondary Metabolites. Human Press, Totowa, New Jersey. p. 130.
Mariz, S.R., Arauojo, W.C., Cerqueira, G.S., Araujo, W.C., Duarte, J.C., Diniz, M.F.F.M., Medeiros, I.A., 2008. Avaliacao histopatologica em ratros apos tratamento agudo com o extrato etanolico de partes aereas de *Jatropha gossypiifolia* L. Braz. J. Pharmacog. 18 (2), 213–216.
Mariz, S.R., Cerqueira, G.S., Arauojo, W.C., Duarte, J.C., Melo, A.F.M., Santos, H.B., Oliveria, K., Diniz, M.F.F.M., Medeiros, I.A., 2006. Estudo toxicologixo agudo do extrato ethanolico de partes aereas de *Jatopha gossypiifolia* L. em ratos. Braz. J. Pharmacog. 16 (3), 372–378.
Odusote, O.M., Abioye, A.O., Rotib, M.O., 2002. *Jatropha curcas* seed oil Linn (Euphorbiaceae) contraceptive activity and on oral formulation. Nig. Quart. J. Hosp. Med. 12 (1–4), 44–47.
Oluwole, F.S., Bolarinwa, F., 1997. *Jatropha curcas* extract causes anemia in rat. Phytother. Res. 11, 538–539.
Rakshit, K.D., Darukeshwara, J., Rathina Raj, K., Narasimhamurthy, K., Saibaba, P., Bhagya, S., 2008. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. Food Chem. Toxicol. 46, 3621–3625.
Sun, R.Y., 1998. NDST (New Drug Statistic) Version 8.0. Wan-Nan Medical College, Wuhu, China.
The State Food and Drug Administration of People's Republic of China, 2005. Regulatory Guide on the Techniques for Drug Researches. Chinese Medical Science and Technology Press, Beijing. pp. 83–93.
Trebien, H.A., Neves, P.C.A., Yunes, R.A., Calixto, J.B., 1988. Evaluation of pharmacological activity of crude hydroalcoholic extract from *Jatropha elliptica*. Phytother. Res. 2 (3), 115–118.
Zhou, H.J., 1988. Statistical Methods for Biological Test. People's Medical Publishing House, Beijing. p. 214.

CHAPTER - 10.2

Occular and dermal toxicity of *Jatropha curcas* phorbol esters

Rakshit K. Devappa, Joy Roach, Harinder P.S. Makkar*, Klaus Becker

Institute for Animal Production in the Tropics and Subtropics, (480b), University of Hohenheim, Stuttgart, Germany.

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Abstract

Jatropha seeds are a promising feedstock for biodiesel production. Jatropha seed oil is toxic due to the presence of phorbol esters (PEs). The ever-increasing cultivation of Jatropha runs the risk of increased human exposure to Jatropha products. In the present study, *in vitro* studies on reconstituted human epithelium (RHE) and human corneal epithelium (HCE) were carried out to evaluate effects of toxic Jatropha oil, purified PEs-rich extract, purified PEs (factors C_1, C_2, $C_{3mixture}$ and (C_4+C_5)) and nontoxic Jatropha oil. The PEs are purified from toxic Jatropha oil. The results demonstrated severe cellular alterations such as marked oedema, presence of less viable cell layers, necrosis and/or partial tissue disintegration in epithelium and expression of inflammatory response (interleukin-1α and prostaglandin E_2 release) by several folds both in RHE and HCE. However, the nontoxic oil exhibited lesser cellular and inflammatory response compared to the toxic oil indicating the severity of toxicity due to PEs. Conclusively, Jatropha PEs are found to be toxic both towards RHE and HCE. The information obtained from the study would aid in the development of safety procedures in the Jatropha biodiesel industry. It is advised to use protective gloves and glasses when handling PEs containing Jatropha products.

1. Introduction

Jatropha curcas is a promising feedstock for biodiesel production (Makkar and Becker, 2009a). By 2015 it is projected to cultivate ~12.8 million hectares worldwide with an oil yield of 26 Mt/annum (GEXSI, 2008). During the biodiesel production, many coproducts such as nitrogen rich seed cake, phytochemicals, glycerol, among others could be obtained (Makkar and Becker, 2009a). The presence of toxic compounds limits the utilization of Jatropha based products. There are many reports describing the toxic effects of Jatropha seed, oil or extracts exhibiting toxicity towards higher animals (mice, rat, rabbit, sheep, among others), microorganisms, insects and humans (Devappa et al., 2010). In humans and animals the exposure to Jatropha based products are purely accidental or force fed (Devappa et al., 2010). In majority of the studies, oil soluble phorbol esters (PEs) were regarded as the active component responsible for toxicity in Jatropha. Generally, the PEs (mg/g dry matter) are distributed in most of the plant parts such as roots (0.55), bark (outer brown skin) (0.39), bark (inner green skin) (3.08), wood (0.09), stems (0.78–0.99), leaves (1.83–2.75), buds (1.18–2.10), flowers (1.39–1.83) and kernels (2–6). However, PEs were not found in the latex (Makkar and Becker, 2009a).

PEs are the most potent and toxic tigliane diterpenes commonly found in the genera *Anthosthema, Croton, Euphorbia, Ostodes, Jatropha, Sapium and Wikstroemia* (Xu et al., 2009). These tigliane diterpenes are diversely oxygenated and hydroxylated in various esterified forms. Most of the PEs found in plants differs in their hydroxylation patterns and in the stereochemistry of their ring systems. The phorbol 12-myristate 13-acetate (PMA; synonym: 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)) isolated from croton oil is the most studied among the PEs in the Euphorbiaceae family. Among the many biological activities of PMA studied (both *in vitro* and *in vivo*) tumour promotion activity is the most investigated one. The PEs promote tumour, following exposure of an initiator chemical such as methylcholanthrene (MCA) or 7,12-dimethylbenzanthracene (DMBA) (Goel et al., 2007). In principle, they act as analogues of diacyl glycerol (DAG), which is a secondary messenger in one of the main cellular signal transduction pathways. Generally, DAG is involved in the activation of enzyme protein kinase C (PKC) which further catalyses the phosphorylation of other proteins involved in signal transduction. The hyper activation of PKC by PEs (DAG analogue) targets multiple sites within a cell resulting in the uncontrolled differentiation of cells, which cumulatively results in the production of tumours (Kinzel et al., 1984; Goel et

al., 2007). In addition to tumour promotion, PEs exhibit wide range of other biochemical and cellular effects; for example they alter cell morphology, serve as lymphocyte mitogen, induce platelet aggregation, elevate cyclic GMP levels, stimulate ornithine decarboxylase and exhibit anti-leukemic activities. The PEs are also found to be involved in the modulation of inflammatory responses, inhibition of virus entry and effects on nociception (Goel et al., 2007).

However, all PEs are not toxic and their activity is strictly structure dependent. Generally, 'α' form of phorbol is inactive when compared to 'β' form (Goel et al., 2007). The position of OH group at ring D (Figure 1) makes phorbol active (β) or inactive (α). The (α) type phorbol have similar physiochemical properties and lipophilicity as that of (β) form, except that they cannot activate PKC due to conformational shifts (Silinsky et al., 2003). The computer model put forth by Jeffrey and Liskamp (1986) suggests the important structural features responsible for the biological activities of PEs includes the presence of (a) polar functional groups near O-3, O-4, O-9, O-20 of PMA, (b) free hydroxyl group at C-20, (c) no steric hindrance near 5 membered ring, and (d) hydrophobic moiety near C-20 (Goel et al., 2007). There are also non-tumour promoting PEs which have at least one of the biological activities of phorbol compounds such as binding to phorbol receptors, but are devoid of tumour promoting properties for example, 12-deoxyphorbol 13-acetate (prostratin), 12-deoxyphorbol 13-propanoate and 12-dexoxy phorbol 13-phenylacetate (Xu et al., 2009).

During Jatropha biodiesel production process, the PEs present in Jatropha oil are completely degraded. However, in early steps of transesterification PEs were still detected in the acid gums and in disposed water washings (Makkar et al., 2009b). This suggests that one should be cautious when using acid gums in animal feed and care is also needed in disposing of water washings containing PEs to avoid ecotoxicity. As the scale of Jatropha production increases, so does the risk of eco-toxicological effects/exposure of Jatropha based products. This has raised some concerns with respect to the consequences of occupational exposure of Jatropha oil (Gressel et al., 2008; Achten et al., 2008: Devappa et al., 2010). In spite of high production and plausible applications of Jatropha, knowledge on the potential impacts of toxic Jatropha oil on human health and environment is still fragmentary. In the present study, possible toxic effects of purified PEs from Jatropha oil were investigated using reconstructed human epithelium (RHE) and human corneal epithelium (HCE). These *in vitro* test methods (RHE and HCE) have been widely used for evaluating toxicity of chemicals. (Cotovio et al., 2007; Alepee et al., 2009; Kishore et al., 2009; Tornier et al., 2009; Goethem et al., 2006).

2. Material ad methods

2.1. Materials

Jatropha curcas seeds (toxic Indian variety) were collected from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. The phorbol 12-myristate-13-acetate (PMA) was obtained from Sigma Aldrich (St. Louis) and all other chemicals/solvents used were of analytical grade.

2.2. Extraction of Jatropha oil

Jatropha curcas seeds (both toxic and nontoxic genotypes) were mechanically pressed using a screw press to obtain oil and seed cake. The oil was centrifuged at 3150 *g* for 20 min to remove residues and the clear oil was collected and stored in a refrigerator (4 °C) until further use.

2.3. Quantification of PEs

The PEs were determined at least in triplicate according to Makkar et al. 2007, based on the method of Makkar et al. (1997). Briefly, 0.5 g of phorbol ester containing samples were extracted with 1−1.5 ml solvent (99 % methanol / 1 % THF) in a ball mill (Retsch MM200, 30 1/s) for 5 minutes. The supernatant (12,500 g for 3 min) was collected and concentrated by using pressurised air. Similarly, the residue was re-extracted 3 times; supernatant was pooled together, concentrated as above and made to a known volume (1 ml). A suitable aliquot was loaded into a high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 x 4 mm i.d., from Merck (Darmstadt, Germany) column and separation was carried out at 23°C. The flow rate was 1.3 ml/min using a gradient elution. The four phorbol ester peaks appeared between 25.5 and 30.5 min and were detected at 280 nm. The concentration was expressed equivalent to both phorbol 12-myristate-13-acetate (PMA) and *Jatropha* factor C_1 (Makkar et al. (2007).

2.4. Crude phorbol ester extraction

Jatropha oil was mixed with methanol (1:2, w/v) in a capped container at room temperature (60 °C) for 15 min using a magnetic stirrer (300 rpm). Thereafter, the mixture

was centrifuged at 3150 x *g* for 5 min to get upper methanolic and lower oily layers. Both the layers were separated. The oily layer was re-extracted 3 more times with the fresh solvent in a ratio of 1:1.5, 1:1 and 1:1 (w/v) respectively. The methanolic layers were pooled together and rotaevaporated (65 °C, 300 mbar) to get oily PEs enriched fraction (PEEF) (Devappa et al., 2010; for details see Chapter 3.).

2.5. Purification of PEs

In brief, the *Jatropha* PEs enriched fraction was subjected to column chromatography (Silica G-60) to get a concentrated PEs-rich extract (Jatropha factors C_1-C_5) (see Chapter 9 for details). The concentrated PEs-rich extract was fractionated by semi-preparative HPLC to obtain individual PEs, which were purified on a Sephadex LH-20 column. The purified PEs (factors C_1 and C_2) were confirmed by 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, TOCSY, HMBC) NMR and the data corresponded to data published by Haas et al. (2002). Whereas, factor C_3 and factors (C_4+C_5) were obtained as a mixture. Consequently, in this study the concentration for *Jatropha* factors used was expressed as *Jatropha* factor C_1 equivalents. The concentrated PEs-rich extract was also used in the study. PEs were stored in ethanol until further analysis at -80 °C.

2.5. Effect of phorbol esters on reconstructed human epidermis (RHE)

The Reconstructed human epidermis (RHE) model closely resembles human epidermis *in vivo* (Doucet et al., 1998). The RHE cultures were purchased from SkinEthic Laboratories (Nice, France). In brief, the fully differentiated RHE was cultured using primary keratinocytes isolated from human foreskin. The culture was carried out on inert microporous polycarbonate filter (0.5 cm^2) at the air–liquid interface for 17 days. The culture were maintained in chemically defined growth medium MCDB 153 (5 mg/ml insulin, 1.5mM calcium chloride, 25 mg/ml gentamycin, and 1 ng/ml epidermal growth factor (EGF) (Rosdy and Clauss, 1990). The work on skin epidermal tissue engineering has been previously described by Rosdy et al. (1997). The fully differentiated and stratified epidermis model obtained on day-17 consisted of main basal, supra basal, spinous and granular layers and a functional stratum corneum (Fartasch and Ponec, 1994; Kandárová, 2006a; Kandárová et al., 2006b). On day 17, tissues were transported from the firm to our laboratory on a nutritive agarose plate enclosed in an aluminium bag. Upon arrival, the tissues were transferred to another 6-well plates consisting of 1 ml growth culture medium. The plates

consisting RHE tissues were incubated (37°C, 5% CO_2 and 95% relative humidity) until test substance application. On day 19, the RHE tissues were transferred into another 24 well plate containing 300 µl pre-warmed maintenance culture medium. The maintenance medium and growth medium was shipped by the company along with the RHE. All the experimental procedure was carried out in sterile conditions. The experiment involves topical application of test materials to surface of the epidermis and subsequent assessment of their effects on histology and inflammatory substances. The test substances (test material, negative and positive controls) are topically applied on RHE tissue and exposed RHE for 42 minutes at room temperature (22 °C). After 42 minutes, the RHE tissue was rinsed with Dulbecco's Phosphate-Buffered Saline (D-PBS), mechanically dried and transferred into fresh medium and then incubated (37 °C, 5% CO_2 and 95% humidified atmosphere) for 42 additional hours.

The following test articles (10 µl) were applied topically on RHE tissues (i.e. 20 µl/cm²): toxic Jatropha oil, nontoxic Jatropha oil, purified PEs (factor C_1, C_2, $C_{3mixture}$ and (C_4+C_5)) and PEs-rich extract (factor C_1 to C_5 in the same proportion as they exist in the oil), 70% ethanol, 5% sodium dodecyl sulphate (5% SDS; positive control), phosphate buffered saline (PBS; negative control) and dimethylsulfoxide (DMSO; vehicle). The application of DMSO (10µl) acted as blank. The aforementioned PEs containing test articles and nontoxic jatropha oil were dissolved in DMSO and its (DMSO) concentration did not exceed 0.1% in the final application (10 µl). To ensure even spreading of test samples, a nylon mesh (8 mm diameter) was deposited onto the RHE tissues before application.

2.6. Effect of phorbol esters on reconstituted human corneal epithelium (HCE)

The HCE model was purchased from SkinEthic Laboratories (Nice, France). The model consists of immortalized human corneal epithelial cells cultured in a chemically defined medium and seeded on a synthetic membrane at the air–liquid interface. The tissue structure obtained was a multilayered epithelium (0.5 cm^2) resembling *in vivo* epithelium (Cotovio et al., 2007) representing about 5–7 cell layers. The HCE (age day 5 – 0.5 cm^2) was transported to our laboratory in a manner similar to the RHE. The inserts containing the HCE were transported in a multiwell plate filled with an agarose-nutrient solution in which they were embedded. The maintenance medium and growth medium was shipped by the Company along with the human tissue models. Upon arrival they were transferred to a new maintenance medium, 1 ml/well in 6 well plates and incubated (37°C and 5% CO_2) in a

humidified incubator. The medium was renewed after 24 h. The tissues were transferred to new 24 well plate containing maintenance medium (300 µl). The test samples (10 µl) were topically applied on a tissue and treated for 1 hour at room temperature. After treatment, the HCE tissues were rinsed three times with sterile D-PBS and the tissues were incubated with new maintenance medium (300 µl /well) for an additional 16-hour period in a humidified incubator (37 °C and 5% CO_2). The experiment was carried out in duplicate. The 0.1% DMSO in physiological saline, 5% SDS and PBS were used as vehicle-blank, positive control and as the negative controls respectively. The same set of test samples as used in Section 2.5 was taken for this evaluation.

2.7. Quality control of RHE and HCE tissues

Both RHE and HCE are prepared in accordance to the ISO9001 quality system of Skinethic Laboratories. According to SkinEthic's quality control criteria, functionality model conditions (viability, barrier function, and histology) were evaluated on each RHE and HCE batches. Both RHE and HCE used in our study were viable and did not show any significant morphological alterations in histology of tissue sections.

2.8. IL-1α and PGE_2 measurements

After incubation (42 h for RHE (Section 2.5) and 16 h for HCE (Section 2.6)), the subnatant underneath the treated RHE and HCE tissues were homogenised by agitating at 300 rpm and separately stored (-20°C) to measure the interleukin and prostaglandin release. The growth medium used for the experiment was also frozen (control). Release of IL-1α was quantified in the RHE and HCE subnatant culture medium by an ELISA (DLA-50, Quantikine, Minneapolis, USA) kit. Similarly, the PGE_2 was quantified in the RHE and HCE subnatant culture medium by an ELISA kit (KGE004B, R&D Systems, Abingdon, UK). The analysis of IL-1α and PGE_2 was carried out as mentioned in the respective manufacturer's kit.

2.9. Histological evaluation of RHE and HCE

For histological studies, the polycarbonate filter was carefully cut out from its plastic ring with a sharp scalpel and transferred immediately into a vial containing 2 ml of buffered formaldehyde 4% (w/v). The vials were tightly capped and sent to SkinEthic Laboratories for histopathological evaluation. In brief, the tissues were embedded in paraffin and the

sections (3-5µm) were stained with haematoxylin and eosin (H&E stain). The stained sections were observed for histological changes under microscope (20X magnification).

3.0. Results and discussion

In the Jatropha biodiesel industry, the accidental contact of workers with Jatropha based products (especially when using oil or extracts from Jatropha products containing PEs) could occur while working. The evaluation of irritational potential for these products is essential in order to develop strategies to prevent their exposure and to develop safety measures. For many years, the tests on animals has played important role in regulatory research. However, the experiments on animals give rise to ethical dilemma and thus the increased use, lately of the alternative animal testing methods such as *in vitro* predictive methods which generally mimic the *in vivo* test systems.

The skin epithelium and corneal epithelium are the primary point of contact for a toxic material (Huhtala et al., 2008). Therefore, we have chosen the RHE and HCE models, which mimic the human skin and corneal epithelium respectively to test the toxicity of the PEs. In addition, release of inflammatory substances such as interleukins and prostaglandins was also evaluated. Interleukins are cytokines, whose production is increased in response to stimuli produced by inflammatory agents, toxins or infections (Dinarello, 1991) and they are not generally produced by cells of healthy individuals with the exception of epithelial cells, keratinocytes and cells from central nervous system (Dinarello, 1991; Oppenheim, 1986; Dinarello, 1993). The cytokine IL-1α is present in the cytoplasm of keratinocytes of all epidermal layers. The IL-1α is only released from leaky cells following cell injury or membrane perturbation (Dinarello, 1998). The passively released IL-1α induces inflammatory cascade by the expression of IL-6 and IL-8. Further, it activates phospholipase A2 (PLA2) which is a key enzyme in the arachidonic acid cascade, resulting in the production of PGE_2 (Luger, 1989; Nickoloff and Naidu, 1994; Corsini and Galli, 1998; Terry et al., 1999). The production of PGE_2 has been observed in a wide variety of tissues and their increased secretion was observed during pathological conditions including inflammation and many cancers. In our study, the Jatropha oil used for the isolation of PEs had 0.11 mg/g of PEs, equivalent to *Jatropha* factor C_1 (4.6 mg/g of PEs, equivalent to PMA).

3.1. Effect of phorbol esters on Reconstituted human epidermis (RHE)

The RHE treated with the vehicle solvent (DMSO) did not show any significant histological changes (Data not shown). Whereas, positive control (5% SDS) used in the experiment exhibited severe alterations in the RHE such as tissue necrosis, marked oedema, less viable cells layers or partial tissue disintegration. These changes were also observed when toxic oil containing 2.42 ng of PEs (*Jatropha* factor C_1 equivalent), PEs-rich extract (factor C_1 to C_5) containing 2.42 ng of PEs, or purified individual PEs [C_1 (1.3 ng), C_2 (0.5 ng), $C_{3mixture}$ (0.4 ng) and $C_4 + C_5$ (0.25 ng); these are the amounts of individual PEs in 2.42 ng of a mixture of PEs as present in the oil] were applied on RHE. The results suggest that PEs are highly active and upon topical application cause tissue damage. Whereas, 70% ethanol (a commonly used hand sanitizer) treated RHE exhibited absence of significant histological changes, slight cellular alterations in the upper layer and partial tissue necrosis in one area of the sample was observed. Similarly, the Jatropha nontoxic oil (equivalent to toxic oil, v/v) exhibited slight epidermal changes, absence of significant histological changes in the viable cell layers and slight modifications of the stratum corneum.

Upon tissue damage RHE is reported to express IL-1α and PGE_2 (Tomaino et al., 2006; Hsia et al., 2008). The PBS and DMSO produced 30 and 35 pg/ml for IL-1α respectively; and expressed 4.9 ng/ml and 5.13 ng/ml of PGE_2 respectively. The application of 70% ethanol (10 µl) on RHE produced 42 pg/ml and 4.67 ng/ml of IL-1α and PGE2 respectively. Generally, the hand sanitizers contain 60−70% alcohol. Similarly, Ahn et al. (2010) reported that the alcoholic hand sanitizer containing plant extracts were non irritant to skin and the cell viability test results were comparable both in *in vitro* and *in vivo*. However, they observed the increased expression of IL-1α. In our study, there was slight increase (1.35 folds) in the IL-1α production by 70% ethanol treated RHE when compared to with PBS treated RHE (1 fold). Further the 70% treated RHE was compared with RHE treated with test samples. Considering the IL-1α (42 pg/ml) and PGE_2 (4.7 ng/ml) production of 70% treated RHE as baseline, the IL-1α production by SDS, toxic oil; purified PEs-rich extract (factor C_1-C_5); C_1, C_2, $C_{3mixture}$ and (C_4+C_5) was higher by 8.9, 2.8, 3.2, 1.8, 2.3, 1.3 and 1.9 folds; and PGE_2 production increased by 1.42, 1.27; 1.66; 1.42, 1.05, 1.50 and 1.60 folds respectively. The histological alterations and production of IL-1α (1.2 fold) and PGE_2 (1.02 fold) were less evident by the nontoxic oil (48.73 pg/ml and 4.77 ng/ml) and these effects were comparable to those produced by 70% ethanol. The epidermal keratinocytes are reported to express inflammatorty mediators (such as IL-1α) upon inflammation (Slivka and Zeigler, 1993).

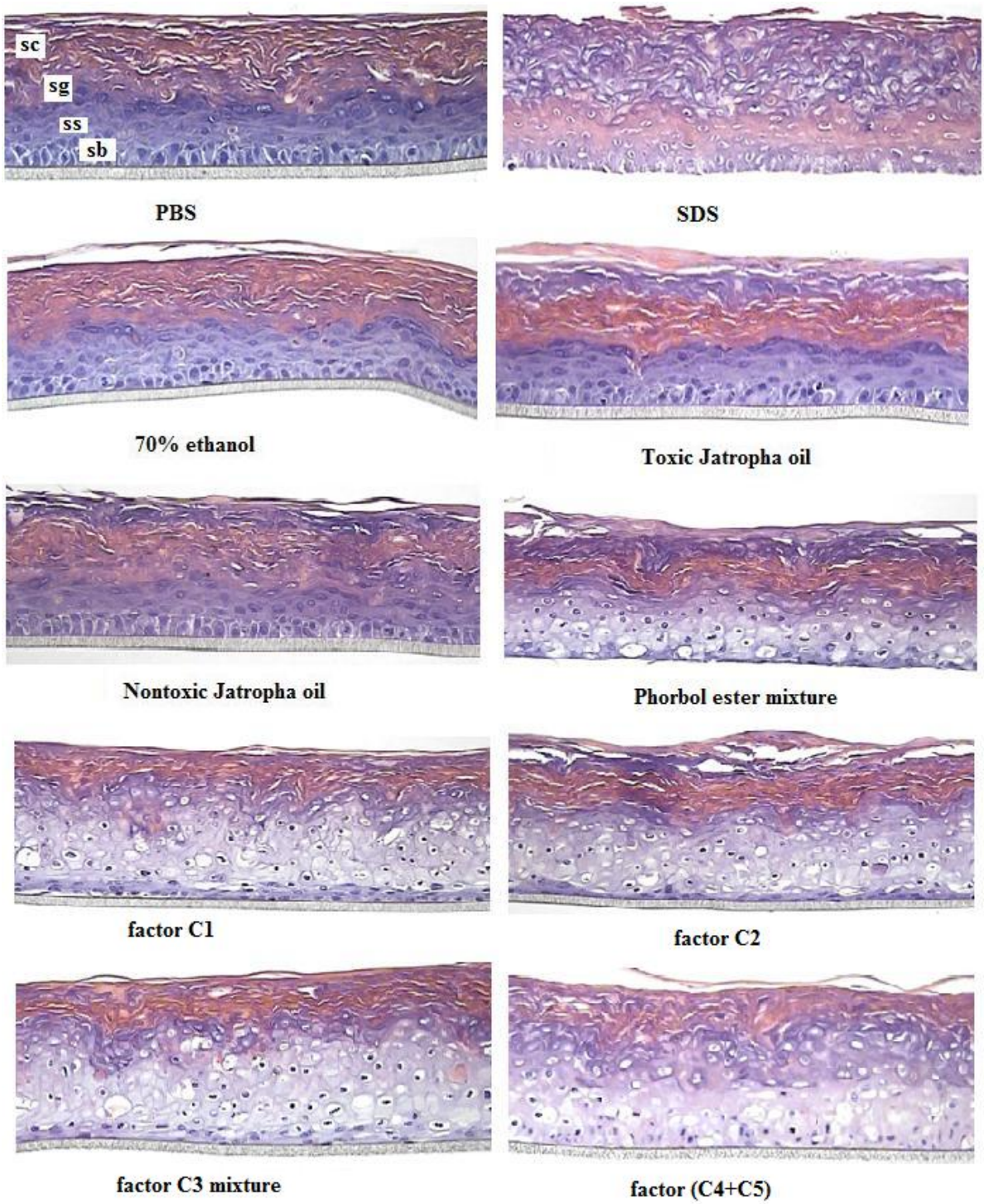

Figure 1. The histology sections of reconstructed human epidermis (RHE) (sc, stratum corneum; sg, stratum granulosum; ss, stratum spinosum; sb, stratum basale) treated with phosphate buffer saline (PBS), Sodium dodecyl sulphate (5% SDS),70% ethanol, toxic Jatropha oil, nontoxic Jatropha oil, PEs-rich extract and Jatropha purified PEs (factor C_1, C_2, $C_{3mixture}$ and (C_4 +C_5)).

In another study, PEs (PMA) induced PGE_2 release in human keratinocytes culture even at non-cytotoxic concentrations (Lawrence and Benford, 1995). Overall, our data from

histological studies suggest that PEs from *J. curcas* are highly bioactive and their *in vitro* topical application on skin induce the production of cutaneous inflammatory substances and cellular alterations. The Jatropha PEs are easily soluble in petroleum ether, methanol, hexane, dichloromethane and they can be extracted using these solvents (unpublished, our observation). The exposure to these PEs containing extracts exhibit similar histological changes *in vivo* as observed in our studies. The petroleum ether extract from Jatropha toxic oil was applied topically on shaved dorsal skin (*in vivo*) of rabbit, mice and rats. In rabbit, the petroleum ether extract (100 µl) produced marked erythema and oedema, which later became necrotic and regenerated. In mice, the extract (50 µl) produced hemorrhagic eyes, swelling of the face, diarrhoea and skin erythema before death. In rats, the extract (50 µl) exhibited edema and erythema, which later formed scaling and thickening of skin. The cellular infiltration in the upper epidermis, parakeratosis and thickening of stratum corneum were also noted. The PEs were suggested to be the active component in the petroleum ether extract (Gandhi et al., 1995). Horiuchi et al. (1987) reported that methanol extract from *J. curcas* oil exhibited tumour promoting properties in mice skin upon initiation by 7, 12-dimethylbenz[a]anthracene (DMBA). Mice (36%) displayed tumours in 30 weeks. They also reported that PEs are the major irritant principle in oil and responsible for tumour promotion. In addition, a Jatropha PEs, DHPB (which was renamed later by Haas et al. (2002) as factor C_1) was reported to be more toxic but weak tumour promoter than the widely studied and potent tumour promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA or PMA). The application of 34 nmol of DHPB induced ornithine decarboxylase in mouse skin (2.8 nmol CO_2/30 min/mg protein) and activated PKC *in vitro* (50% effective dose being 36.0 nM). DiGiovanni et al. (1988) reported that the events that best correlate with TPA's tumour promoting properties in skin are the induction of epidermal hyperplasia, dark basal keratinocytes, dermal inflammatory response and induction of epidermal ornithine decarboxylase activity which is followed by increased polyamine levels.

3.2. Effect of phorbol esters on human corneal epithelium (HCE)

The HCE treated with the vehicle chemical (0.1% DMSO in physiological saline) showed moderate cellular alterations mainly located in upper layer of the cells and partial tissue necrosis in some area of HCE. Sakaguchi et al. (2011) have reported that the vehicle (0.1% DMSO in physiological saline) used did not affect cell viability (>92% viability) even at 0.5% level. The positive control (5% SDS) used in our study exhibited severe alterations and

complete disintegration of the HCE tissue. Similar results were observed when 0.5% and 1.0% SDS were tested on the HCE (Seaman et al., 2010) wherein the SDS, exhibited time and dose dependent increase in causing injury to the HCE and the treatment with 1% SDS for 10 min found to decrease cell viability to 52% (Seaman et al., 2010).

In our study, we used higher concentration of SDS (5%) and that resulted in complete disintegration of tissue (histology picture not shown). Except tissue necrosis in some area of the tissue and slight epidermal changes, the PBS (negative control) treated HCE showed absence of significant histological changes. At 2.42 ng equivalent of PEs, both toxic Jatropha oil and PEs-rich extract (all 5 factors in the proportion present in the oil) elicited severe alterations in some areas such as marked cellular alterations/less viable cell layers, partial tissue necrosis and/or partial tissue disintegration of supra basal cell layers were observed in the HCE. Similar effects were observed when purified individual PEs [C_1 (1.3 ng), C_2 (0.5 ng), $C_{3mixture}$ (0.4 ng) and C_4+C_5 (0.25 ng); these are the amounts of individual PEs in 2.42 ng of a mixture of PEs as present in the oil] were tested. Although the severity of toxicity was less compared to the toxic oil and PEs, the nontoxic oil (equivalent to volume used for toxic oil) did exhibit alterations (necrosis) in some areas and marked cellular alterations. The results indicate that the Jatropha oil even if it is nontoxic causes damage to corneal epithelial cells and the severity of toxicity increases with the presence of PEs. In addition, all the PEs caused tissue damage in the HCE.

The HCE model is reported to express IL-1α upon exposure to chemicals (Seaman et al., 2010; Huhtala et al., 2008). In our study, the subnatants (the medium collected after treatment with HCE) were also evaluated for inflammatory substances (IL-1α and PGE_2). Application of PBS (10 µl) on the HCE produced 30.2 pg/ml IL-1α and 5.3 ng/ml PGE_2 (these being the baseline values). When compared to PBS, the IL-1α production in the tested samples increased by several folds - 0.1% DMSO (1.6 fold), SDS (9.7 fold), toxic oil (5.8 fold), nontoxic oil (3.8 fold), purified PEs-rich extract (6.9 fold), C_1 (4.0 fold), C_2 (5.2 fold), $C_{3mixture}$ (4.6 fold) and (C_4+C_5) (3.7 fold)). The DMSO is reported to be irritant (Conquet et al., 1977) and its use as a vehicle in our study even at 0.1% might have caused slight cellular alterations as well as increase in the inflammatory response. The order of toxicity observed was SDS > PEs-rich extract > toxic oil > C_2 > $C_{3mixture}$ > C_1 > nontoxic oil > (C_4+C_5) > vehicle (0.1% DMSO). However, the PGE_2 production in HCE could not be conclusively correlated with the test samples as there was high variability between the treated HCE cultures (data not shown). As far as our knowledge goes, there are no published data on the

effect of Jatropha PEs on corneal epithelium. However, the TPA was found to stimulate a receptor mediated active ion transport (Cl- transport) in the corneal epithelium and suggested the involvement of PKC in the regulation of membrane Cl- transport in the mammalian corneal epithelium (Crosson et al., 1986). Similarly, Watsky and Guan et al. (1997) have reported the PEs to be harmful to the corneal endothelial function resulting in increased endothelial permeability, cellular changes in endothelial cells and swelling of cornea. They also suggested that these effects are mediated by PKC pathway. Wink et al. (2000) have reported that DHPB isolated from Jatropha (renamed later by Haas et al. (2002) as factor C_1) and TPA increased the PKC activity *in vitro*.

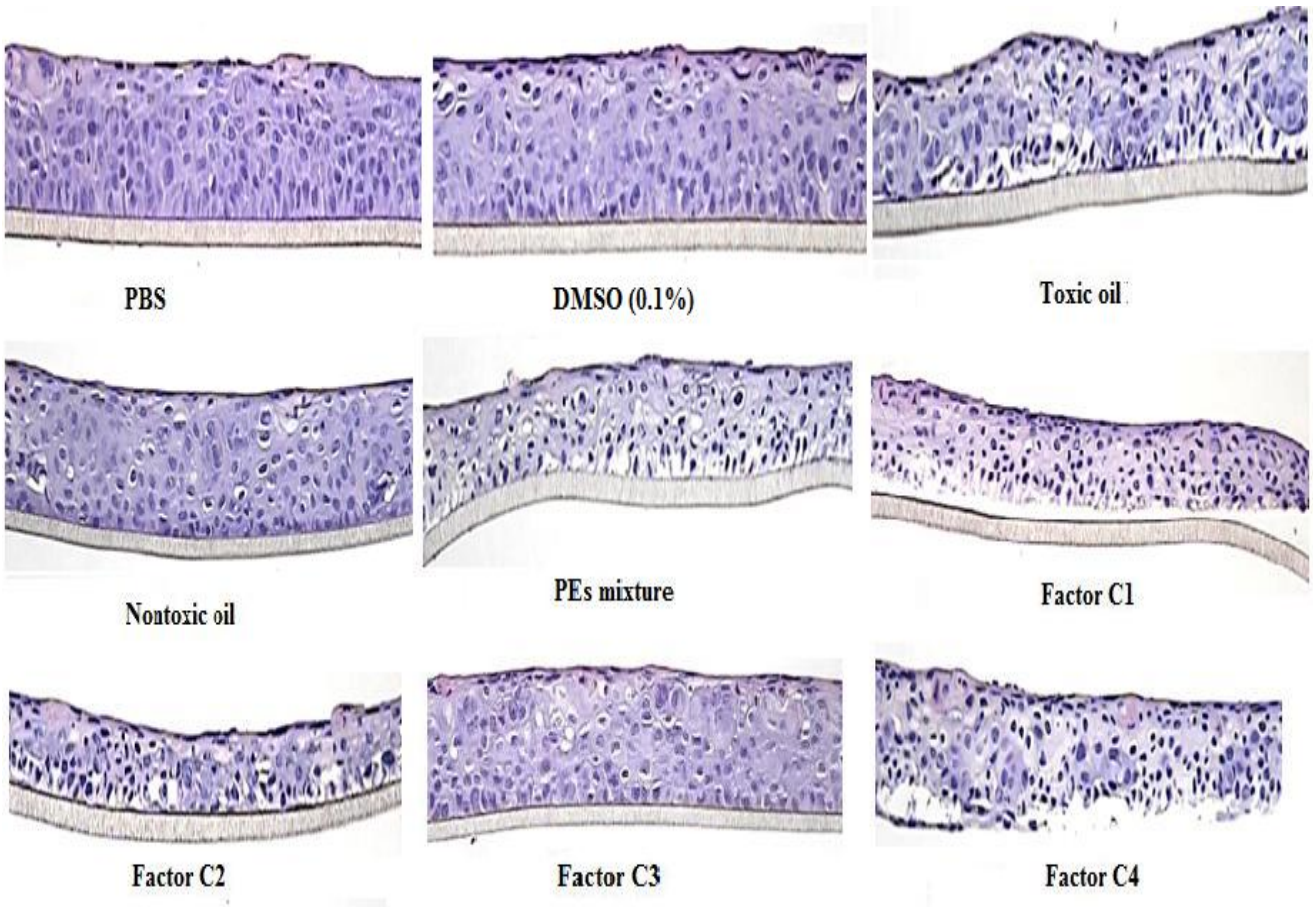

Figure 2. The histology sections of human corneal epithelium (HCE) treated with phosphate buffer saline (PBS), vehicle chemical (DMSO 0.1%), toxic Jatropha oil, nontoxic Jatropha oil, PEs-rich extract and Jatropha purified PEs (factor C_1, C_2, $C_{3mixture}$ and (C_4 +C_5)).

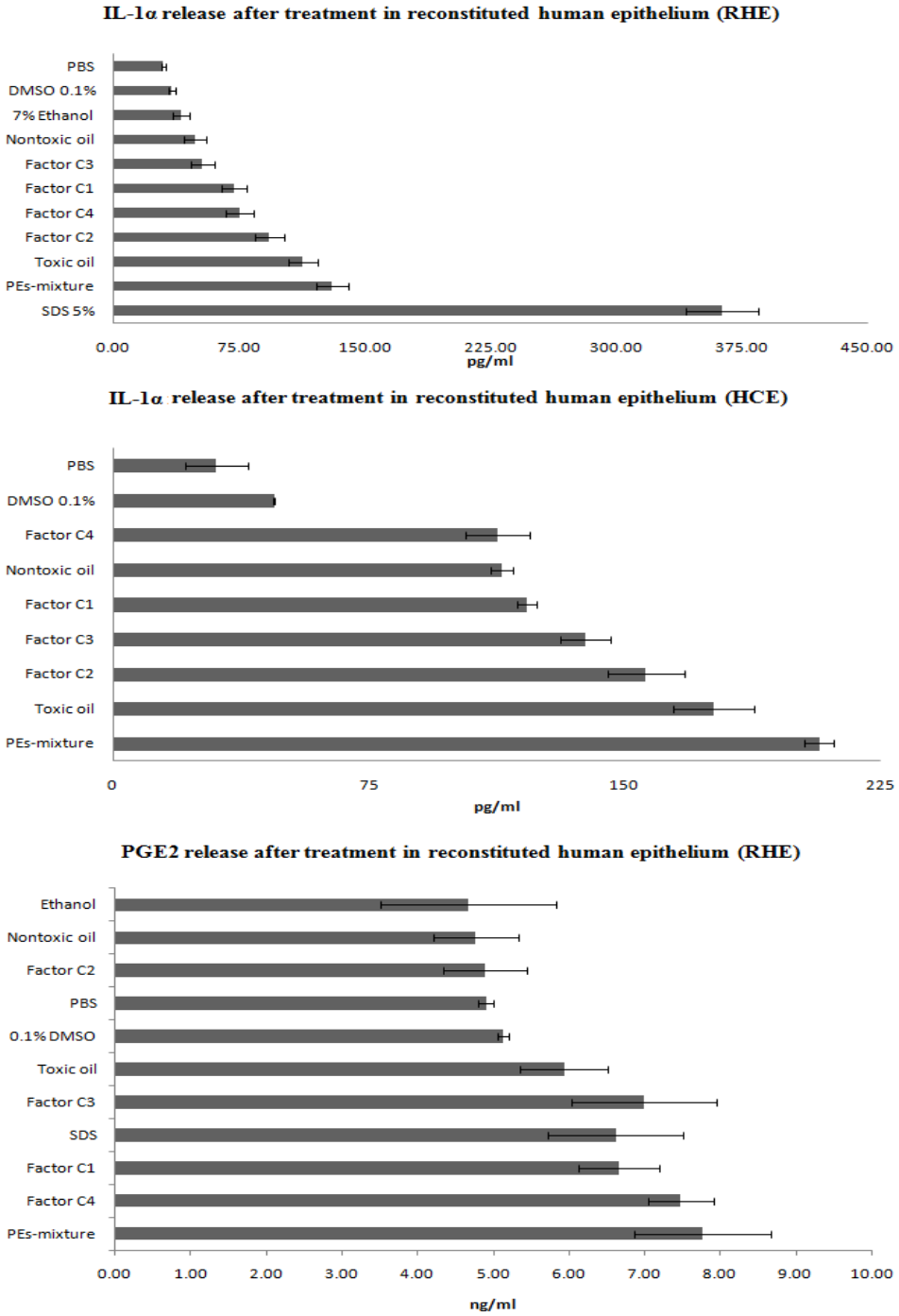

Figure 3. Effect of phorbol esters on IL-1α and PGE_2 release in the incubation medium of RHE and HCE samples after exposing to different samples; toxic Jatropha oil (2.42 ng phorbol esters mixture equivalent), nontoxic Jatropha oil (equivalent to toxic Jatropha oil, v/v), PEs-rich extract ((2.42 ng) containing factor C_1 to C_5), purified individual PEs [factor C_1 (1.3 ng), factor C_2 (0.5 ng), factor $C_{3mixture}$ (0.4 ng) and factor C_4+C_5 (0.25 ng); these are the amounts of individual PEs present in 2.42 ng of a mixture of PEs as present in the toxic oil], 5% SDS (positive control), phosphate buffer saline (PBS), vehicle (0.1% DMSO). Data are expressed as mean ± SD (n=4, $P < 0.05$).

Overall, in our study the results indicate that the presence of PEs in oil increases its toxicity (upon exposure) towards tissues (both RHE and HCE) and we presume that the activation of PKC could be one of the mode of action through which Jatropha PEs exhibits cellular alterations and inflammatory response.

4. Conclusions

Jatropha based products may contain PEs which are toxic principles. Upon topical exposure to reconstructed human epidermis and human corneal epithelium, the toxic Jatropha oil exhibited severe histological alterations and inflammatory responses; while these responses were minor with the nontoxic Jatropha oil. Similarly, the purified PEs also elicited severe histological alterations and inflammatory responses, suggesting their role in Jatropha toxicity. Thus, the direct contact with toxic Jatropha oil or phorbol ester containing Jatropha products should be avoided. It is advised to use the protective gloves and glasses when handling phorbol ester containing Jatropha products.

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References

1. Achten WMJ, Verchot L, Franken YJ, Mathijs E, Singh VP, Aerts R, Muys B. 2008. *Jatropha curcas* bio-diesel production and use. Biomass Bioenerg 32:1063–1084.
2. Ahn JH, Eum KH, Kim YK, Oh SW, Kim YJ, Lee M. Assessment of the dermal and ocular irritation potential of alcohol hand sanitizers containing aloe vera with *in* vitro and *in vivo* methods. Mol Cel Toxicol 6:397−404.
3. Alépée N, Tornier C, Robert C, Amsellem C, Roux MH, Doucet O, Pachot J, Méloni M, de Brugerolle de Fraissinette A. 2009. A catch-up validation study on reconstructed human epidermis (SkinEthic RHE) for full replacement of the Draize skin irritation test. Toxicol In Vitro 24:257–66.

4. Conquet P, Durand G, Laillier J, Plazonnet B. 1977. Evaluation of ocular irritation in the rabbit: objective versus subjective assessment. Toxicol Appl Pharmacol 39:129–139.
5. Corsini E, Galli CL. 1998. Cytokines and irritant contact dermatitis. Toxicol Lett 102–103:277–282.
6. Cotovio J, Grandidier MH, Lelièvre D, Bremond C, Flamand N, Loisel-Joubert S, Van Der Lee A, Capallere C, Meunier JR, Leclaire J. 2007. The use of the reconstructed Human Corneal Model (HCE) to assess in vitro eye irritancy of chemicals. AATEX 14, Special Issue, 343–350. Proc. 6th World Congress on Alternatives & Animal Use in the Life Sciences
7. Crosson CE, Klyce SD, Bazan HEP, Bazan NG. 1986. The effect of phorbol esters on the chloride secreting epithelium of the rabbit cornea. Curr Eye Res *5*:535–541.
8. Devappa RK, Makkar HPS and K. Becker. 2010. Optimization of conditions for the extraction of phorbol esters from Jatropha oil. Biomass and Bioenerg 34:1125−1133.
9. Devappa RK, Makkar HP, Becker K. 2010. Jatropha toxicity-a review. J Toxicol Environ Health B Crit Rev 13:476–507.
10. DiGiovanni J, Kruszewski FH, Coombs MM, Bhatt TS, Pezeshk A. 1988. Structure-activity relationships for epidermal ornithine decarboxylase induction and skin tumour promotion by anthrones. Carcinogen 9:1437–1443.
11. Dinarello CA, Wolff SM. 1993. New Engl J Med 328:106.
12. Dinarello CA. 1991. Interleukin-1 and interleukin-1 antagonism. Blood 77:1627.
13. Dinarello CA. 1998. Interleukin-1, interleukin-1 receptor and interleukin-1 receptor antagonist. Intern Rev Immunol 16:457–499.
14. Doucet O, Garcia N, Zastrow L. 1998. Skin culture model: a possible alternative tothe use of excised human skin for assessing *in vitro* percutaneous absorption. Toxicology in Vitro 12:423–430.
15. Fartasch M, Ponec M. 1994. Improved barrier structure formation in air-exposed human keratinocyte culture systems. J Invest Dermatol 102: 366–374.
16. Gandhi VM, Cherian KM, Mulky MJ. 1995. Toxicological studies on ratanjyot oil. Food Chem Toxicol 33:39–42.
17. GEXSI. 2008. Available at http://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf

18. Goel G, Makkar HPS, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 26:279–288.
19. Goethem VF, Adriaens E, Alépée N, Straube F, De Wever B, Cappadoro M, Catoire S, Hansen E, Wolf A, Vanparys P. 2006. Prevalidation of a new in vitro reconstituted human cornea model to assess the eye irritating potential of chemicals. Toxicol In Vitro 20:1–17.
20. Gressel J. 2008. Transgenics are imperative for biofuel crops. Plant Sci 174:246–263.
21. Haas W, Strerk H, Mittelbach M. 2002. Novel 12 deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J Nat Prod 65:1434–1440.
22. Horiuchi T, Fujiki H, Hirota M, Suttajit M, Suganuma M, Yoshioka A, Wongchai V, Hecker E, Sugimura T. 1987. Presence of tumour promoters in the seed oil of *Jatropha curcas* L. from Thailand. Jpn J Cancer Res 78:223–226.
23. Hsia E, Johnston MJ, Houlden RJ, Chern WH, Hofland HE. 2008. Effects of topically applied acitretin in reconstructed human epidermis and the rhino mouse. J Invest Dermatol 128:125–30.
24. Huhtala A, Salminen L, Tähti H, Uusitalo H. 2008. Corneal Models for the Toxicity Testing of Drugs and Drug Releasing Materials. Topics in Multifunctional Biomaterials and Devices, Ed. N Ashammakhi © 2008. http://www.oulu.fi/spareparts/ebook_topics_multifunctional/abstracts/huhtala.pdf
25. Jeffrey AM, Liskamp RMJ. 1986. Computer-assisted molecular modelling of tumour promoters: rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin. Proc Natl Acad Sci USA 83:241–245.
26. Kandárová H, Liebsch M, Schmidt E, Genshow E, Traue D, Meyer K, Spielmann H, Steinoff C, Tornier C, De Wever B, Rosdy M. 2006a. Assessment of the skin irritation potential of chemicals by using the SkinEthic reconstructed human epidermal model and the common skin irritation protocol evaluated in the ECVAM skin irritation study. Alt Lab Anim 34:393–406.
27. Kandárová H, Liebsch M, Spielmann H, Genshow E, Schmidt E, Traue D, Guest R, Whittingham A, Warren N, Gamer AO, Remmele M, Kaufmann T, Wittmer E, De Wever B, Rosdy M. 2006b.Assessment of the human epidermis model SkinEthic RHE for in vitro skin corrosion testing of chemical according to new OECD TG431. Toxicol In Vitro 20:547–59.

28. Kinzel V, Richards J, Goerttler K, Loehrke H, Furstenberger G, Marks F. 1984. Interaction of phorbol derivatives with replicating cells. IARC Sci Publ 56:253–264.
29. Kishore AS, Surekha P, Murthy PB. 2009. Assessment of the dermal and ocular irritation potential of multi-walled carbon nanotubes by using in vitro and in vivo methods. Toxicol Lett 191:268–74
30. Lawrence JN, Benford DJ. 1995. Comparison of tumour promoter-induced prostaglandin E2 release in human and rat keratinocytes. Carcinogen 16:1247–51.
31. Luger TA. 1989. Epidermal cytokines. Acta Derm Venereol Suppl (Stockh) 151, pp. 61–76 discussion 106–110 .
32. Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 45:3152−3157.
33. Makkar HPS, Becker K. 2009b. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J lipid Sci Technol 111:773–787.
34. Makkar HPS, Siddhuraju P and Becker K, A laboratory manual on quantification of plant secondary metabolites, Humana press. Totowa, New Jersey, pp. 130 (2007).
35. Makkar HPS, Maes J, De Greyt W, Becker K. 2009a. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J Am Oil Chem Soc 86:173–181.
36. Nickoloff BJ, Naidu Y. 1994. Pertubation of epidermal barrier function correlates with initiation of cytokine cascade in human skin. J Am Acad Dermatol 30:535–546.
37. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. 1986. There is more than one interleukin 1. Immunol Today 7:45–56.
38. Rosdy M, Bertino B, Butet V, Gibbs S, Ponec M, Darmon M. 1997. Retinoic acid inhibits epidermal differentiation when applied topically on the stratum corneum of epidermis formed *in vitro* by human keratinocytes grown on defined medium. In Vitro Toxicol 10:39–47.
39. Rosdy M, Clauss LC. 1990. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. J Invest Dermatol 95: 409–414.
40. Sakaguchi H, Ota N, Omori T, Kuwahara H, Sozu T, Takagi Y, Takahashi Y, Tanigawa K, Nakanishi M, Nakamura T, Morimoto T, Wakuri S, Okamoto Y, Sakaguchi M, Hayashi T,Hanji T, Watanabe S. 2011. Validation study of the

Short Time Exposure (STE) test to assess the eye irritation potential of chemicals. Toxicol In Vitro 25:796–809.

41. Seaman CW, Whittingham A, Guest R, Warren N, Olson MJ, Guerriero FJ, Adriaens E, De Wever B. 2010. An evaluation of a cultured human corneal epithelial tissue model for the determination of the ocular irritation potential of pharmaceutical process materials. Toxicol In Vitro 24:1862–1870.
42. Silinsky EM, Searl TJ. 2003. Phorbol esters and neurotransmitter release: more than just protein kinase C? British Journal of Pharmacology 138:1191–1201.
43. Slivka SR, Zeigler F. 1993. Use of an *in vitro* skin model for determining epidermal and dermal contributions to irritant responses. Cut Ocul Toxicol 12:49–57.
44. Terry, CM, Clikeman JA, Hoidal JR, Callahan KS. 1999. TNF-alpha and IL-1alpha induce heme oxygenase-1 via protein kinase C, Ca2+, andphospholipase A2 in endothelial cells. Am J Physiol 276(5 Pt 2):H1493–501.
45. Tomaino A, Cristani M, Cimino F, Speciale A, Trombetta D, Bonina F, Saija A. 2006. *In vitro* protective effect of a Jacquez grapes wine extract on UVB-induced skin damage. Toxicol *In Vitro* 20:1395–1402.
46. Tornier C, Amsellem C, Fraissinette Ade B, Alépée N. 2010. Assessment of the optimized SkinEthic Reconstructed Human Epidermis (RHE) 42 bis skin irritation protocol over 39 test substances. Toxicol In Vitro 24:245–56.
47. Watsky MA, Guan Z. 1997. Phorbol ester modulation of rabbit corneal endothelial permeability. Invest Ophthalmol Vis Sci 38:2649–2654.
48. Wink M, Grimm C, Koschmieder C, Sporer F, Bergeot O. 2000. Sequestration of phorbolesters by the aposematically coloured bug *Pachycoris klugii* (Heteroptera: Scutelleridae) feeding on *Jatropha curcas* (Euphorbiaceae). Chemoecology 10:179–184.
49. Xu R, Zhao W, Jiang C. 2009. Ester prodrugs of prostratin and related phorbol compounds. United States Patent Application 2009016358.

CHAPTER -11

In vitro tumour promotion studies on *Jatropha curcas* phorbol esters

Rakshit K. Devappa[a], Joy Roach[a], Giridhar Kanuri[b], Harinder P.S. Makkar[a]*, Klaus Becker[a]

[a]*Institute for Animal Production in the Tropics and Subtropics, (480b), University of Hohenheim, Stuttgart, Germany.*

[b]*Department of Nutritional Medicine, University of Hohenheim, D-70599 Stuttgart, Germany*

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Abstract

Jatropha seed oil is a potential feedstock for biodiesel production. The oil is toxic due to the presence of phorbol esters (PEs). The increasing Jatropha cultivation runs the risk of increased human exposure to Jatropha products. In the present study, *in vitro* tumour promotion studies were carried out to evaluate whether all the phorbol esters present in Jatropha oil are tumour promoters or not. The PEs are purified from toxic Jatropha oil. The purified Jatropha PEs (factor C_1, C_2, $C_{3mixture}$, (C_4+C_5) and PEs-rich extract (containing factors C_1–C_5) were evaluated in *in vitro* cell transformation tests (Bhas 42 cells). The PEs-rich extract (0.00005–2 µg/ml) and individual purified Jatropha PEs (factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5)) (0.0005–1 µg/ml) exhibited tumour promoting properties by forming statistically significant increase in the transformed foci with the increase in concentration. The order of transformed foci/well formation was PEs-rich extract > factor (C_4+C_5) > factor $C_{3mixture}$ > factor C_1 > factor C_2. However, all the Jatropha PEs did not exhibit tumour initiation properties. In addition, PEs-rich extract (2 µg), factor C_1 (1 µg), factor C_2 (1 µg), factor $C_{3mixture}$ (1 µg) and factor (C_4+C_5) (1 µg) induced the hyper activation of PKC. Conclusively, all the Jatropha PEs in purified form exhibited tumour promotion activity *in vitro*. The tumour promotion activity was mediated by the activation of PKC. The use of protective gloves to avoid direct contact with Jatropha oil or Jatropha based products/coproducts containing PEs is recommended.

1. Introduction

Phorbol esters (PEs) are group of tigliane diterpenes which are oxygenated and hydroxylated in various esterified forms. They are commonly found in the plants belonging to Euphorbiaceae family. The first phorbol ester was identified from croton oil (phorbol myristate 13-acetate (PMA); synonym: 12-O-tetradecanoylphorbol-13-acetate (TPA)) and it is the most widely studied among a variety of PEs present in the whole Euphorbiaceae family. The TPA exhibits multitude of biological activities *in vitro* and *in vivo* (Goel et al., 2007). The most noted biological activity of TPA is its ability to promote tumour following the exposure of initiator chemicals such as 7, 12-dimethylbenzanthracene (DMBA) or methylcholanthrene (MCA). The tumour promoting properties have also been reported for PEs other than TPA and this activity is mediated through enzyme protein kinase C (PKC), which is involved in the phosphorylation of other proteins in the signal transduction. Generally, PKC is activated by diacyl glycerol (DAG), which acts as a secondary messenger in the signal transduction pathways. Phorbol esters such as TPA or other active PEs act as an analogue of DAG. The TPA binds to C1 domain of PKC and activates its kinase activity. Among the many roles of PKC, it regulates proliferation and differentiation process in the cells. Thus, tumour promoting activities of PEs are generally attributed to PKC activation. The hyper activation of PKC by PEs causes uncontrolled or undesired regulation of PKC (e.g. by TPA) leading to mis-regulation of cell proliferation. These processes cumulatively results in the production of tumours (Kinzel et al. 1984; Silinsky and Searl, 2003). There are also non PKC enzyme receptors for PEs which include (a) chimaerins, (b) RasGRP, (c) *Caenorhabditis elegans*, Unc-13 and mammalian Munc13s (Kazanietz, 2000).

In addition to tumour promotion, PEs alter cell morphology, acts as a lymphocyte mitogen, induce platelet aggregation, elevate cyclic GMP levels, modulate inflammatory response, affect nociception and exhibit antileukemic activity, among others (Goel et al., 2007). However, all PEs are not toxic and their biological activity depends on their structural configuration. The position of OH group at ring D makes phorbol active (β form) or inactive (α form). The inactive phorbols (‘α’ form) also have similar physiochemical properties and lipophyllicity to that of β-phorbols, but they cannot activate PKC due to change in their structural confirmation (Silinsky and Searl, 2003). The non tumour promoting PEs have also been reported. They have atleast one activity of phorbol compounds such as binding to PKC receptors but they do not have tumour promoting properties. The examples of non-tumour

promoting PEs are 12-deoxyphorbol 13-acetate (prostratin), 12-deoxyphorbol 13-propanoate and 12-dexoxy phorbol 13-phenylacetate (Xu et al., 2009).

The Jatropha plant provides high quality seed oil as a feedstock for biodiesel production. During the biodiesel production, many value added byproducts (seed cake, glycerol and phytochemicals, among others) could be obtained (Makkar et al., 2009). The presence of toxic PEs in the oil used for biodiesel production and in the byproducts raises environmental health and human exposure concerns. In *J. curcas*, 6 types of PEs have been identified (Haas et al., 2002). However, knowledge on their biological activity and potential applications are limited. The present study was undertaken to evaluate whether all the PEs present in Jatropha seed are tumour promoters or not?

2. Material and methods

2.1. Materials

J. curcas seeds (toxic Indian variety) were collected from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. All other chemicals/solvents used were of analytical grade.

2.2. Extraction of Jatropha oil

J. curcas seeds were mechanically pressed to obtain oil and it was centrifuged at 3150 x *g* (20 min) to obtain clear oil. The clear oil was stored in a refrigerator (4 °C) until further use.

2.3. Quantification of phorbol esters

Phorbol esters were determined according to Makkar et al. (2007), based on the method of Makkar et al. (1997). Briefly, 0.5 g of oil or purified Jatropha PEs was extracted for 5 minutes with 1.5 ml of solvent (99 % methanol / 1 % THF) in a ball mill (Retsch MM200, 30 1/s). The supernatant was collected and solvent was removed under pressurised air. The procedure was repeated thrice with the subnatant and solvent from each extraction was pooled together and solvent was removed under pressurised air. The extract was made to known volume and injected into high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 x 4 mm i.d., from Merck (Darmstadt, Germany) column and separation was carried out at 23 °C. The flow rate was 1.3 ml/min using a gradient elution. The phorbol esters (four peaks) were detected at 280 nm and they appeared between 25.5 and 30.5

min. The concentration of phorbol esters were expressed equivalent to phorbol 12-myristate-13-acetate (PMA) or Jatropha factor C_1 (Makkar et al., 2007).

2.4. Purification of phorbol esters

In brief, Jatropha oil was extracted with methanol (1:2, w/v; 60 °C, 15 min, 300 rpm) using a magnetic stirrer. The upper methanolic layer and lower oily layer were separated by centrifugation (3150 × *g*, 5 min). The oily layer was re-extracted thrice with the fresh methanol in a ratio of 1:1.5, 1:1 and 1:1 (w/v) respectively. All the upper methanolic layers were pooled together and rotaevaporated (65 °C, 300 mbar) to get oily PEs enriched fraction (PEEF) (Devappa et al., 2010). The PEEF was further purified by column and high performance liquid chromatography to get purified phorbol esters rich extract (PEs-rich extract) (for details See chapter 9). The purified PEs was confirmed by 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, TOCSY, HMBC) NMR and the data was found similar to the data published by Haas et al. (2002). The Jatropha PEs, factor C_1 and C_2 are purified to homogeneity. Whereas, factor $C_{3mixture}$ and factor (C_4+C_5) was obtained as a mixture. Further in the study, the concentration for all the Jatropha factors were expressed as *Jatropha* factor C_1 equivalents. The purified PEs (factors C_1, C_2, $C_{3mixture}$ and (C_4+C_5)) and PEs-rich extract (factors C_1 to C_5) are stored in ethanol until further analysis at -80 °C.

2.5. Experiment of Bhas 42 cells

The cell culture and the experiment were carried out as described by the Sakai et al. (2010). The cryo-preserved Bhas 42 cells (mouse embryo cell line) were purchased from Health Science Research Resources Bank (Osaka, Japan). In a humidified incubator (37°C, 5% CO_2), the proliferation of cells were done using Eagle's minimum essential medium (PAN Biotech Gmbh) supplemented with 10% foetal bovine serum (medium-A). After reaching 70% confluence, cells were subcultured using 0.25% trypsin (Sigma). The proliferated cells were stored in a 0.5 ml medium (~5×10^5 cells/ml) containing 5% dimethyl sulfoxide (DMSO) at −80 °C.

2.5.1. Transformation assay

The test was carried out as described by Sakai et al. (2010)

2.5.1.1. Test chemicals used in the assay

The purified phorbol esters isolated from Jatropha oil (factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5) and their mixtures (factors C_1 to C_6) designated as PEs-rich extract (for details see chapter 9), TPA, MCA (positive control) and 0.1% DMSO (vehicle and negative control) were used in the transformation assays.

The transformation assay involves two separate assays (initiation assay and promotion assay) to screen carcinogenic and tumour promoting compounds respectively.

2.5.1.2. Initiation assay

The experiment was carried out in sterile conditions. In brief, the frozen Bhas 42 cells were thawed and cultured in a medium-A until 70% confluence was reached. Further, the cells were cultured in medium-B (Eagle's minimum essential medium supplemented with 5% foetal bovine serum). The trypsinized cells were diluted (2000 cells/ml) and an aliquot (2 ml) was added into each well of the 6 well plates, marked as day zero. After 24 h of seeding, the plates containing cultures were treated with test samples at least in triplicates. The test samples were premixed with the medium and added to the culture plates. The plates were incubated (37 °C, 95% humidity and 5 % CO_2) until 72 h. After 72 h of treatment period, the medium was changed with medium-B (without test samples; day-4) and further cultured with medium-B until day 21. In between, the medium was changed on day-7, day-11 and day-14. After day-21, the cells were fixed in methanol and stained with Giemsa's stain. Along with Jatropha PEs, the MCA (positive control) and 0.1% DMSO (negative control) was used for the assay.

2.5.1.2. Promotion assay

In brief, cells were treated similar to the initiation assay except the following changes. Each well in the 6 well plate was seeded with 14,000 cells/well (2 ml aliquot) at day-0 and cultured for 4 days with no medium change. The medium in the plates were changed with the fresh culture medium (2 ml) containing test samples on day-4, day-7 and day-11. The treatment was carried out till day-14 and thereafter normal medium (without test samples) was added and cultured till day-21. Along with the Jatropha PEs, the TPA and 0.1% DMSO was used as positive and negative controls respectively.

2.5.1.3. Analysis for transformed foci

The transformed foci were evaluated as described by Sakai et al. (2010). The morphological characteristics observed were, more than 100 cells, deep basophilic staining,

random orientation of cells at the edge of foci, dense multi layering of cells, spindle-shaped cells different from the contact-inhibited monolayer cells and dense multi layering of cells and invasive growth into the monolayer of surrounding contact-inhibited cells. The foci in each well was counted and statistically evaluated for the increase in transformed foci as affected by different concentrations**.**

2.5.1.4. Evaluation of results

The assays were judged positive when there was statistically significant increase in the transformed foci/well between the tested doses. If the statistically significant increase was observed at only one dose, then the result was considered equivocal and the assay was repeated with appropriate doses.

2.5.2. Cell viability assay

The concentration range applicable for the Bhas 42 transformation assay (initiation and promotion assay) was first determined using crystal violet (CV) staining method. The assay was also conducted in parallel with the every transformation assay for each test sample. The cells were treated as mentioned in the initiation and transformation assay, but the cells were incubated till day-7 and stained with 0.1% CV solution. The test was carried out in triplicates. The CV was extracted with 50% ethanol containing 0.03 M sodium citrate and 0.02 M hydrochloric acid. The absorbance of the extract was measured at 540 nm and viability of the cells was expressed as percentage of the control cells.

2.6. Activation of protein kinase C

Activation of protein kinase C was assessed using nonradioactive peptag assay (Promega). The assay was carried out as described in the protocol with some modifications. In brief, the assay uses brightly pink coloured fluorescent peptide (P-L-S-R-T-L-S-V-A-A-K), which are highly specific for protein kinase C. The assay was carried out as described by Wink et al. (2000). The phosphorylation by PKC alters the peptides charge (+1 to -1), which can be separated by horizontal agarose gel electrophoresis. The phosphorylated species migrates toward the positive electrode, while the nonphosphorylated substrate migrates toward the negative electrode. The presence of activators such as phorbol esters, activates PKC which in turn phosphorylates the peptide. In the assay, blank (DMSO), negative and positive assay

controls, PEs-rich extract (factors C_1-C_5 as a mixture), individual factors C_1, C_2, $C_{3mixture}$ and (C_4+C_5) were tested.

3. **Results**

Jatropha oil used for the isolation of PEs had 3−4.6 mg/g of PEs (PMA equivalent or 0.072−0.11 mg/g as factor C_1 equivalent). The purified Jatropha PEs (factor C_1, C_2, $C_{3mixture}$, (C_4+C_5) and PEs-rich extract containing factors C_1–C_5 were subjected to *in vitro* cell transformation tests. When compared with other transformation assays such as SHE cells, BALB/c 3T3 cells or C3H10T1/2 cells, the Bhas 42 assay does not require initiator treatment for cell transformation (e.g. MCA or DMBA) and has shorter assay time (3 weeks compared to 6 weeks in other tests). In addition, the Bhas 42 assay has initiation and promotion stages that mimic the two stages involved in the animal carcinogenicity tests.

3.1. Dose response study or selection of test concentration

The concentration range selected for the cell transformation assays was assessed by cell viability assay (data not shown), before conducting initiation and transformation assay. The dose range included in the study was selected as described by the Sakai et al. (2010).

3.2. Cell toxicity and cell transformation activity in the initiation assay

The effects of Jatropha PEs and MCA on cell viability and cell transformation activity at the initiation stage are shown in Figure 1 and 2. On day 1 to 4, the cells were exposed to different concentrations of PEs, MCA and the cell viability was expressed by taking the value for solvent control (DMSO) as 100% (Figure 1). For PEs-rich extract (0.00005−2 μg/ml) the cell viability was ≥ 75%, except at a concentration 0.5 μg/ml (55%). Whereas, factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5) at a concentration of 0.0005–1 μg/ml exhibited ≥ 90%, ≥ 91%, ≥ 99% and ≥ 96% of cell viability respectively. At the same concentration level (0.001-1 μg/ml), the TPA used in our study exhibited ≥ 82% cell viability when compared with the reported values for TPA (≥ 75%) (Sakai et al., 2010). The cell viability of MCA treated cells decreased with the increased concentrations and the trend was similar as reported by Sakai et al. (2010). At the same concentration level (0.001–2 μg/ml), the MCA used in our study exhibited higher (≥ 64%) cell viability than the reported values (≥ 35%) (Sakai et al., 2010). In our study, PEs-rich extract seems to be more cytotoxic when compared with other purified phorbol esters (factors C_1, C_2, $C_{3mixture}$ and (C_4+C_5)).

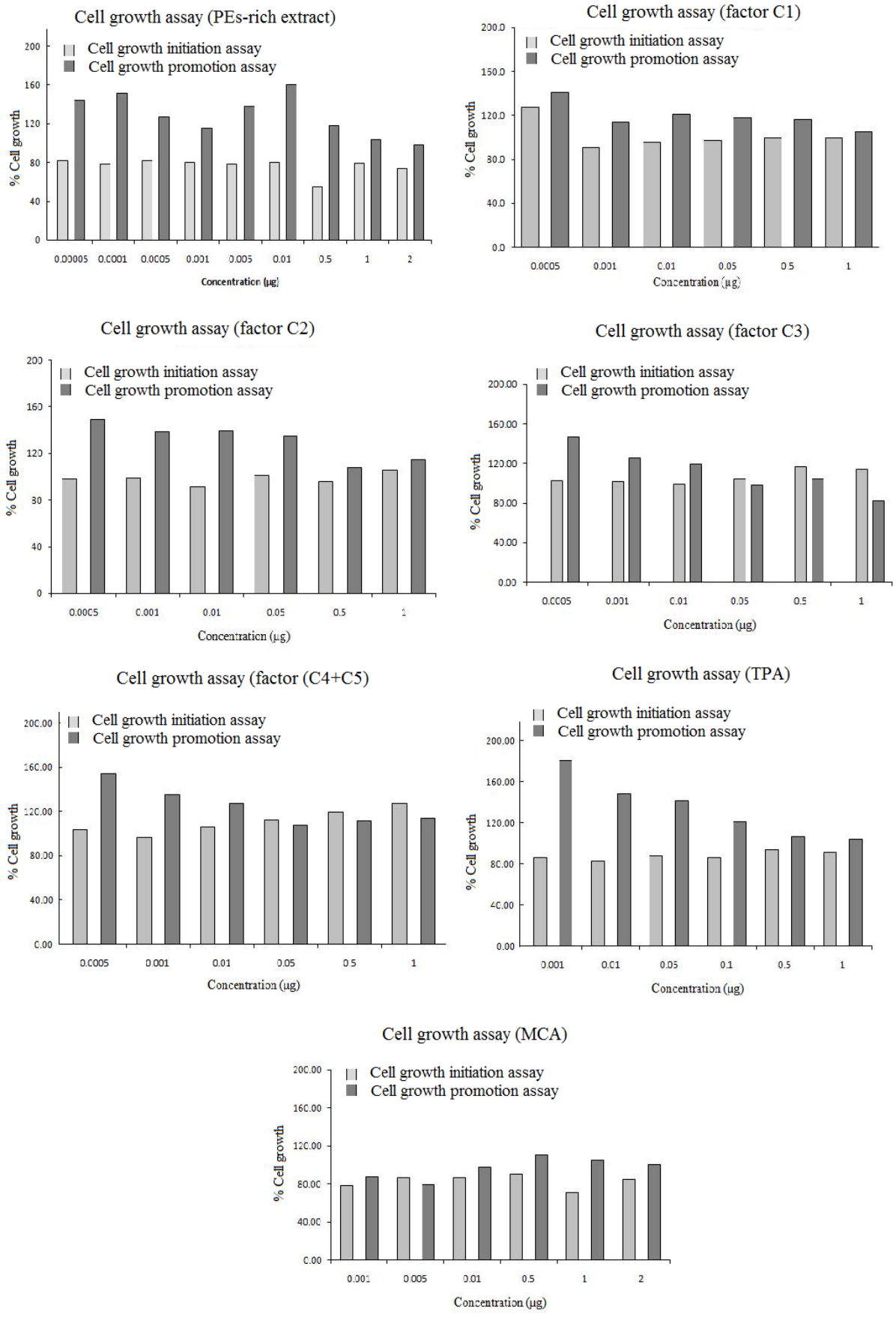

Figure 1. Cell growth of Bhas 42 cells treated with Jatropha phorbol esters, TPA and MCA during transformation assay (initiation assay and promotion assay).

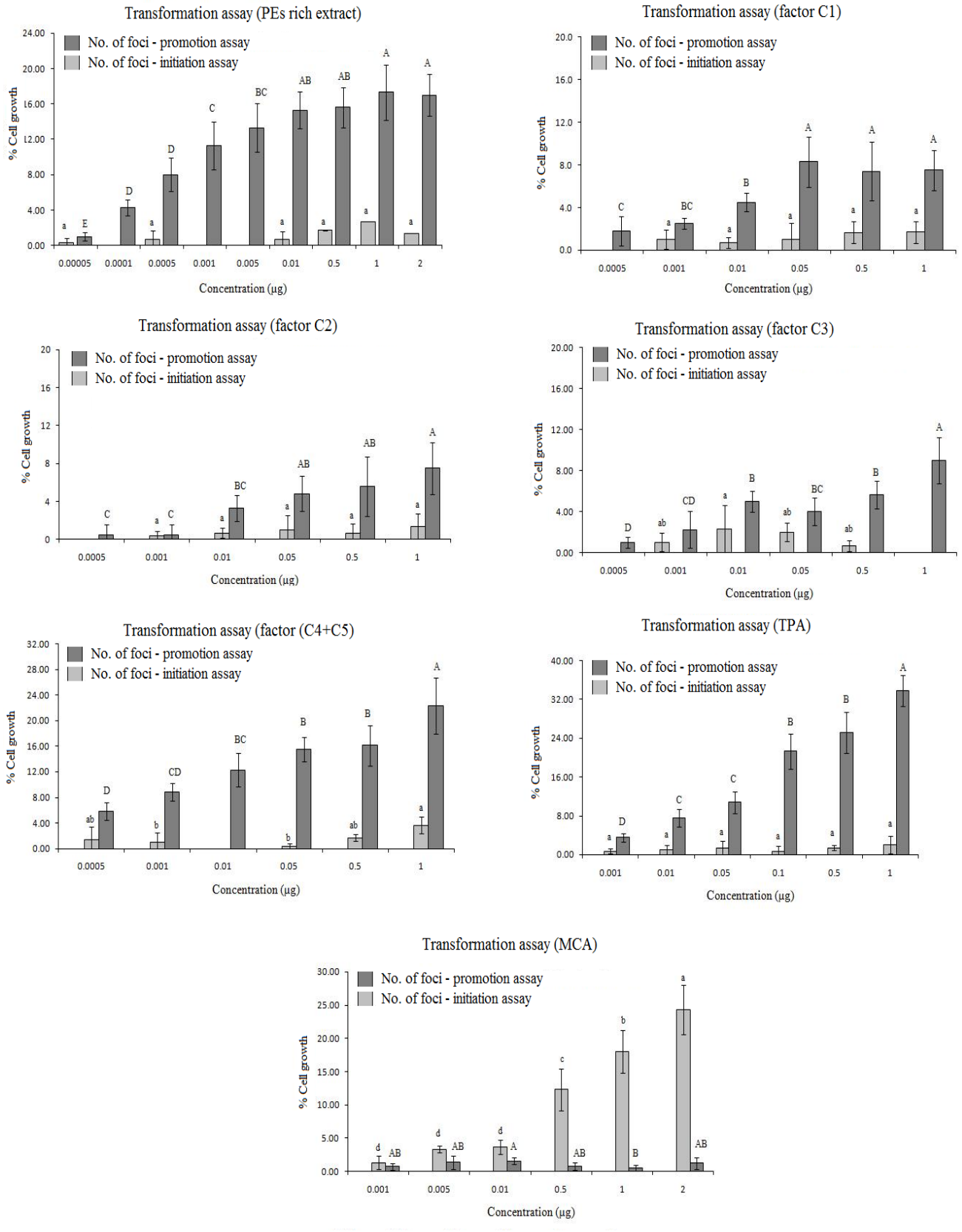

Figure 2. Formation of transformed foci in Bhas 42 cells treated with Jatropha phorbol esters, TPA and MCA during transformation assay (initiation assay and promotion assay).

In the initiation stage, TPA, PEs-rich extract, factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5) did not increase ($P<0.05$) the cell transformation frequencies. The above tested compounds were assigned as negative in the initiation assay as there was no dose showing statistically significant increase in the formation of transformed foci/well (Sakai et al., 2010). The positive control (MCA) significantly increased ($P<0.05$) the formation of transformed foci. At the same concentration level (2 µg/ml), the average number of transformed foci/well observed for MCA- treated cells was slightly lower (24.33) compared with the reported values (28.7) (Sakai et al., 2010). In the present study, MCA was taken as the positive initiator compound.

3.3. Cell viability and cell transformation activity at promotion stage

The effects of Jatropha PEs and MCA towards cell viability and cell transformation activity at the promotion stage are shown in Figure 1 and 2. The TPA was used as a reference tumour promoter compound and the effects were compared with those of Jatropha PEs. On day 4 to 7, the cells were exposed to different concentrations of Jatropha PEs and MCA; and the cell viability was expressed relative to that of the solvent (control 100%) (Figure 1). For PEs-rich extract (0.00005–2 µg/ml), the cell viability was 98−144%. Whereas, factor C_1, factor C_2, factor $C_{3mixture}$, factor (C_4+C_5) at a concentration of 0.0005–1 µg/ml exhibited 105–140%, 107–148%, 82–146% and 107–154% of cell viability respectively. At the same concentration level (0.001–1 µg/ml), the TPA used in our study exhibited lesser cell viability (103–180%) when compared to reported values (112–231%) (Sakai et al., 2010). The cell viability increased with the increased concentrations of Jatropha PEs and TPA; and the trend was similar to that reported by Sakai et al. (2010). Whereas, the MCA (0.001–2 µg/ml) used in our study exhibited decrease in cell viability (100–87%) with the increase in concentration. But, the values were higher when compared with the reported values (95–69%) for MCA at same concentration levels (Sakai et al., 2010).

In the cell transformation test conducted at promotion stage, TPA (positive control), PEs-rich extract, factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5) exhibited significant increase in the formation of average number of transformed foci/well (Figure 2). The above tested compounds were assigned as positive in the promotion assay as there was dose dependent increase ($P<0.05$) in the formation of transformed foci (Sakai et al., 2010). For PEs-rich extract (0.00005–2 µg/ml), the average number of transformed foci/well formed was 1–17.

Whereas, average number of transformed foci/well formed after treatment with the factor C_1, factor C_2, factor $C_{3mixture}$ and factor (C_4+C_5) at a concentration of 0.0005–1 µg/ml were 1.8−7.5, 0.5−7.5, 1−9 and 5.8−22.3 respectively. At the same concentration level (0.001−1 µg/ml), TPA used in our study formed lesser average number of transformed foci/well (3.5−33.8) when compared with the reported values for TPA (2.7−34.8) (Sakai et al., 2010). Overall, the formation of transformed foci/well increased with the increased concentrations of Jatropha PEs, TPA and the trend was similar as reported by Sakai et al., 2010. The order of transformed foci/well formation was TPA > PEs-rich extract > factor C_4 > factor $C_{3mixture}$ > factor C_1 > factor C_2. However, MCA (negative control) did not produce any significant increase in transformed foci/well. In the promotion assay, Jatropha phorbol esters and TPA were judged as positive promoter compounds, whereas MCA was taken as a negative promoter compound.

3.4. Protein kinase C activity

The compounds judged positive in the promotion assay (Section 3.3) was evaluated for protein kinase C activity. In the assay, phorsphoylated fluorescent peptide obtained as an enzymatic reaction product indicated the PKC activity. The presence of PKC activators increases the PKC activity. In the assay, positive control (cAMP as a PKC activator), TPA (1 µg), PEs-rich extract (2 µg), factor C_1 (1 µg), factor C_2 (1 µg), factor $C_{3mixture}$ (1 µg) and factor (C_4+C_5) (1 µg) induced phosphorylation of fluorescent peptide, which was visualised in an agarose gel indicating the PKC activity (Figure 3). The negative control (absence of PKC activator) and DMSO (1 µl, vehicle) did not induce any phosphorylation of the fluorescent peptide.

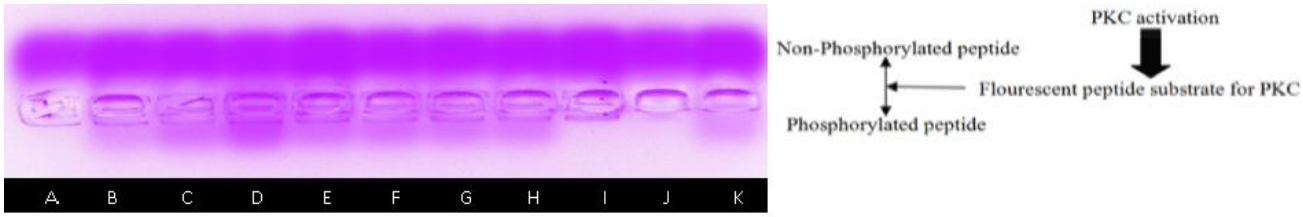

Figure 3. Protein kinase C activity of Jatropha phorbol esters and TPA: (A) negative control (DMSO), (B) DMSO+ positive control, (C) positive control + TPA, (D) PEs-rich extract, (E) factor C_1, (F) factor C_2, (G) factor $C_{3mixture}$, (H) factor (C_4+C_5), (I) control without PKC activator or PEs, (J) negative control (DMSO) and (K) TPA

4.0. Discussion

Jatropha oil is projected as one of the promising feedstocks for biodiesel production. Recently, there has been large scale cultivation of Jatropha plant in tropical/subtropical countries such as India, China and Indonesia, among others. By 2015, the projected Jatropha oil yield is 12.8 million tons/annum (GEXSI, 2008). As the oil contains toxic PEs, exposure to humans either by skin, eye or oral route could take place. During biodiesel production there is a chance for the workers coming in contact with the toxic oil containing PEs. Generally, mouse skin tumour promotion is well defined involving two stages of carcinogenesis, initiation stage and promotion stage (Berenblum, 1975). Exposure to single dose of an initiator compounds (at low doses) or of a promoter compound does not cause tumour formation. But tumour formation occurs with the repeated application of the promoter compound after a single application of initiator compound, indicating that proper order of exposure to initiator and promoter compounds is necessary to cause tumour. As the humans are exposed to different environmental factors in their life time, often in low doses, it is possible that similar synergism may occur causing tumours. The potential of a compound for causing tumour is very much dependent on the type and dose of other substances (e.g. MCA) that an individual is exposed to (Yamasaki and Weinstein, 1985).

In the present study, all the purified PEs and PEs-rich extract isolated from Jatropha and TPA did not show tumour initiation activity; however they exhibited the tumour promotion activity *in vitro*. In the same *in vitro* transformation assay PEs such as phorbol 12-13 di decanoate exhibited tumour initiation properties (Sakai et al., 2010). However, the basic skeleton of phorbol esters such as phorbol or 4α phorbol did not exhibit initiation or promotion activity in the Bhas 42 assay (Tanaka et al. 2009; Ohmori, 2009). This clearly indicates that the toxicity and potency of tumour promotion of PEs are structure dependent. The computer model put forth by Jeffrey and Liskamp (1986) suggests the important structural features responsible for the biological activities of PEs, which includes the presence of (a) polar functional groups near O-3, O-4, O-9, O-20 of TPA, (b) free hydroxyl group at C-20, (c) no steric hindrance near 5 membered ring, and (d) hydrophobic moiety near C-20 (Goel et al., 2007). Generally, in majority of the reported animal studies the active PEs (e.g. TPA) did not act as an initiator compound. Horiuchi et al. (1987) observed that the methanol extract from Jatropha oil caused tumour promotion in mice (36%) following treatment of an initiator compound (7, 12-dimethylbenz[a]anthracene (DMBA)) in 30 weeks. Further, they purified PEs and termed one

of them as DHPB (which was later renamed as factor C_1 by Haas et al. (2002) and applied (34 nmol) on mouse skin which resulted in the induction of ornithine decarboxylase (2.8 nmol CO_2/30 min/mg protein) and activation of PKC *in vitro* (50% effective dose being 36.0 nM). In addition, they also reported that DHPB or factor C_1 is more toxic, but has weak tumour promoter activity compared with TPA. In our study in addition to factor C_1, all other Jatropha factors exhibited tumour promotion properties in vitro. In addition to tumour promotion, the Jatropha PEs also exhibited toxicity in laboratory animals at higher concentrations. In mice the purified PEs mixture (factor C_1 to C_6) (PMA equivalent) exhibited an LD_{50} of 27.34 mg/kg body mass (Li et al., 2010). The PEs from Jatropha can easily be extracted in organic solvents such as ethanol, methanol, diethyl ether and petroleum ether among others (our unpublished data). Gandhi et al., 1995 reported that application of petroleum ether extract (100 µl) obtained from *J. curcas* oil on shaved dorsal rabbit skin exhibited marked erythema and oedema, which later became necrotic and regenerated. In mice, topical application (50 µl) of the petroleum ether extract of *J. curcas* oil on the shaved dorsal skin elicited swelling of the face, hemorrhagic eyes, diarrhoea, and skin erythema before death, whereas in rats (50 µl), topical application on the shaved dorsal skin produced oedema and erythema at 4 h of the application, which afterwards led to severe scaling and thickening of the skin. The stratum corneum of skin demonstrated parakeratosis and thickening, and cellular infiltration in the upper dermis; and the toxicity of Jatropha PEs in other organisms is discussed in detail elsewhere (Devappa et al., 2010). Majority of the *in vivo* tumour promotion and toxicity studies conducted using Jatropha PEs either in purified form or in extracts, suggested that the activity is mediated by the activation of PKC enzyme. In our study, similar to TPA, the purified Jatropha PEs and PEs-rich extract increased PKC activity. Similar observation was made by Wink et al. (2000) using DHPB (factor C_1). A number of reports suggest that the effects of TPA are through the mediation of PKC (especially in skin) and the events that best correlate with skin tumour promoting properties of TPA are dermal inflammatory response, induction of epidermal ornithine decarboxylase activity followed by increased polyamine levels, induction of epidermal hyperplasia and dark basal keratinocytes (Di Giovanni et al., 1988). It has been reported that among the many genes up-regulated upon the exposure of TPA, matrix metalloproteinase 10 (mmp-10) could be considered as the target gene (FDSC, Japan). In the Bhas 42 assay system used in the present study it would be interesting to evaluate the effect of Jatropha PEs on gene expression.

5.0. Conclusion

Jatropha PEs when tested in purified form exhibited tumour promotion activity *in vitro*. The tumour promotion activity was mediated by the activation of PKC. Further validation of these *in vitro* results using other models should be conducted. In the meantime care should be taken while handling the toxic oil or Jatropha based products/coproducts. The use of protective gloves to avoid direct contact with Jatropha oil is suggested.

References

1. Berenblum, I. 1975. Sequential aspects of chemical carcinogenesis: Skin. In: Becker, F.F., ed., Cancer: A Comprehensive Treatise, Vol. 1, New York, Plenum Press, pp. 323−344.
2. Devappa RK, Makkar HPS, Becker K. 2010d. Jatropha toxicity – A review. J Toxicol Environ Health B Crit Rev 13:476−507.
3. DiGiovanni J, Kruszewski FH, Coombs MM, Bhatt TS, Pezeshk A. 1988. Structure-activity relationships for epidermal ornithine Decarboxylase induction and skin tumour promotion by anthrones. Carcinogen. 9:1437−1443.
4. FDSC (Hatano Research Institute, Food and drug Safety centre, Japan). http://www.fdsc.or.jp/hatanoken/gakkai/Asada/asada.pdf
5. Gandhi VM, Cherian KM, Mulky MJ. 1995. Toxicological studies on Ratanjyot oil. Food Chem Toxicol 33:39−42.
6. GEXSI. 2008. http://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf.
7. Goel G, Makkar HPS, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 26:279−288.
8. Griner EM, Kazanietz MG. 2007. Protein kinase C and other diacylglycerol effectors in cancer. Nature Reviews Cancer 7:281−294.
9. Haas W, Strerk H, Mittelbach M. 2002. Novel 12 Deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J Nat Prod 65:1434−1440.

10. Horiuchi T, Fujiki H, Hirota M, Suttajit M, Suganuma M, Yoshioka A, Wongchai V, Hecker E, Sugimura T. 1987. Presence of tumour promoters in the seed oil of *Jatropha curcas* L. from Thailand. Jpn J Cancer Res 78:223–226.
11. Jeffrey AM, Liskamp RMJ. 1986. Computer-assisted molecular modelling of tumour promoters: rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin. Proc Natl Acad Sci. USA 83:241–245.
12. Kazanietz MG. 2000. Eyes wide shut: protein kinase C isozymes are not the only receptors for the phorbol ester tumour promoters. Mol Carcinog 28:5−11.
13. Kinzel V, Richards J, Goerttler K, Loehrke H, Fürstenberger G, Marks F. 1984. Interaction of phorbol derivatives with replicating cells. IARC Sci Publ 56:253–264.
14. Li CY, Devappa RK, Liu JX, Lv JM, Makkar HP, Becker K. 2010. Toxicity of *Jatropha curcas* phorbol esters in mice. Food Chem Toxicol. 48:620−625.
15. Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on Nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 45:3152−3157.
16. Makkar HPS, Becker K. 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J lipid Sci Technol 111:773 – 787.
17. Makkar HPS, Siddhuraju P, Becker K. 2007. A laboratory manual on quantification of plant secondary metabolites. Humana Press, New Jersey, p. 130.
18. Ohmori K. 2009. *In Vitro* assays for the prediction of tumourigenic potential of non-genotoxic carcinogens –minireview. J Health Sci 55:20−30.
19. Sakai A, Sasaki K, Muramatsu D, Arai S, Endou N, Kuroda S, Hayashi K, Lim YM, Yamazaki S, Umeda M, Tanaka N. 2010. Bhas 42 cell transformation assay on 98 chemicals: the characteristics and performance for the prediction of chemical carcinogenicity. Mutat Res 702:100−122.
20. Silinsky EM, Searl TJ. 2003. Phorbol esters and neurotransmitter release; more than just protein kinase C?. Br J Pharmacol 138:1191–1201.
21. Tanaka N, Sasaki K, Hayashi K, Sakai A, Asada S, Muramatsu D, Kuroda S, Mizuhashi, Nagai M, Suzuki H, Imamura T, Asakura M, Satoh H, Sakamoto A, Nakao R, Hirose H, Ishii N, Umeda M. 2009. An Interlaboratory Collaborative Study on a Cell Transformation Assay Using Bhas 42 Cells. AATEX 14:831−848.

22. Wink M, Grimm C, Koschmieder C, Sporer F, Bergeot O. 2000. Sequestration of phorbolesters by the aposematically coloured bug Pachycoris klugii (Heteroptera: Scutelleridae) feeding on *Jatropha curcas* (Euphorbiaceae). Chemoecol 10:179–184.
23. Xu R, Zhao W, Jiang C. 2009. Ester prodrugs of prostratin and related phorbol compounds. United States Patent Application 2009016358.
24. Yamasaki H, Weinstein IB. 1985. Cellular and molecular mechanisms of tumour promotion and their implications for risk assessment. Methods for estimating risk of chemical injury: human and non-human biota and ecosystems. Edited by V.B. Vouk, GC Butler, DG Hoel and DB Peakall.

CHAPTER -12

Pharmaceutical potential of phorbol esters from *Jatropha curcas* oil

Rakshit K. Devappa [a], Chandi C. Malakar [b], Harinder P.S. Makkar [a*] and Klaus Becker [b]

[a]*Institute for Animal Production in the Tropics and Subtropics, (480b), University of Hohenheim, Stuttgart-70599, Germany;* [b]*Institut fuer Chemie, Universitaet Hohenheim, Stuttgart, Germany*

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Abstract

Phorbol esters (PEs) are diterpenes, present in *Jatropha curcas* seeds and have a myriad of biological activities. Since PEs are toxic, they are considered to be futile in *Jatropha* based biodiesel production chain. In the present study, the extracted PEs from *Jatropha* oil was used as a starting material to synthesize pharmacologically important compound, prostratin. The prostratin synthesized from *Jatropha* showed identical mass with that of the reference standard prostratin, as determined by Nano-LC-ESI-MS/MS. Considering the rapid growth in *Jatropha* biodiesel industry, potential exists to harness large amount of PEs which can be further utilized to synthesize prostratin as a value added product.

Keywords: *Jatropha*; phorbol esters; toxicity; prostratin; by-product

1. Introduction

Phorbol esters (PEs) are tigliane diterpenes present in the genus Euphorbiaceae. These groups of compounds have an array of biological activities, exhibiting toxicity at higher doses and beneficial or pharmaceutical effects at lower doses (Goel et al., 2007). In *Jatropha curcas* (Euphorbiaceae), 6 different types of PEs (*Jatropha* factors C_1 to C_6) are present (Haas, Sterk & Mittlebach, 2002). The Jatropha kernel, screw pressed oil and screw pressed cake generally contains phorbol esters in the range of 2−6 g kg^{-1}, 3−6 g kg^{-1} and 0.5−4 g kg^{-1} respectively (Makkar et al., 2009; and unpublished data from our laboratory). Although present in lower concentrations, they exhibit severe toxicological symptoms upon oral and topical exposure to different parts of *Jatropha* plant including seeds. Several studies report the need to remove these compounds to increase the potential use of byproducts such as protein rich seed cake (Makkar et al., 1997; Goel et al., 2007). Due to toxicity, these compounds are generally regarded as futile compounds in the *Jatropha* biodiesel production chain. In addition, the removal of PEs from *Jatropha* based products and their further utilization in agropharmaceutical applications could enhance the economic sustainability of the *Jatropha* biodiesel production chain. In the present study, the pharmaceutical potential of PEs obtained from *Jatropha* oil was evaluated by converting them into a high-valued compound which is nontoxic and has high biological activity. The studies were focused on the synthesis of prostratin from *Jatropha* PEs.

Prostratin (12-deoxyphorbol-13-acetate) is a tigliane diterpene, and its presence is reported in plants such as *Pimelea prostrate, Euphorbia cornigera* and *Homalanthus nutans* (Miana, Bashir & Evans, 1985). Similar to phorbol-12-myristate-13-acetate (PMA; synonym, 12-O-tetradecanoylphorbol-13-acetate (TPA)), the prostratin is a protein kinase C (PKC) activator. However, prostratin is a potent anti tumour compound while PMA a tumour promoter. In addition, prostratin was found to inhibit PMA-stimulated tumour formation (Szallasi & Blumberg, 1991; Szallasi, Krausz & Blumberg, 1992; Szallasi, Krsmanovic & Blumberg, 1993). Thus, it belongs to a discrete subclass of PKC activators differing in its biological activities when compared with the tumour-promoting PEs, such as PMA (Ventura & Maioli, 2001). Prostratin has also been found to be a promising adjuvant in antiviral therapy. Many antiviral treatments have been successful in decreasing the active viral pool in AIDS patients (HAART). However, the persistence of latent viral reservoirs limits the complete viral eradication. Prostratin activates this latent virus pool (Wender, Kee & Warrington, 2008) in turn acting as an adjuvant in HIV therapy. Prostratin have been found to induce HIV expression in latently infected cell lines and primary cells *in vitro* and also to inhibit HIV entry

into target cells by down regulating CD4 and CXCR4 receptors (Kulkosky et al., 2001; Gustafson et al., 1992).

The prostratin was reported to be the active component present in Samoan mamala tree bark extract which is used for treating hepatitis (Cox et al., 2001; Johnson, Banack & Cox, 2008). In *H. nutans*, its concentration has been found to vary from 0.2 − 52.6 μg g^{-1} fresh stem wood (Johnson, Banack & Cox, 2008). However, presence of low amounts of prostratin in plants, difficult purification steps and the lesser yield limits its potential to economically harvest in large quantities. In addition, establishment of large scale plantation by replacing the native ecosystem to obtain substantial amounts of prostratin could have significant ecological effects (Johnson, Banack & Cox, 2008; Wender, Warrington & Kee., 2009).

Alternatively, prostratin could be obtained by synthetic methods by utilizing PEs as a starting material. Industrial oil crop such as *Jatropha curcas* provide a promising feedstock of PEs for prostratin synthesis. Wender, Kee & Warrington (2008) have reported the synthesis of prostratin from phorbol (obtained from croton oil), which is a basic skeleton in the PEs such as PMA. They also presumed that prostratin could be synthesized from *Jatropha* PEs by hydrolysis, but further investigation has not been carried out. However, the *Jatropha* PEs do not contain phorbol as a basic skeleton, instead they contain 12-deoxy phorbol as a basic skeleton. Wender, Kee & Warrington (2008) suggested that 12-deoxy 16-hydroxy phorbol obtained from the *Jatropha* PEs could be converted into crotophorbolone, which can be further subjected to synthetic reactions to obtain prostratin (Figure 1). In the present study, the possibility of converting *Jatropha* phorbol esters into crotophorbolone and then into prostratin was investigated.

2. Material and methods

2.1. Materials

Jatropha curcas seeds (toxic Indian variety) were collected from wild trees (mature, approx. age 15 years) existing in places around Jaipur (geographical coordinates: 26°55′0″ N, 75°49′0″ E), Rajasthan, India. Phorbol 12-myristate-13-acetate (PMA) and the reference prostratin were obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals/solvents used were of analytical grade.

2.2. Extraction of phorbol ester

J. curcas seeds were mechanically pressed using a screw press to obtain oil. The oil was centrifuged at 3150 × *g* for 20 min to remove residues. The phorbol ester enriched fraction (PEEF) was obtained from *Jatropha* oil by following the procedure reported by Devappa, Makkar & Becker (2010). In brief, the oil was mixed with methanol (1:2, w/v) and the mixture was stirred using a magnetic stirrer (55 °C for 15 min, 300 rpm). Thereafter, the mixture was centrifuged (3150 x *g* for 5 min) to get upper methanolic and lower oily layers. The methanol layer was rotaevaporated to get oily PEEF. The oily PEEF was stored at -80 °C until further analysis.

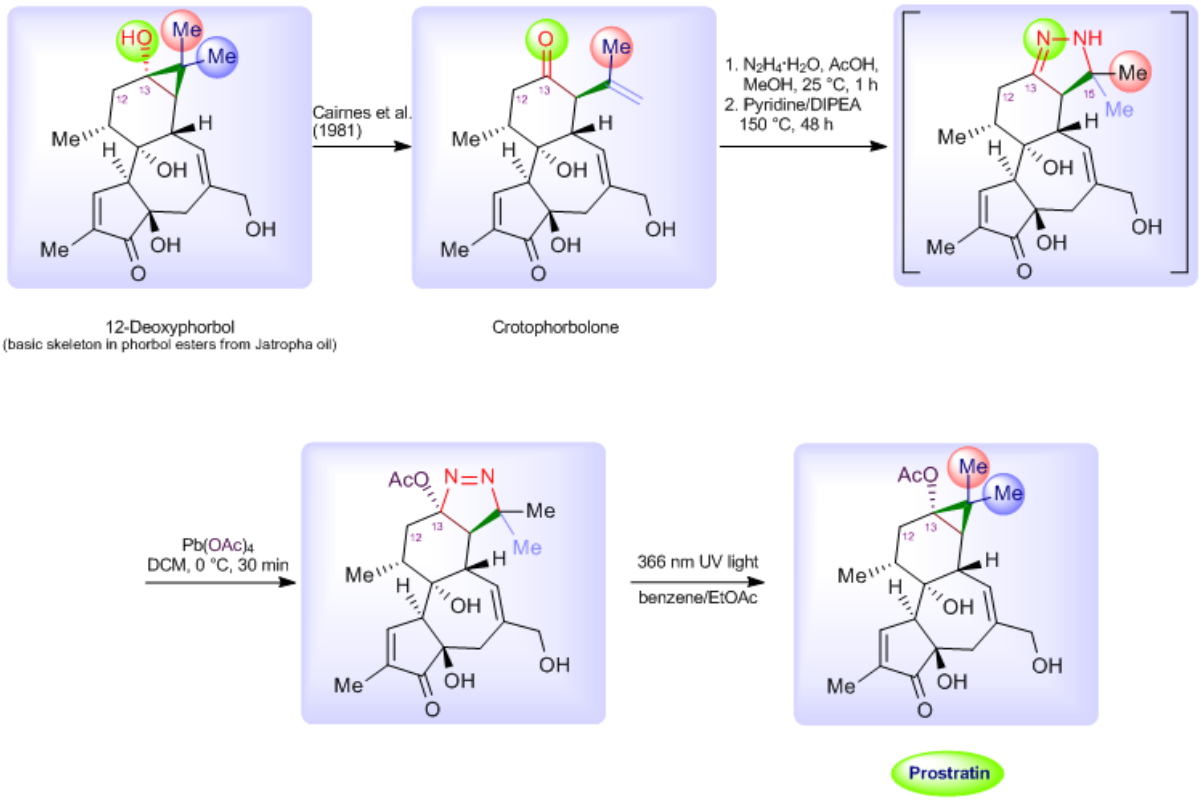


Figure 1. Synthesis of prostratin from Jatropha phorbol esters (adopted from Wender, Kee & Warrington, 2008).

2.3. Phorbol ester analysis

Phorbol esters were determined at least in triplicate according to Makkar, Siddhuraju & Becker (2007), based on the method of Makkar et al. (1997). Briefly, 0.5 g of oil or PEEF was extracted four times with 2% tetra hydrofuran in methanol. A suitable aliquot was loaded into

a high-performance liquid chromatography (HPLC) fixed with a reverse-phase C_{18} LiChrospher 100, 5 mm (250 x 4 mm id, from Merck (Darmstadt, Germany) column. The column was protected with a head column containing the same material. The separation was performed at RT (23°C) and the flow rate was 1.3 mL min^{-1} using a gradient elution. The four phorbol ester peaks (containing 6 PEs) appeared between 25.5 and 30.5 min were detected at 280 nm. The spectra of each peak were taken using Merck-Hitachi L-7450 photodiode array detector. Phorbol-12-myristate 13-acetate was used as an external standard which appeared between 31 and 32 min. The area of the four phorbol ester peaks was summed and the concentration was expressed equivalent to PMA or factor C_1. The PEs detection limit in the HPLC was 3 − 4 μg.

2.4. Synthesis of Prostratin

The PEs from PEEF were converted to crotophorbolone by using the method of Cairnes et al. (1981) and further, the crotophorbolone was converted into prostratin using the method of Wender, Kee & Warrington (2008). The methods for the isolation of crotophorbolone and synthesis of prostratin from crotophorbolone is briefly mentioned in the following subsections.

2.5. Procedure for isolating crotophorbolone

The crotophorbolone was extracted as reported by Cairnes et al. (1981). The methanol (1.5 L) was refluxed with 41.25 g of $Ba(OH)_2$ and filtered. The resulting solution (1.3 L) was mixed with the PEEF (400 g) on a magnetic stirrer under nitrogen, at room temperature for 20 h. After extraction, the barium soap was separated by vacuum filtration and the filtrate was collected. Methanol was removed from the filtrate under vacuum using a rotary evaporator (280 mbar). The resulting brown residue was redissolved in water (1 litre) and extracted with diethyl ether (3 × 700 mL) to remove nonpolar impurities. The aqueous phase was separated and its pH adjusted to 5 using 4N H_2SO_4 and then saturated sodium sulphate (Na_2SO_4, 35 mL) was added to this aqueous solution. This was kept overnight in a refrigerator (4 °C). The precipitated barium sulphate ($BaSO_4$) was filtered off, and the pH of the filtrate was readjusted to 7.0 using 2N NaOH. From this solution the nonpolar impurities were removed by extraction with ethyl acetate (2 × 250 mL) and diethyl ether (1 × 250 mL). The aqueous phase was concentrated under vacuum until its volume was approximately 15 mL. It is important to note that during evaporation the pH of the residual solution was thoroughly checked every 10 – 15 min and the pH was maintained at 7 by adding 4N H_2SO_4. The residue thus obtained was

mixed with warm ethanol (200 mL), and finally the crude reaction mixture was achieved followed by removing the solvent using a rotary evaporator (280 mbar). The yield of the crude reaction mixture was not recorded.

The crude reaction mixture was dissolved in chloroform and loaded onto a column (50 × 10 cm; with 1 kg silica gel-60). Then the crude mixture was eluted successively with 2.5, 5.0, 7.5, 12.5, 30% methanol in chloroform and each fraction (2L) was collected. All fractions were checked for crotophorbolone using a TLC and it was found that eluents 7.5 − 12.5% methanol in chloroform contained crotophorbolone in considerable amounts. These farctions were combined and concentrated using a rotary evaporator (vacuum 280 mbar). Acetone (50 mL) was added to the residue and it was kept overnight in a refrigerator. The fractions having the precipitate were collected and centrifuged to collect the precipitate, "crotophorbolone".

2.6. Procedure for the synthesis of prostratin

The procedure described by Wender, Kee & Warrington (2008) and Wender, Warrington & Kee (2009) was used. The synthesis reaction of prostratin from crotophorbolone is shown in Figure 1. Crotophorbolone (10 mg) in methanol was mixed at room temperature. Acetic acid (18 µl) and hydrazine hydrate (6 µl) were added and the reaction mixture was allowed to stir for 1.5 h at room temperature. After completing the reaction, ethyl acetate (5 mL) and basic alumina (0.7 g) were added and stirred for 10 min at room temperature. The resulting mixture was passed through a celite pad (Sigma) and concentrated under vacuum. The residue was transferred to a vial followed by re-dissolving in pyridine (0.9 mL) and N, N-Diisopropylethylamine (DIPEA) (0.1 mL). The vial was sparged with argon and sealed with a teflon cap. The sealed vial was heated at 150 °C for 50 h. After incubation, the reaction mixture was concentrated under vacuum, sparged with argon and resealed quickly to avoid oxidation of pyrazoline. The crude reaction mixture was dissolved in chloroform maintaining 0 °C using an ice bath. Then a suspension of lead acetate (20 mg) in chloroform (3 mL) was added to the reaction mixture at 0 °C. The reaction was allowed to stir for 30 min and quenched by adding saturated $NaHCO_3$ (2.5 mL). The reaction mixture was extracted with ethyl acetate (4 ×10 mL) by using a separating funnel and combined organic layers were dried over anhydrous sodium sulphate. Solvent was removed in vacuum (280 mbar) to afford the crude product which followed by further purification using flash column chromatography over silica gel afforded a residue. The residue was dissolved in benzene/ethyl acetate (1:1) in a 25 mL round bottom flask, sparged with argon and tightly sealed with a septum. The reaction

mixture was then allowed to stir under UV radiation (366 nm) at room temperature for 1 hour to afford the crude product. Flash column chromatography of the crude reaction mixture over silica gel (80% ethyl acetate in pentane) afforded the mixture of compounds (referred as reaction mixture-B) along with prostratin.

2.7. Recovery of prostratin by preparative thin layer chromatography (TLC)

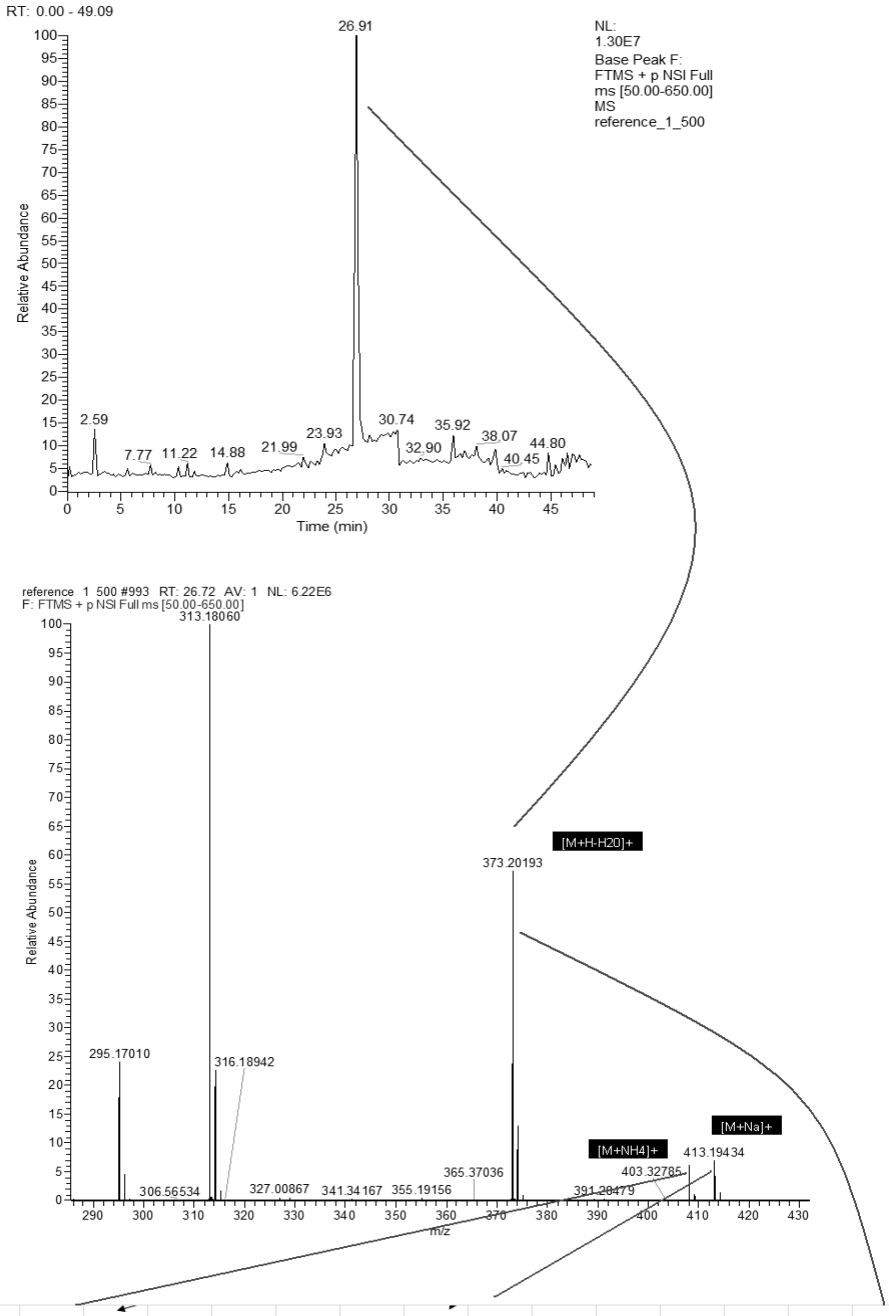
The preparative TLC was carried out to separate prostratin from the mixture. The reference prostratin was also subjected to TLC (65% ethylacetate/pentane (v/v)). The retention factor (R_f) value (0.33) similar to reference prostratin was scraped out from the TLC (multiple runs for the same sample), washed with ethyl acetate and the eluate was rotaevaporated (280 mbar) to get prostratin (Figure 4). In the same solvent system, the R_f value of TPA, *Jatropha* factor C_1, *Jatropha* factor C_2 was 0.55, 0.45 and 0.49 respectively. The R_f value for *Jatropha* PEs-mixture, *Jatropha* factor $C_{3mixture}$ and *Jatropha* factor (C_4+C_5) was not calculated as it was a mixture.

2.8. Analysis of molecular mass by Nano-LC-ESI-MS/MS

The experiments were performed on an ACQUITY nano-UPLC system (Waters, USA) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Germany). The sample was separated on a 20 cm × 75 μm BEH 130 C18 reversed phase column (1.7 μm particle size, Waters, USA). Gradient elution was performed from 10% acetonitrile (ACN) to 80% ACN in 0.1% formic acid (FA) within 30 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.0.7 software. Survey spectra (m/z = 50 − 650) were detected in the Orbitrap at a resolution of 30.000 at m/z = 400. The reference prostratin (1 mg mL^{-1}) and the test samples (reaction mixture-B and prostratin obtained after preparative TLC from the *Jatropha* PEs) were prepared by dissolving 1 μl in 9 μl of methanol. From this, 2 μl was injected in to the system.

3. Results and Discussion

Wender, Kee & Warrington (2008) postulated that *Jatropha* PEs could be a good candidate for producing highly bioactive compound prostratin. The present study has illustrated that prostratin could be synthesised from *Jatropha* PEs. The experimental section is described in the supplementary material (online only). The synthesis reaction of prostratin from crotophorbolone is shown in Figure 1. As this was a preliminary study to assess whether



m/z	Theo. Mas	Delta (ppm	RDB equiv	Composition	m/z	Theo. Mas	Delta (ppm	RDB equiv	Composition	m/z	Theo. Mas	Delta (ppm	RDB equiv	Composition
408.2392	408.2381	2.71	6.5	C22 H34 O6 N	413.1943	413.1935	2.13	7.5	C22 H30 O6 Na	373.2019	373.201	2.62	8.5	C22 H29 O5
	408.2354	9.27	2	C19 H36 O9		413.1959	-3.69	10.5	C24 H29 O6		373.1986	9.07	5.5	C20 H30 O5 Na
	408.2448	-13.71	15	C30 H32 O		413.1961	-4.36	12	C25 H28 O3 N Na		373.2068	-13.11	-0.5	C15 H33 O10
	408.2322	17.1	15.5	C29 H30 O N		413.1986	-10.18	15	C27 H27 O3 N		373.1951	18.36	17.5	C29 H25
	408.2295	23.66	11	C26 H32 O4		413.1892	12.53	2	C16 H31 O11 N		373.1927	24.81	14.5	C27 H26 Na
	408.2506	-28.1	6	C23 H36 O6		413.1876	16.34	16.5	C29 H26 O Na		373.2138	-31.81	9.5	C24 H30 O2 Na
	408.2533	-34.66	10.5	C26 H34 O3 N		413.2017	-17.9	1.5	C17 H33 O11		373.2162	-38.25	12.5	C26 H29 O2
	408.2228	40.08	2.5	C18 H34 O9 N		413.1868	18.35	-1	C14 H32 O11 N Na		373.1857	43.5	4.5	C18 H29 O8
	408.2592	-49.05	1.5	C19 H38 O8 N		413.202	-18.57	3	C18 H32 O8 N Na		373.2197	-47.55	0.5	C17 H34 O7 Na
	408.2169	54.47	11.5	C25 H30 O4 N		413.2044	-24.39	6	C20 H31 O8 N		373.1833	49.95	1.5	C16 H30 O8 Na

Figure 2. LCMS/ESI chromatograms of reference prostratin obtained from sigma.

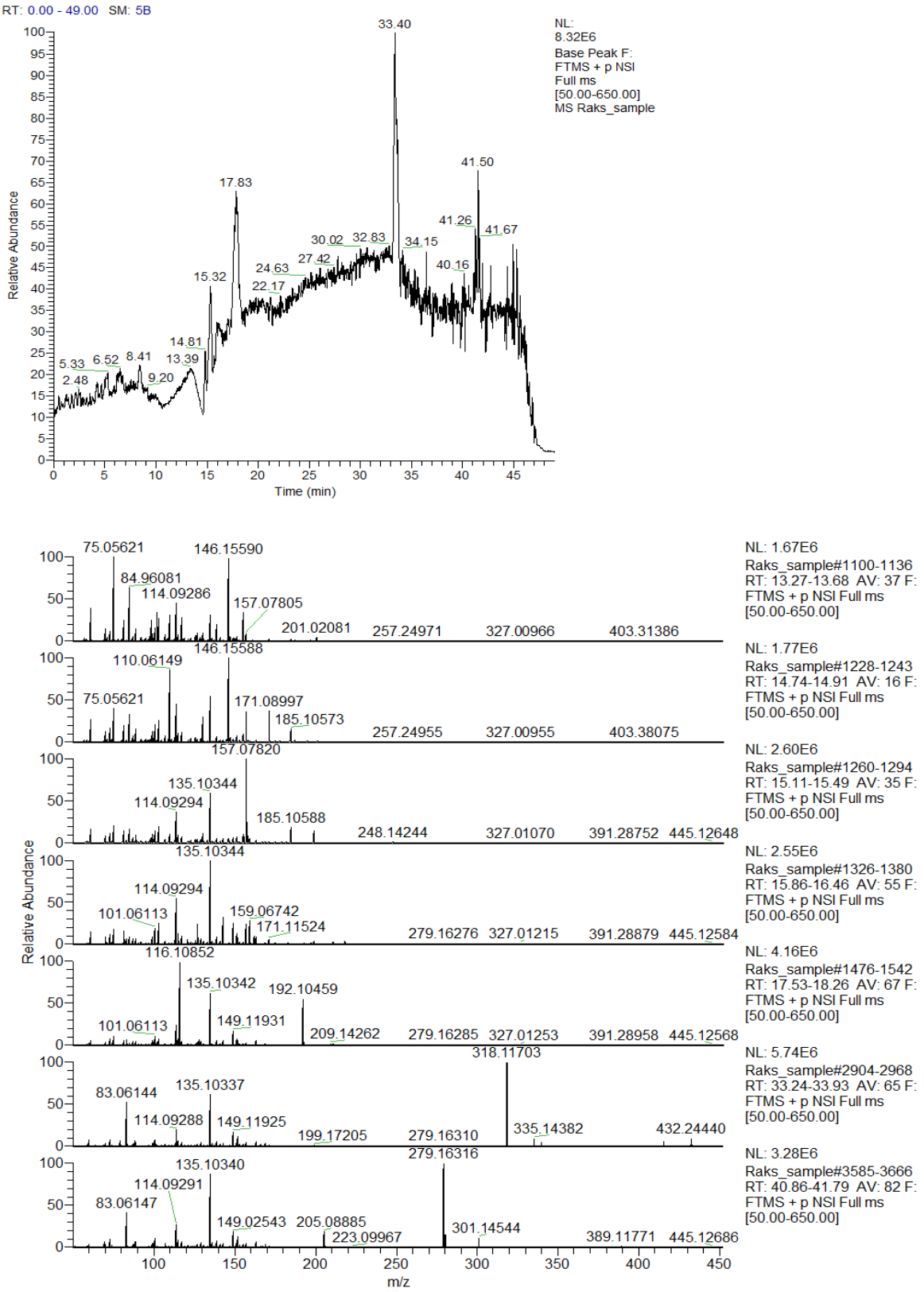


Figure 3. LCMS/ESI chromatograms showing degradation of prostratin synthesized by using Jatropha phorbol esters.

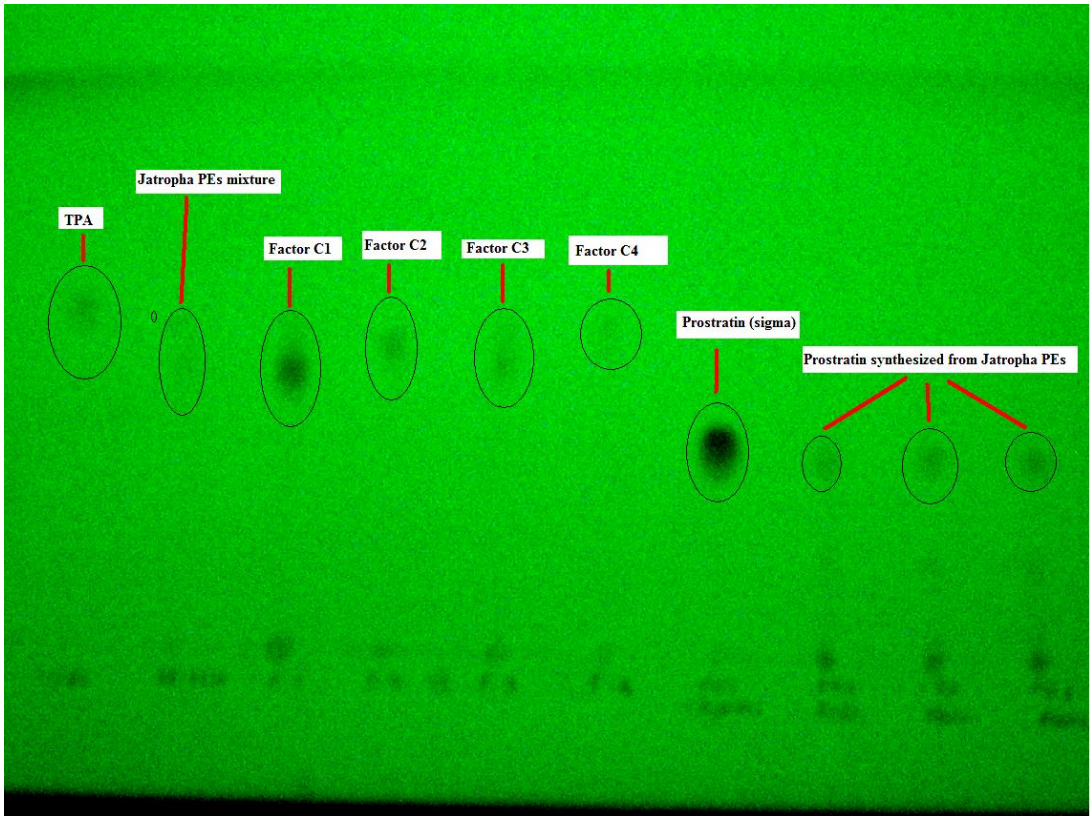


Figure 4. The TLC chromatogram (UV-254 nm) showing Jatropha phorbol esters mix (factor C_1 to C_6), factor C_1 to C_5, phorbol 12-myristate 13-acetate, prostratin (reference compound obtained from Sigma) and the reaction mixture-B of the present study containing prostratin.

Jatropha PEs from PEEF could be used as an intermediate to synthesize prostratin, the material balance was not considered. The oil and the PEEF had 4.7 mg g^{-1} and 53 mg g^{-1} PEs (PMA equivalent) (or 0.11 mg g^{-1} or 1.3 mg g^{-1} as factor C_1 equivalent). After treating the PEEF with barium hydroxide in methanol, crotophorbolone was obtained (~6 − 10 mg). Similarly, the crotophorbolone was also obtained from *Jatropha* PEs by reacting with methanolic sodium methoxide treatment for 2 h at room temperature (Hirota et al., 1988). The crotophorbolone was the starting material for the reaction to synthesise prostratin. In our study, prostratin synthesized from crotophorbolone was recovered after separation by preparative TLC, stored at 4 °C for 3 weeks and then subjected for detection of the mass by the ESI/LCMS. The result showed complete degradation of prostratin obtained from *Jatropha* PEs (Figure 2 and 3). The reason for the degradation was not clear. However, we presume that

degradation might have occurred during the elution from preparative TLC or while storing the sample in a dried form for a long time (3 weeks). Due to limited sample availability to test the mass, we further subjected the 'reaction mixture-B' (which was stored in -80 °C) directly to Nano-LC-ESI-MS/MS. The results showed similar chromatographic peak pattern and had a similar molecular weight to that of the reference prostratin obtained from Sigma (Figure 1 and Figure 5).

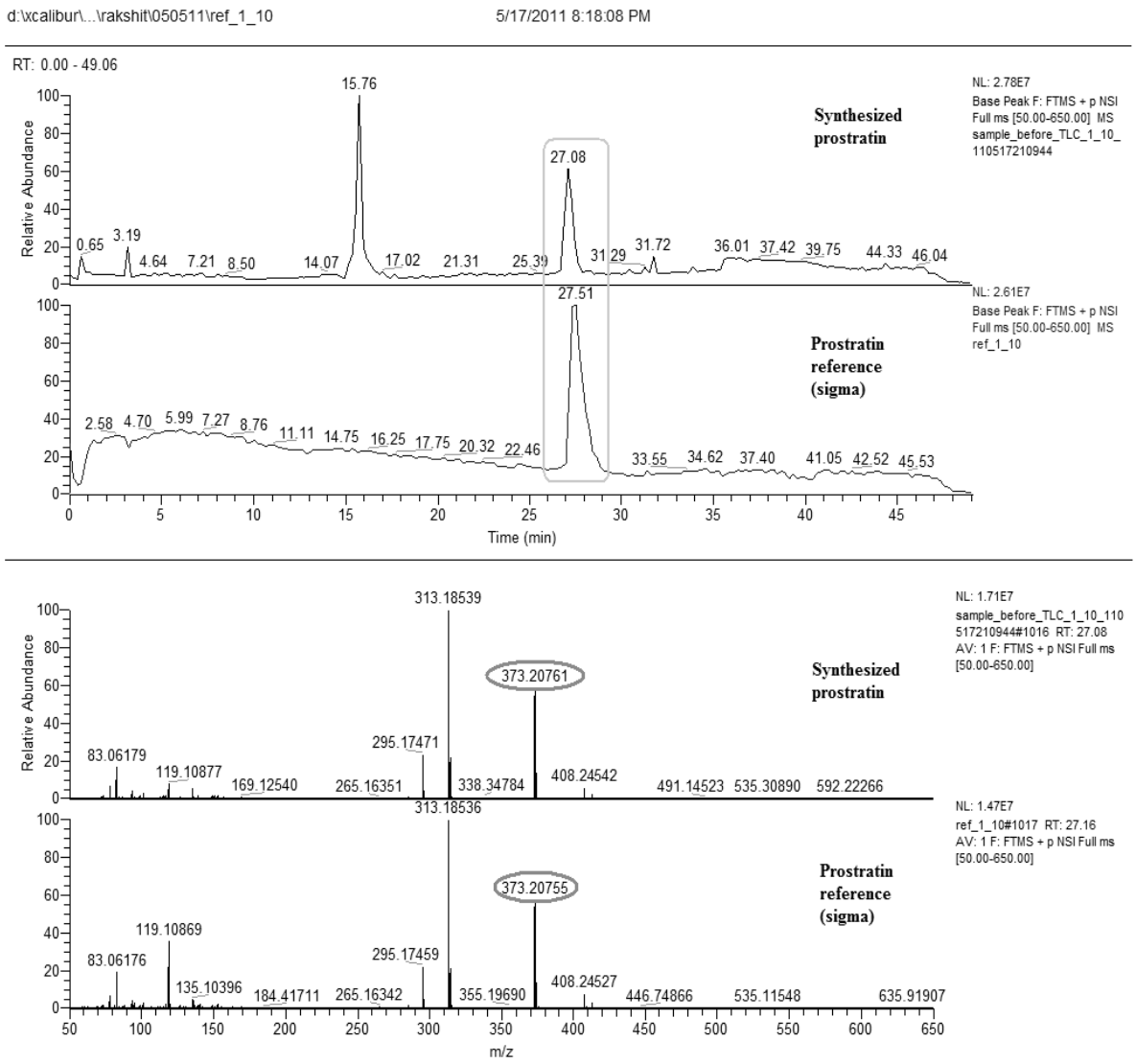


Figure 5. LCMS/ESI chromatograms of reference (prostratin obtained from sigma) and reaction mixture-B containing prostratin (obtained from Jatropha phorbol esters).

4. Conclusion

The results suggest that prostratin can be synthesised from *Jatropha* PEs. However, further optimization of conditions to have higher yield is needed. Our assumption is that the rapid growing *Jatropha* biodiesel industry could yield high amount of oil, about 26 million tons/annum by 2015 (GEXSI, 2008). Considering the average PEs content (our laboratory observation) of 3 − 6 g kg^{-1} oil (PMA equivalent) (or 73 − 145 mg kg^{-1} oil, equivalent to *Jatropha* factor C_1), potential exists for obtaining 79500 − 15900 tonnes (or ~1900 − 3850 tonnes of PEs, equivalent to *Jatropha* factor C_1). Overall, the PEs could be obtained as a byproduct in the process of biodiesel production. This means, there will be no extra burden on the ecosystem for disposing toxic PEs and even the prostratin synthesized from *Jatropha* PEs could be a value added product to the *Jatropha* biodiesel industry.

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References

Cairnes DA, Mirvish SS, Wallcave L, Nagel DL, Smith JW. 1981. A rapid method for isolating phorbol from croton oil. *Cancer Letters, 14,* 85 − 91.

Cox PA. 2001. Ensuring equitable benefits: The Falealupo Convenant and the isolation of anti-viral drug prostratin from a Samoan Medicinal Plant. *Pharmaceutical Biology, 39*, 33 − 40.

Devappa RK, Makkar HPS, Becker K. 2010. Optimization of conditions for the extraction of phorbol esters from *Jatropha* oil. *Biomass Bioenergy*, 34, 1125−1133.

GEXSI. 2008. Available at http://www.*Jatropha*-platform.org/documents/GEXSI_Global-*Jatropha*-Study_FULL-REPORT.pdf

Goel G, Makkar HPS, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity, and toxicity in animals. *International Journal of Toxicology, 26,* 279 − 88.

Gustafson KR, Cardellina JH 2nd, McMahon JB, Gulakowski RJ, Ishitoya J, Szallasi Z, Lewin NE, Blumberg PM, Weislow OS, Beutler, JA, Buckeit RW Jr, Crag GM, Cox PA, Bader JP, Boyd MR. 1992. A nonpromoting phorbol from the Samoan medicinal plant *Homalanthus nutans* inhibits cell killing by HIV-1. *Journal of Medicinal Chemistry, 35,* 1978 – 1986.

Haas W, Sterk H, Mittlebach M. 2002. Novel 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropha curcas. Journal of Natural Products, 65,* 1434 − 1440.

Hirota M, Suttajit M, Suguri H, Endo Y, Shudo K, Wongchai V, Hecker E, Fujiki H. 1988. A new tumour promoter from the seed oil of *Jatropha curcas* L., an intramolecular diester of 12-deoxy-16-hydroxyphorbol. *Cancer Research, 48,* 5800 − 5804.

Johnson HE, Banack SA, Cox PA. 2008. Variability in content of the anti-AIDS drug candidate prostratin in Samoan populations of *Homalanthus nutans*. *Journal of Natural Products, 71,* 2041 − 2044.

Kulkosky J, Culnan DM, Roman J, Dornadula G, Schnell M, Boyd MR, Pomerantz RJ. 2001. Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. *Blood, 98,* 3006 − 3015.

Makkar HPS, Becker K. 2009. *Jatropha curcas,* a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of lipid Science and Technology*, 111, 773 – 787.

Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. *Journal of Agricultural Food Chemistry, 45,* 3152 − 3157.

Makkar HPS, Siddhuraju P, Becker K. 2007. A laboratory manual on quantification of plant secondary metabolites, Humana press. Totowa, New Jersey, pp. 130.

Miana GA, Bashir M, Evans FJ. 1985. Isolation of Prostratin from *Euphorbia cornigera*. *Planta Medica, 51,* 353 − 354.

Szallasi Z, Blumberg PM. 1991. Prostratin, a nonpromoting phorbol ester, inhibits induction by phorbol 12-myristate 13-acetate of ornithine decarboxylase, edema, and hyperplasia in CD-1 mouse skin. *Cancer Research, 51,* 5355 – 5360.

Szallasi Z, Krausz KW, Blumberg PM. 1992. Non-promoting 12-deoxyphorbol 13-esters as potent inhibitors of phorbol 12-myristate 13-acetate-induced acute and chronic biological responses in CD-1 mouse skin. *Carcinogenesis, 13,* 2161 – 2167.

Szallasi Z, Krsmanovic L, Blumberg PM. 1993. Nonpromoting 12-deoxyphorbol 13-esters inhibit phorbol 12-myristate 13-acetate induced tumour promotion in CD-1 mouse skin. *Cancer Research, 53,* 2507 – 2512.

Ventura C, Maioli M. 2001. Protein kinase C control of gene expression. *Critical Review in Eukaryotic Gene Expression, 11,* 243 – 267.

Wender PA, Kee JM, Warrington JM. 2008. Practical synthesis of prostratin, DPP, and their analogs, adjuvant leads against latent HIV. *Science, 320,* 649 − 652.

Wender PA, Warrington J, Kee J. 2009. Process to produce prostratin and structural or functional analogs thereof. United States patent publication number – US 2009/0187046.

CHAPTER - 13

Overall Discussion

This work was initiated to answer the question, whether the phorbol esters (PEs), present in Jatropha oil as the main toxic agent for livestock and aquaculture species, could be utilized for agro-pharmaceutical applications; and if yes, how?

We considered interdisciplinary approaches utilizing chemistry and toxicology in quest to answer the afore-raised questions. The potential of *Jatropha curcas* seed oil as a promising feedstock for biodiesel production is widely accepted (Makkar et al. 1998a; Carels, 2009). In addition to Jatropha oil, the utilization of coproducts such as glycerol, fatty acid distillate and seed cake, among others obtained during biodiesel production could enhance the economic viability of the Jatropha based biofuel industry. However, the possible presence of PEs in these coproducts has been one of the main concerns. The removal of PEs from the Jatropha based products would provide considerable opportunities to better utilize these products. The seed cake, a major coproduct obtained during the biodiesel production is also non edible. The detoxification of seed cake has been achieved and the proteins from detoxified Jatropha kernel meal (DJKM, 60% protein) and detoxified protein isolate (DPI, 90% protein) could replace at least 50% of the protein contributed by the high-quality fish meal (65% protein) in standard fish diet. At this level of DJKM or DPI incorporation, the growth response and nutrient utilization in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) were better than those obtained using soybean meal at the same level of fish meal protein replacement (Devappa et al., 2010a; Kumar et al., 2009). In addition, Jatropha seeds also contain a variety of phytochemicals such as diterpenes, proteins/peptides, alkaloids and lignans (Devappa et al., 2010a; Devappa et al., 2010b; Devappa et al., 2011). Harnessing these highly bioactive phytochemicals and utilizing them in agro-pharmaceutical applications could further increase profitability, economic viability and sustainability of the Jatropha biodiesel industry. In the present work, a group of closely related phytochemicals called phorbol esters (PEs, a tigliane diterpene) was chosen to evaluate their agro-pharmaceutical potential. The reason for choosing this group of compounds (PEs) was that, (a) they exhibit myriad of biological activities in different organisms even at micro molar to nano molar concentrations, (b) they are toxic unwanted biomaterial in the Jatropha oil used for biodiesel

production and its removal from the oil is necessary to avoid occupational exposure or to limit the uncontrolled environmental disposal, and, (c) they can be harnessed in huge quantities, considering the recent growth in cultivation of Jatropha plant. In this thesis, work has been carried out on *J. curcas* phorbol esters, unless it is specified for other species, the term Jatropha stands for *J. curcas* and used throughout in the following sections.

The following discussion covers information about the localization of PEs in the kernel and effective extraction method used to isolate them as a phorbol ester enriched fraction (PEEF). The quality of the biodiesel prepared from the residual oil (after extraction of PEs) is also discussed. In the first part of the discussion, agricultural potential of PEEF has been described wherein information about the biological activity, stability during storage conditions, and soil biodegradability and insecticidal potential of PEEF have been discussed. In the second part of the discussion, the pharmaceutical potential of Jatropha PEs has been described wherein the purification of PEs, their biological effects *in vivo* (mice) and *in vitro* (tissue culture) models and tumour promoting properties have been described. In addition, a procedure to synthesize a potent antitumour and anti-HIV compound (prostratin) from Jatropha PEs has been discussed.

13.1. Localization of phorbol esters and major antinutrients in the Jatropha kernel

To better harness the potential of PEs from Jatropha oil an array of questions were addressed. The first question was: where are the PEs and other major antinutrients localised in Jatropha kernel? And is there a way to rapidly identify PEs?

The knowledge on distribution of toxic and antinutritional components within a seed helps to assess their possible *in situ* roles. Also the distribution information assists in isolation of these components from a seeds for various possible applications. The phytate, trypsin inhibitor and PEs were considered as a major antinutrients and toxic factors in the Jatropha seed (Makkar et al., 1998a and 1998b; Makkar et al., 2008b). Phytate was highest in the endosperm portion comprising 96.5% of the total phytate present in whole kernel, suggesting that the majority of phosphate supply during germination and metabolic activities was contributed by phytate present in the endosperm. It was interesting to note that the trypsin inhibitor was present in all parts of the kernel (cotyledon, kernel coat, hypocotyl and endosperm contained 0.63, 0.002, 0.55 and 24.17 mg/g TIA respectively) and its concentration was also highest in the endosperm region, contributing nearly 95.3% TIA in the

kernel (Chapter 2). The presence of high amounts of phytate and trypsin inhibitors in the seed was also considered responsible for protection against predatory organisms such as insects or microorganisms (Chen Dayi et al., 1995; Ryan, 1990; Wilson, 1980; Halim et al., 1973; Mosolov et al., 1976; Mosolov et al., 1979). The concentration of PEs was highest in kernel coat (1416 mg/100 g kernel coat). The kernel coat constitutes least proportion in the kernel when compared with other kernel parts, contributing minimal amount to the total pool of PEs present in the kernel. The endosperm and seedcoat portion had the highest share in the total pool of PEs present in the kernel with 86% and 11.3% respectively. However, the hard shell surrounding the kernel does not contain PEs but it acts as a primary line of defence against predatory attacks. The localisation of PEs in high concentrations in the endosperm and kernel coat regions suggests that these compounds act as a potent second line of defence to the Jatropha seed, especially during post harvest storage. Overall, endosperm portion was the richest source of major antinutritional factors and toxic factor (PEs) in the kernel.

Based on the presence or absence of PEs in the seed, *J. curcas* was designated as toxic and nontoxic varieties (Makkar et al. 1998a). Currently, PEs are determined using the chromatographic (HPLC) procedures which is time consuming, expensive and requires special piece of equipment and skills. Considering the widespread commercial cultivation of toxic varieties of *J. curcas* and the upcoming cultivation of nontoxic varieties clearly emphasize the need for rapid, cost effective and accurate method to differentiate between toxic and nontoxic genotypes of Jatropha species. In our study, we developed a qualitative method to differentiate between toxic and nontoxic Jatropha genotypes (Chapter 2). It was based on passing the methanol extract from raw kernels/defatted kernel meal through a solid phase extraction (SPE) column and measuring the absorption of SPE-eluate at 280 nm using a UV spectrophotometer. After screening the Jatropha seeds (both toxic and nontoxic genotypes) from different parts of the world, a cut-off absorbance was set up. The cut-off absorbance with ≥0.056 for the SPE eluate from raw kernels was considered as toxic and the cut of absorbance with ≤0.032 s was considered as nontoxic. However, the absorbance of SPE eluate from raw kernels between 0.056 and 0.036 should be confirmed for toxic/nontoxic variety by analysing PEs in HPLC, because at this absorption range both toxic and nontoxic genotypes having low PEs exhibited absorption at 280 nm. Similarly, the cut-off absorbance with ≥0.059 for the SPE eluate from defatted kernels was considered as toxic. And the cut of absorbance with ≤0.043 for the SPE eluate from defatted kernels was considered as nontoxic. The absorbance of SPE eluate from defatted kernels between 0.059

and 0.043 should be confirmed for toxic/nontoxic variety by analysing PEs in HPLC. The method was developed using toxic (from different parts of the world) and Mexican nontoxic genotypes and the validation of the results was done using the pre-established HPLC method. The advantages of the developed qualitative method are that it is easy, affordable and cost effective, and a large number of samples could be analyzed in a short period of time. The developed method could be easily applied for screening the toxicity of products and coproducts obtained from Jatropha biodiesel industry. However, further confirmation of toxic and nontoxic genotypes, especially for food applications must be carried out using the pre-established HPLC method (Makkar et al., 1997) for the identification and quantification of PEs.

13.2. Optimization of phorbol esters extraction and quality of the residual oil as a feedstock for biodiesel production

The Jatropha PEs exhibit myriad of biological activities. Various aqueous and organic solvent extracts of the seeds have shown a wide range of activities from microorganisms to higher animals. For example, many organic solvent extracts (e.g. alcoholic and ether extracts) produced rodenticidal, molluscicidal, insecticidal, antimicrobial, cytotoxic and anti-tumour properties. In addition, oral consumption of the seeds and leaves caused adverse effects in higher animals such as sheep, goat, pig and chicken. In majority of the studies, the biological activity is attributed to the presence of PEs (Devappa et al., 2010b). Considering the high bioactivity of PEs, we further developed methods to harness them from the Jatropha oil. The oil obtained after mechanical extraction of seeds is non edible due to the presence of PEs and cannot be used for edible purposes without detoxification, making it attractive for biodiesel production. The oil is the rich source of PEs. During mechanical extraction, majority of PEs (~70-75%) present in the seed get extracted with the oil fraction (Makkar et al., 2009a). The oil is subjected to many treatments (stripping, degumming and esterification) during biodiesel production wherein PEs present in oil undergo partial or complete destruction depending on the deodorisation conditions (Makkar et al., 2009a). Instead of losing the PEs, if a suitable method can be adopted to extract these esters before the oil is taken to biodiesel production, the PEs could be a valued coproduct, which could contribute to enhancing economic viability and sustainability of Jatropha oil based biodiesel production chain. This is subject to the condition that the extraction of PEs from the oil does not adversely affect the quality of

biodiesel produced. The PEs could find various applications in agriculture, medicine and pharmaceutical industries. In the recent years, increased consumer preference along with global appeal for using renewable natural sources as bio-control agents in conventional agricultural practices has propelled the search for new raw materials. The PEs obtained could meet these requirements for some of the agricultural applications.

In our study, we evaluated the effects of mechanical extractions combined with organic solvent extraction on the yield and biological activity of extracted PEs. In addition the quality of biodiesel produced from the PEs-reduced residual oil was also evaluated. The aim was to integrate the developed method into the established Jatropha biodiesel production processes (Chapter 3). The solvent (methanol), was chosen on the basis of its low cost, easy availability, higher extractability and environmental compatibility. The magnetic bead and ultra turrax were used as tools for the extraction. The methanolic layer obtained after extraction contained PEs, which was further condensed to obtain phorbol ester enriched fraction (PEEF) and the residual oil was virtually free of PEs. The virtually PEs free oil was nontoxic in the snail bioassay (see Chapter 3 and discussion below demonstrating that the snail bioassay is the most sensitive bioassay for detecting PEs) even in the concentrated form (30 times the untreated Jatropha oil, which is equivalent to 30 ppm of PEs (equivalent to PMA; synonym, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)) indicating that recovering PEs from Jatropha oil before processing it to biodiesel would make it innocuous. Both the tools used for the extraction were effective in extracting PEs from Jatropha oil. The PEs present in PEEF were unaffected by the extraction methods and exhibited high biological activity as determined by snail bioassay with an LC_{100} of 1ppm. However, complete removal of PEs from oil takes 60 min, which might be considered long for the industry. So, further studies were considered with the aim to extract maximum PEs in the shortest possible time. This was achieved with a single extraction step wherein 78−80% of PEs were extracted with methanol using both magnetic bead and ultraturrax as a tool at 5 min (55 °C) and 2 min (22−23 °C) respectively. Further, the residual oil (20−22% PEs) obtained by magnetic bead and ultraturrax extraction methods had as good feedstock quality as the untreated Jatropha oil for biodiesel production (Chapter 4). The fatty acid composition of residual oils did not change and both the extraction methods did not exhibit deleterious influence on the fatty acid composition. The biodiesel produced from both the residual oils met European (EN 14214:2008) and American biodiesel standard (ASTM D6751-09) specifications. The oxidative stability indices for both the biodiesels were well the permitted limit. The

advantage of using ultraturrax method over magnetic bead extraction method is that it takes lesser time, lesser solvent and can be done at room temperature. The disadvantage of using ultraturrax is that the method is slightly harsh when compared with the mild extraction as done by magnetic stirring. The biological activity of PEEF obtained by both the shortened methods was unaffected by the extraction methods and exhibited high biological activity in the snail bioassay (L_{100}, 1 ppm).

Conclusively, the study demonstrated that PEs could be easily extracted (up to 78−80%) from Jatropha oil in a short time (2 and 5 min) depending on the tools used for the extraction. The obtained residual oil after PEs extraction is still fit for processing into biodiesel. The extraction method did not affect the biological activity of PEs present in PEEF.

13.3. Approaches used to harness the potential of phorbol ester enriched fraction (PEEF)

After obtaining PEEF from the above mentioned studies (Chapters 2 and 3), we followed two approaches to evaluate, (1) the potential of PEEF as a bio control agents in agriculture, and (2) the potential of utilizing PEEF in pharmaceutical applications.

The preliminary requirement for any successful biocontrol agent is that it should have high bioactivity on the target, prolonged shelf-life and easy biodegradability of active compound for example in soil. In addition, to utilize them at industrial scale the bioactive phytochemical should be available in large quantities and should be easily extractable using environmental friendly methods.

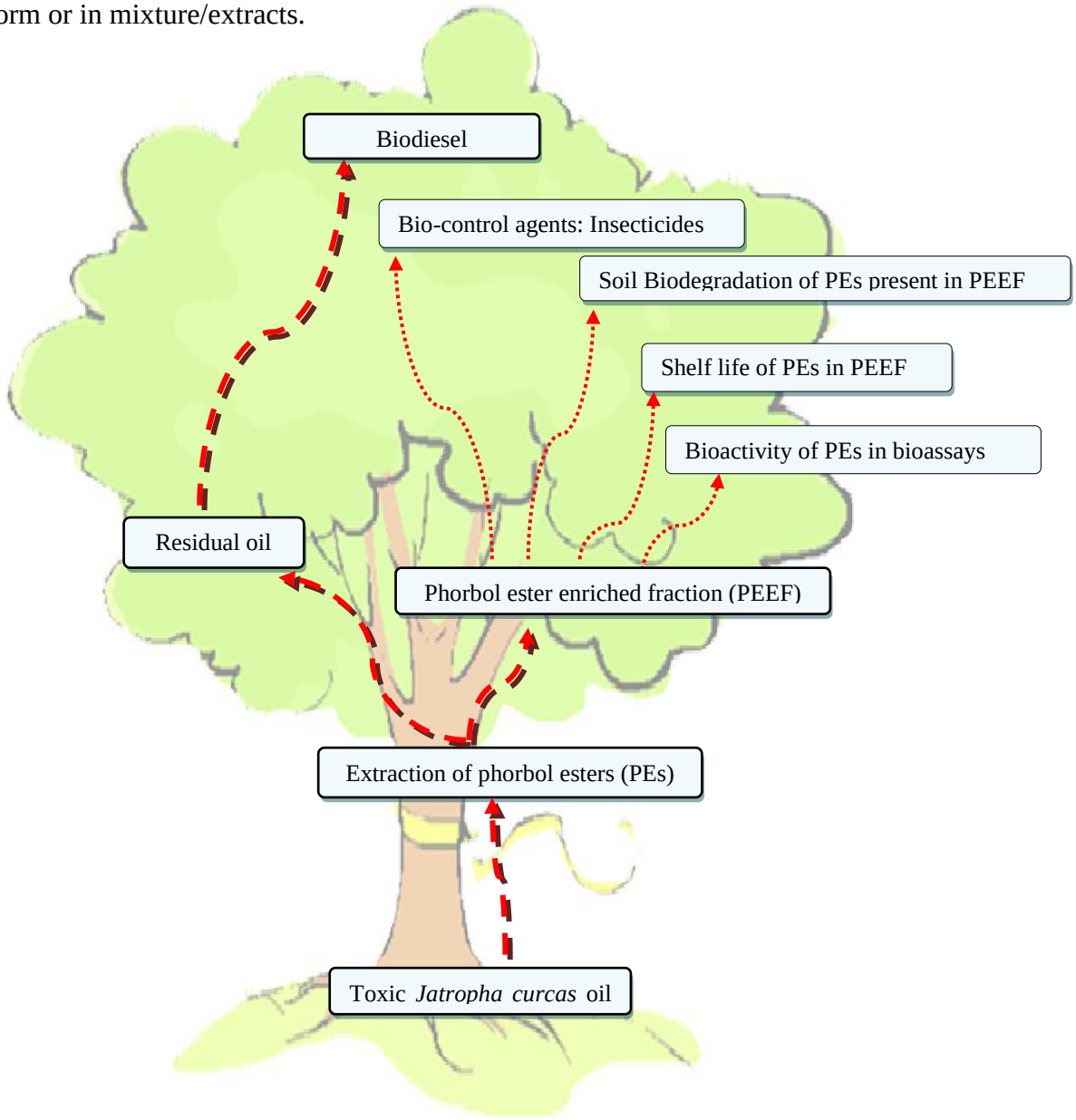
13.4. Approach 1: Agricultural potential of PEEF as an insecticide

In this section the potential of PEEF is discussed for its biological activity, shelf life, degradability in soil and insecticidal activity.

13.4.1. Biological activity of phorbol ester enriched fraction (PEEF)

Despite the advances in chromatographic and spectroscopic techniques, majority of the natural product chemists or quality control specialists in developing countries involved in Jatropha biodiesel production chain either lack expertise/infrastructure for biological screening or often long waiting time is required for screening of samples for biological

activity when sent to other laboratories. It is therefore highly desirable to use simple bioassays that are specific, rapid, accurate, valid, reproducible, cost effective, and low-skill requiring. Most of the aforesaid requirements for rapid screening of phytochemicals are better met by *in vitro* tests than the time consuming *in vivo* tests (Gutleb et al., 2004; Vanden berghe and Vlietinck, 1991). The biological test systems/bioassays may provide useful and rapid screening methods for measuring potency of toxic phytochemicals either in purified form or in mixture/extracts.



Approach 1

In our study, the biological activity of the extracted PEEF was evaluated in three aquatic bench top bioassays (snail (*Physa fontinalis*), brine shrimp (*Artemeia salina*), daphnia (*Daphnia magna*)) and microorganisms (Chapter 5). The PEEF was potent in all the bioassays and the potency increased with increase in the concentrations of PEs, but sensitivity of the bioassays for PEs varied among the tested organisms. The PEEF exhibited an EC_{50} (48 h, PMA equivalent) of 0.33, 26.5 and 0.95 ppm PEs for snail, artemia and daphnia respectively. The susceptibility of bacterial and fungal species was low towards PEs (EC_{50}>50 ppm) when compared with the aquatic bioassays. The minimum inhibitory concentration (MIC) for *Streptococcus pyogenes* and *Proteus mirabilis* was 215 ppm PEs. When compared with bacterial species, *Fusarium* species of fungi was more sensitive with an EC_{50} of 58 ppm PEs. Among the tested bioassays, the snail bioassay was most sensitive with 100% snail mortality at 1 µg of PEs/ml. Similarly, many studies have been reported wherein organic solvent extract from Jatropha seed or oil exhibited molluscicidal, antibacterial and antifungal activities. The activity was attributed to the presence of PEs (Rug and Ruppel, 2000; Liu et al., 2003; Devappa et al., 2009; Saetae and Suntornsuk, 2009)

All the bioassays (*P. fontinalis*, *A. salina*, *D. magna* and micro organisms) used in our study were easy to handle and can be conducted with little resources. The snail bioassay has been the most sensitive. This bioassay could be used to monitor the presence of PEs in various Jatropha derived products such as oil, biodiesel, kernel meal, cake and latex. It could also be used for detecting the presence of PEs in soil and other matrices in the ecosystem interacting with Jatropha production and use chain. In addition, the study has also demonstrated that the PEs exhibit moluscicidal, antifungal and antibacterial activities. Thus, the extracted PEEF could also serve as a natural molluscicide, fungicide and bactericide against snails, fungal phytopathogens, pathogenic bacteria or as insecticide, which in turn could be used for agricultural and pharmaceutical applications.

13.4.2. Shelf life of phorbol ester enriched fraction (PEEF)

Despite the potency and possible applicability of PEs present in the PEEF as a bio-control agent, the stability of these compounds (PEs) is an important characteristic for their use in various applications. Generally, the activity of biochemicals either in pure form or in formulations tends to decrease gradually with time. Therefore, it is essential to monitor and improve the storage stability of any phytochemicals for its effective use. The stability is

determined for a wide variety of pure substances or their end user products. It is well documented that the biological activity of PEs differs widely from one source to another. Jatropha PEs are reported to be unstable and difficult to isolate in the pure form (Haas et al., 2002). However, we anticipated that the stability of PEs in the form of an enriched fraction would be higher than that of the purified PEs. Our long term aim was to utilize PEEF extracted from *J. curcas* oil in suitable agricultural applications as a biocontrol agent. In our study (Chapter 6), the stability and efficacy of the extracted PEEF was evaluated over a long period of storage time under defined laboratory conditions; at room temperature (RT), 4 °C and -80 °C for 870 days. The PEEF was unstable at room temperature exhibiting 50% degradation of PEs after 132 days when compared with PEEF stored at 4 °C and -80 °C, wherein 9% and 4% degradation was observed at the same time. There was rapid reduction in the biological activity of PEEF when stored at RT, becoming ineffective after 260 days. Whereas, biological activity decreased slowly for the PEEF stored at 4°C and -80°C (at day-870), retaining up to 27.5% and 32.5% of bioactivity respectively. However, the biological activity values did not correlate with the PEs content in the PEEF. The results indicated that even though PEs are present in the PEEF (as quantified by the HPLC), there was a gradual reduction in the bioactivity of the PEEF. The reduction in the efficacy or biological activity of the PEEF may be due to alterations in the chemical nature or structural features responsible for biological activities of the PEs. The loss in biological activity was largely attributed to the degradation of PEs. The degradation of PEs was found to be due to auto-oxidation. The auto-oxidation of PEs was reflected with the changes in fatty acid composition, increase in peroxide value and reduction in inherent free radical scavenging activity. Similarly, Schmidt and Hecker (1975) reported that purified PMA (in acetone) when stored at cold conditions (4 °C) decomposed within 3 months and the degradation was higher under diffused daylight (25 °C) within 3 months. The 7-hydroperoxide was formed as a major decomposed product. They suggested that solutions stored in the refrigerator be wrapped in black foil.

In our study, the supplementation of synthetic antioxidants was found to increase the protection of PEs present in the PEEF against degradation. The potency of synthetic antioxidants was initially screened with a newly developed chemical oxidation assay wherein chemical Vazo-67 was mixed with the PEEF to achieve rapid PEs degradation. The main advantage of this method is that the efficacy of antioxidants can be evaluated in a shorter time and under conditions which are closer to the actual storage conditions when compared with

generally used the rancimat or oxidative stability instruments (Shah et al., 2009) to determine oxidative stability index (OSI), wherein the test samples are exposed to stream of air at elevated temperatures (100−130 ºC). Our developed assay was used as a tool to eliminate ineffective antioxidants. The PEEF supplemented with effective antioxidants (as screened by chemical oxidation assay (Vazo-67)) was observed for stability of PEs up to 132 days (wherein 50% degradation of PEs was observed without the addition of antioxidants). The effective concentration (in mg) to protect one mg of PEs against oxidation were in the order: butylated hydroxy anisole (BHA; 0.09) > baynox (0.11) > α-tocopherol (0.13) > Tertiary butylhydroquinone (TBHQ; 0.18) > butylhydroxytoluene (BHT; 0.25). However, the effective concentrations of antioxidants used to protect PEs against oxidation in the PEEF were well above the usual concentrations (500−1000 ppm) used.

The PEs present in the PEEF were most susceptible to oxidation at room temperature when compared with cold temperatures (4 °C and -80 °C). The stability of PEs could be increased by supplementation of effective additives such as butylated hydroxyanisole (BHA), baynox and α-tocopherol. However, further stability studies should be carried out with additive formulations containing combinatorial antioxidants which can stabilize the PEs present in the PEEF at low concentrations.

13.4.3. Biodegradation of phorbol ester enriched fraction (PEEF) in soil

The Jatropha seed cake is rich in nitrogen and could be used as a fertilizer. However, the users and experts have shown concern about the application of seed cake as a fertilizer due to presence of PEs in the cake and the possible impact of the applied cake as a fertilizer and its disposal in the environment on the microbial communities, insects, invertebrates and plant/animal communities. Rug and Ruppel, (2000) quoted a report by Koschmieder (personal communication) that PEs of *J. curcas* decomposes completely within 6 days; however, no quantitative information was provided nor the experimental conditions reported. There is no authentic study describing the fate of Jatropha PEs in the environment. In addition, when toxic Jatropha oil or cake is exposed to environment there will always be a chance of leaching toxic components into the soil, which can cause damage to local biota. Thus, studying the persistence of the PEs in soil was necessary. In our study, the biodegradability of PEs was evaluated using (a) Jatropha expeller pressed seed cake (10% residual oil) containing PEs and, (b) PEEF containing PEs adsorbed to silica (Chapter 7).

The PEEF (oily extract) was adsorbed to silica to mix homogenously with the soil. Both the test samples were mixed with soil and observed for PEs degradation at different temperature and moisture levels. At zero day, concentration of PEs (PMA equivalent) in soil was 2.6 and 0.37 mg/g for PEEF adsorbed silica and seed cake in soil respectively. PEs from silica bound PEEF were completely degraded after 19, 12, 12 days (at 13% moisture) and after 17, 9, 9 days (at 23% moisture) at room temperature (RT), 32 °C and 42 °C respectively. Similarly at these temperatures PEs from seed cake were degraded after 21, 17 and 17 days (at 13% moisture) and after 23, 17, and 15 days (at 23% moisture).

In the snail (*Physa fontinalis*) bioassay, mortality by PE-amended soil extracts decreased with decrease in PEs concentration in soil. However, the biological activity of both seed cake and PEEF bound to silica at 32 °C and 42 °C was reduced by <50% within 2−3 days (at both 13% and 23% moisture level in the soil). The biological activity of both seed cake and PEEF bound to silica in soil was reduced by <50% at 8−9 and 2−3 days respectively at RT (both at 13% and 23% moisture level). Our study demonstrated that PEs in soil are completely degraded by microbial enzymes involving oxidation as one of the intermediate steps and the produced intermediate oxidized products are further broken down rapidly to form nontoxic products. The degradation pattern of peaks as observed in the HPLC was similar to the degradation products observed by chemical oxidation assay (see Chapter 6). The results demonstrate that oxidation is one of the intermediate steps involved in microbial degradation. However, from the results it was plausible to conclude that the degradation of PEs would be greater in tropical humid soils when compared with the temperate soils.

Overall, the study indicates that Jatropha PEs are easily biodegradable in soil and the degraded products are innocuous. If the cake or the PEEF is applied as a fertilizer or used as a bio-control agent on a repeated basis, rapid microbial degradation is more likely due to build up of the PE-degrading microbes. As the population of these organisms increases, degradation would further accelerate and the amount and exposure of the toxic moieties available for causing toxicity to living organisms will be limited. Further studies on evaluation of ecotoxicity of PEs, particularly extent of leaching of PEs from different soils, effects of PEs and their degraded products on water channels and water bodies should be conducted.

13.4.4. Potential of phorbol ester enriched fraction (PEEF) as an insecticide

The pests infesting economically important crops inflict marked losses in the agrarian production. The presence of insects has been recognised as one of the most serious agricultural problems. In agriculture, insect control is usually achieved by using agrochemicals (insecticides), often belonging to different classes of organic compounds, in large amounts. This poses serious problems to human/animal health and aggravates environmental pollution. Conversely, the usage of biological control agents offer the advantage of being compatible with the environment, often with high specificity, and represent a long-term solution for controlling insects that are particularly resistant to organic chemical based controlling agents. Therefore, several efforts have been made to control insects using natural bio-control agents, such as plant phytochemicals. In our study, preliminary work on the potential of the PEEF as an insecticide was investigated. The insect (*Spodoptera frugiperda*) was chosen as a pest because of its wide presence in corn fields in the tropical/subtropical countries such as Mexico and Brazil. In addition it is known to cause considerable damage to maize crop.

The PEEF exhibited contact toxicity with an LC_{50} of PEEF of 0.83 mg/ml (w/v; PMA equivalent) (Chapter 8). However, no effect was observed for the PEs-rich extract (purified phorbol ester mixture, see Chapter 9) at the tested concentrations (0−20 mg/ml). The ineffectiveness of the PEs-rich extract was due to degradation of PEs (results are discussed in chapter 9) when stored as dried residue (4 months at 4 °C). Since the PEs-rich extract was ineffective, only the PEEF was evaluated for antifeedant activity against *S. frugiperda*. When compared with control, dietary intake by *S. frugiperda* was severely affected upon feeding corn leaves treated with the PEEF (0.0625 to 0.25 mg/ml). At higher concentration of the PEEF (0.25 mg/ml, w/v), food consumption and relative growth rate, food conversion efficiency (FCE) was reduced by 33%, 42% and 38% respectively. The relative consumption rate (RCR) was slightly decreased (6%) at 0.25 mg/ml of PEEF; but higher decline was observed at 0.625 mg/ml and 0.125 mg PE/ml of PEEF (39 and 45% respectively). In our study, the adverse effect of the PEEF on feeding and growth of *S. frugiperda* was evident (Chapter 8) indicating the potential of PEEF as a bio-control agent. Similarly, several studies have reported the potential of aqueous or solvent extracts from Jatropha (leaves, root, bark and oil) as bio control agents (Devappa et al. 2011; Devappa et al., 2010b)). The application of Jatropha oil or its organic solvent extracts was found insecticidal against many insect

species such as *Callosobruchus maculates, Lipaphis erysimi*, *Preris rapae, S. oryzae* L., and *S.zeamais, Busseola fusca* and *Sesamia calamistis, Phthorimaeaoperculefla* larvae (potato tuber moth), *Manduca sexta* (tobacco hornworm larvae), *Coptotermes vastator* (Philippine milk termite), *Mycus persicae, Tetranychus urtica*e (spider mite), *Periplaneta Americana* (American cockroach), *Blatella germanica* (German cockroach) and *Oncopeltus fasciatus* (milkweed bug) (Solsoloy, 1995; Jing et al., 2005; Makkar et al., 2007; Shelke et al., 1985 and 1987; Sauerwein et al., 1993; Wink et al., 1997; Acda, 2009). In all the aforementioned reported studies wherein the organic solvent extracts and Jatropha oil was tested, the PEs are suggested to be the active component.

It is evident from our study that the PEEF has potential for use as a bio-control agent. Further in depth field experiments on the effects of the PEEF on *S. frugiperda* will pave the way for its use under field conditions.

13.5.1. Approach 2: pharmaceutical potential of Jatropha phorbol esters

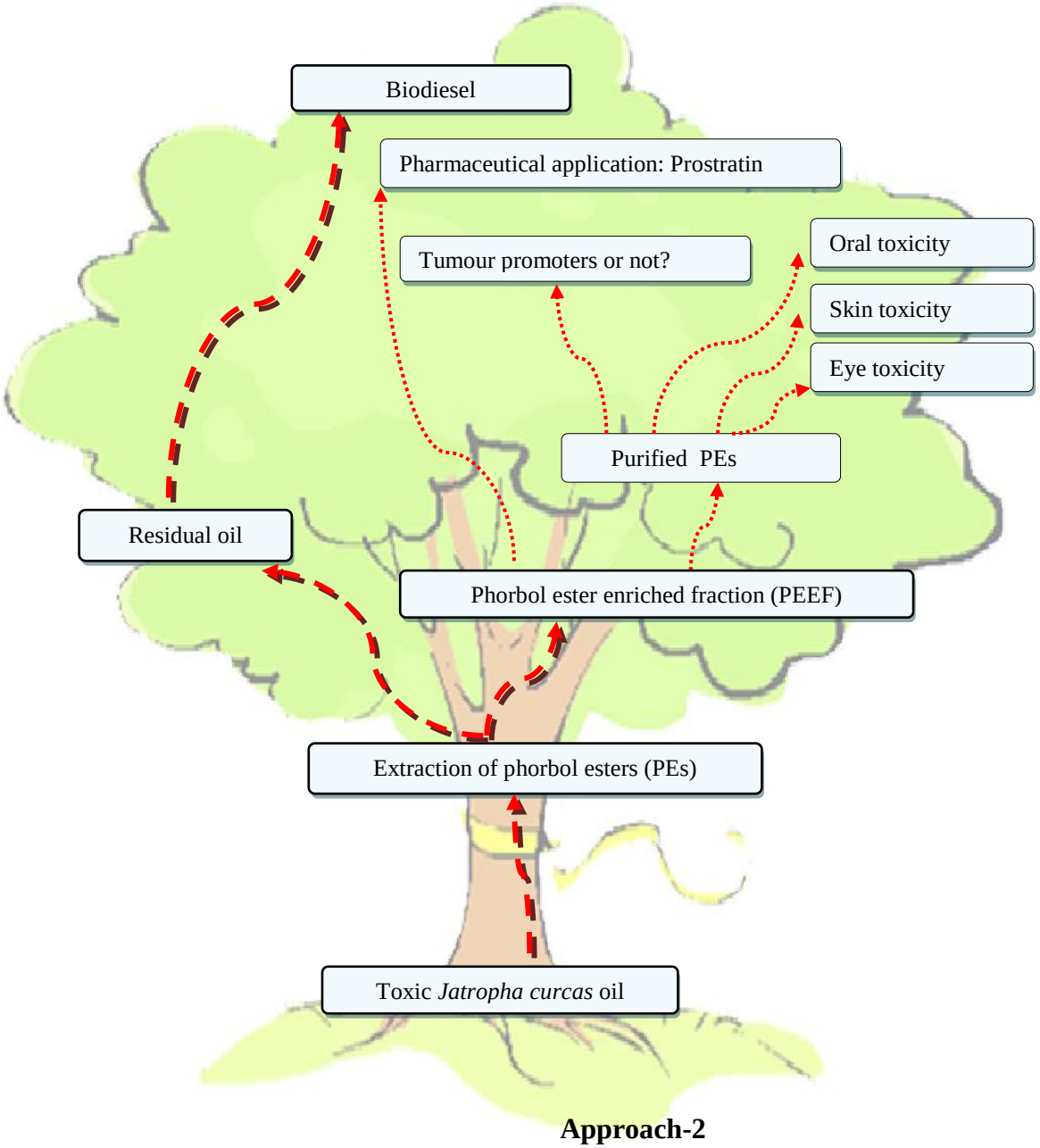
The work addressed a number of questions: whether we can easily purify PEs from the Jatropha oil? If yes, are they (PEs) biologically active and stable in purified form?, Are all purified Jatropha PEs from Jatropha oil is toxic and tumour promoters? Can these be used in pharmaceutical formulations? If not, how can we better utilize the Jatropha PEs?.

We considered interdisciplinary approaches utilizing chemistry, toxicology and biochemistry in quest to answer the afore-raised questions.

13.5.2. Isolation and characterization of phorbol esters from Jatropha curcas oil

The Jatropha oil (904 g) was extracted with methanol to get the PEEF, which was further passed through silica gel column to obtain PEs-rich extract (0.26 g). Further, the PEs-rich extract was subjected to semi-preparative HPLC (reverse-phase C_{18}) and sephadex LH-20 column to obtain purified PEs (Chapter 9). From the PFE (2.0 g), the *Jatropha* factors C_1 (63 mg) and C_2 (18 mg) obtained were confirmed by 1D (^{1}H, ^{13}C) and 2D (HSQC, COSY, TOCSY, HMBC) NMR and compared with data published by Haas et al. (2002). The Jatropha factors $C_{3mixture}$ (35 mg) and the (C_4+C_5) mixture (58 mg) needed further purification. Generally, PEs are quantitatively expressed equivalent to PMA, which is obtained from croton oil. However, comparison of peak areas for PMA and *Jatropha* factor

C_1 in the HPLC gradient elution method (Makkar et al., 1997) showed a difference in sensitivity of absorption at 280 nm by a factor of 41.3. In the percentage of total area contributed by total Jatropha PEs, the factor C_1 is the predominant PE (51%), and hence it is suggested that the concentration of Jatropha PEs should be expressed equivalent to factor C_1. Consequently, the data from here onwards are expressed as *Jatropha* factor C_1 equivalents.



Approach-2

In snail bioassay, the order of activity based on LC_{50} (µg/litre) was: Jatropha factor (C_4+C_5) > factor C_1> factor C_2 > factor $C_{3mixture}$ > PEs- rich extract. In the artemia bioassay

the order of activity based on LC_{50} (ppm) was: factor (C_4+C_5) > factor C_1> factor $C_{3mixture}$ > factor C_2. The high biological activity in snail and artemia bioassay indicated that there was no drastic influence of the extraction methods used during the purification on the PEs. In addition all the Jatropha PEs exhibited platelet aggregation *in vitro* and the order of potency based on ED_{50} (μM) was: Jatropha factor (C_4+C_5) > factor C_1 > factor $C_{3mixture}$ > factor C_2 > PMA.

In our earlier studies (Chapter 6), the PEEF containing PEs exhibited low stability when stored at room temperature and slow degradation at cold temperatures (4 °C and -80 °C). The degradation of PEs in PEEF was due to autooxidation. However, the stability was increased with the addition of antioxidants. Further, we presumed that purification of PEs from PEEF (or methanol extract of oil) would increase the bioactivity as well as stability. The PEs-rich extract was obtained from the PEEF (or methanol extract of oil) and its shelf life was conducted both in dried form and by dissolving in ethanol. When stored as a dried form, the degradation of PEs was severe and reached 100% within 3 months. At -20 °C, the degradation of PEs was slow (20% after 1 year). When the PEs-rich extract was stored in ethanol, there was no change in the PEs content even after 1 year. However, the evaporation of solvent was observed in our study. Therefore, tightly sealed and preferably brown vials should be used for storage.

Conclusively, the PEs could be easily purified from Jatropha oil, retaining high biological activity. When stored as dried form, the stability of PEs-rich extract was low. Alternatively, the storage should be done by dissolving the PEs-rich extract in organic solvents such as ethanol and preferably stored at cold conditions (4 °C and -20 °C).

13.5.3. Pharmaceutical potential of purified Jatropha phorbol esters

Generally, the potential medicinal compounds are exposed through oral, topical, ocular or intravenous routes. Thus, finding out the compatibility or toxicity of Jatropha PEs (factor C_1, factor C_2, factor $C_{3mixture}$, factor (C_4+C_5) and PEs-rich extract) towards skin, eye or oral exposure is essential. In the following subsections the results from oral exposure studies in mice, *in vitro* compatibility studies in reconstructed human epithelium (RHE) and human corneal epithelium (HCE) models and *in vitro* tumour promotion assay using Bhas 42 cells are discussed.

13.5.4. Effect of purified Jatropha phorbol esters in mice – intragastric exposure

In the past, all the LD_{50} studies, toxicity studies or the histopathological studies were conducted using either *J. curcas* oil, seed, defatted seed cake or crude fractions from them; and the results were assumed to be due the presence of PEs. Our study was aimed to evaluate the oral compatibility of Jatropha PEs in mice. The purified PEs-mixture from *J. curcas* oil (collected by HPLC) was toxic to mice on intra gastric administration. The LD_{50} limits for male mice was 27.34 mg/kg body mass (PMA equivalent; 0.66 mg/kg body mass as Jatropha factor C_1 equivalent) (Chapter 10.1). The symptoms and toxicity of PEs-mixtures varied and increased with increase in the exposed dose. The major symptoms observed in the affected animals were depression, languishment, loss of body mass, closing of eyes, humidity of anus. Both small and large intestines were filled with black digesta. It is supposed that the gastro-intestinal tract haemorrhage may result in the black digesta in intestines. The findings showed that the digestive system of mice is sensitive to the presence of PEs.

The prominent pathological symptoms included congestion of sinus hepaticus, haemorrhage of spleen, glomerular atrophy, congestion of the pulmonary alveolar capillaries, few mice exhibited haemorrhage and burst of alveolus. At the highest dose of 36.00 mg/kg (PMA equivalent or 0.87 mg/kg body mass as Jatropha factor C_1 equivalent), multiple abruptions of cardiac muscle fibres and anachromasis of cortical neurons appeared. Other histopathological changes included the frequent appearance of fatty vacuoles in the liver cells, hyperaemia and exudate in spleen, diffuse haemorrhage and exudates in lung, and glomerular sclerosis. Overall, the prominent pathological symptoms were mainly observed in lung and kidney. In addition, all the reported feeding studies on *J. curcas* showed severe clinical and pathological symptoms. Among the important symptoms observed was transient loss of body mass and mild to severe macroscopic/microscopic changes in the kidney, lungs, heart, liver, and spleen in a dose dependent manner (Adam, 1974; Liberalino et al., 1988; Rakshit et al., 2008). The toxicity results from our studies support the findings of Makkar et al. (1997 and 1998ab) that PEs are the main toxins in *J. curcas* oil and seeds. Generally, PEs are present in leaves, stems, flowers , bark and roots of *J. curcas* (Makkar and Becker, 2009b) and therefore the consumption of *J. curcas* in any form, oil, seeds, seed cake or extracts is toxic to animals, and elicits severe pathological symptoms.

The result demonstrated that Jatropha PEs are toxic and the major target organs are lungs, kidney and digestive system. The oral exposure to PEs either accidentally or in the form of

drug should be avoided. In addition, the data obtained in this study would aid in developing: (i) emergency safety procedures, (ii) guidelines for the use of appropriate safety clothing and equipment, and (iii) transport regulations. It would also help in establishing occupational exposure limits and in developing material safety data sheets for various byproducts obtained during biodiesel production from *J. curcas.*

13.5.5. Effect of purified Jatropha phorbol esters in cultured skin and eye tissue (in vitro) – topical exposure

During the process of biodiesel production, the PEs present in Jatropha oil are completely degraded. However, in early steps of transesterification PEs were still detected in the acid gums and in disposed water washings (Makkar et al., 2009a). Considering the rapid growth in Jatropha cultivation and in biodiesel production from Jatropha oil, the accidental contact of workers with Jatropha based products (especially when using oil or extracts from Jatropha products containing PEs) could occur in the working environment. In spite of high production and plausible applications of Jatropha, knowledge on the potential impacts of toxic Jatropha oil on human health and environment is still fragmentary. The study was conducted using reconstructed human epithelium (RHE) and human corneal epithelium (HCE) to assess the possible effects of purified PEs from Jatropha oil on skin and eyes (Chapter 10.2). These models mimic the human skin and corneal epithelium respectively.

In RHE, all the tested PEs containing samples [toxic oil containing 2.42 ng of PEs (*Jatropha* factor C_1 equivalent), PEs-rich extract (contains factor C_1 to C_5; collected from HPLC, see chapter 10.1) containing 2.42 ng of PEs, or purified individual PEs [C_1 (1.3 ng), C_2 (0.5 ng), $C_{3mixture}$ (0.4 ng) and $C_4 + C_5$ (0.25 ng); these are the amounts of individual PEs in 2.42 ng of a mixture of PEs as present in the oil] exhibited severe tissue alterations such as tissue necrosis, marked oedema, less viable cells layers or partial tissue disintegration. In addition, increase in the inflammatory responses, such as IL-1α and PGE_2 were observed. Generally, the production of IL-1α and PGE_2 has been observed in a wide variety of tissues and their increased secretion was observed during pathological conditions including inflammation and many cancers. The order of toxicity observed for IL-1α was: purified PEs-rich extract > toxic oil > factor C_2 > factor (C_4+C_5) > factor C_1 > factor $C_{3mixture}$. While the order of toxicity observed for PGE_2 was: purified PEs-rich extract > factor (C_4+C_5) > factor $C_{3mixture}$ > factor C_1> toxic oil > factor C_2. When compared with toxic Jatropha oil, the

histological alterations and production of IL-1α and PGE_2 were less evident by the nontoxic oil and these effects were comparable to those produced by 70% ethanol (a commonly used hand sanitizer). In general, PEs present in Jatropha seed are easily soluble in petroleum ether, methanol, hexane, dichloromethane and they can be extracted using these solvents. These PEs containing extracts exhibited similar histological changes *in vivo* (Gandhi et al., 1995) as observed in our studies.

In HCE, all the tested PEs containing samples at the same concentrations as for the RHE exhibited severe alterations such as marked cellular alterations/less viable cell layers, partial tissue necrosis and/or partial tissue disintegration of supra basal cell layers. Although the severity of toxicity was less compared with the toxic oil and purified PEs, the nontoxic oil (equivalent to the volume used for toxic oil) did exhibit alterations (necrosis) in some areas and marked cellular alterations in HCE. The results indicate that the Jatropha oil even if it is nontoxic caused damage to corneal epithelial cells and the severity of toxicity increased with the presence of PEs. In addition, all the PEs treated tissue increased the IL-1α production. The order of inflammatory response was: PEs-rich extract > toxic oil > factor C_2 > factor $C_{3mixture}$ > factor C_1 > nontoxic oil > factor (C_4+C_5) > vehicle (0.1% DMSO). However, the PGE_2 production in HCE could not be conclusively correlated with the test samples as there was high variability between the treated HCE cultures.

Our results showed that the presence of PEs in oil increases its toxicity (upon exposure) towards exposed tissues (both RHE and HCE) and we presume that the activation of PKC could be one of the mode of action through which Jatropha PEs exhibits cellular alterations and inflammatory response. Thus, the direct contact with toxic Jatropha oil or PEs containing Jatropha products should be avoided. It is advised to use the protective gloves and glasses when handling PEs containing Jatropha products. The study also demonstrated that application of Jatropha PEs in medicinal formulation either by topical or oral route could cause adverse effects at tissue and cellular levels. In addition, the data obtained in this study would aid in developing safety procedures, guidelines and establishing occupational exposure limits for processing and handling various coproducts obtained during biodiesel production from *J. curcas* oil.

13.5.6. Are all purified Jatropha phorbol esters from Jatropha oil are tumour promoters?

Despite the plausible applications of Jatropha products and coproducts, the presence of toxic PEs in the oil used for biodiesel production and in the coproducts raises environmental health and human exposure concerns. In *J. curcas*, six PEs have been identified (Haas et al., 2002). Several reports indicate that generally all PEs are not toxic and their biological activity depends on their structural configuration (Silinsky and Searl, 2003). The non tumour promoting PEs have also been reported. They have at least one activity of phorbol compounds such as binding to PKC receptors but they do not have tumour promoting properties. The examples of non-tumour promoting PEs include 12-deoxyphorbol 13-acetate (prostratin), 12-deoxyphorbol 13-propanoate and 12-dexoxy phorbol 13-phenylacetate (Xu et al., 2009). In *in vivo*, the extracts from Jatropha oil containing PEs exhibited toxicity and tumour promotion properties. However, knowledge on the biological activity and potential applications of purified individual PEs are limited. We presumed that all Jatropha PEs might not be tumour promoters. Therefore, we conducted *in vitro* studies to identify whether all the PEs present in Jatropha oil are tumour promoters or not. Generally, mouse skin tumour promotion is a well defined model involving two stages of carcinogenesis, initiation stage and promotion stage (Berenblum, 1975). Exposure to single dose of an initiator compounds (at low doses) or of a promoter compound does not cause tumour formation. But tumour formation occurs with the repeated application of the promoter compound after a single application of initiator compound, indicating that proper order of exposure to initiator and promoter compounds is necessary to cause tumour. As the humans are exposed to different environmental factors in their life time, often in low doses, it is possible that similar synergism may occur, causing tumours. The potential of a compound for causing tumour is very much dependent on the type and dose of other substances (e.g. 3-methylcholanthrene (MCA)) that an individual is exposed to (Yamasaki and Weinstein, 1985).

In our study an *in vitro* Bhas 42 cells transformation assay was used to evaluate the tumour promoting potential (Chapter 11). The advantage of using this assay is that it does not require initiator treatment like other assays (e.g. SHE and Balbct3 cells) do and it involves two stages carcinogenesis steps as the *in vivo* models (Berenblum, 1975). The concentration of Jatropha PEs in this study was expressed equivalent to Jatropha factor C_1. In this assay, the PEs-rich extract (0.00005–2 µg/ml; collected from HPLC method, for details see Chapter 10.1) and individual purified Jatropha PEs (factor C_1, factor C_2, factor $C_{3mixture}$ and factor

(C_4+C_5)) (0.0005–1 µg/ml) exhibited tumour promoting properties. Similarly, the reference compound PMA also exhibited tumour promoting properties. The order of transformed foci/well formation was: PMA > PEs-rich extract > factor (C_4+ C_5) > factor $C_{3mixture}$ > factor C_1 > factor C_2. However, none of the Jatropha PEs exhibited tumour initiation properties. Majority of the reported *in vivo* tumour promotion and toxicity studies conducted using Jatropha PEs or PEs from other plant sources, either in purified form or in extracts, suggested that the activity is mediated by the activation of PKC enzyme. In our study, PMA (1 µg), PEs-rich extract (2 µg), factor C_1 (1 µg), factor C_2 (1 µg), factor $C_{3mixture}$ (1 µg) and factor (C_4+C_5) (1 µg) induced the hyper activation of PKC, as measured by nonradioactive peptag protein kinase C assays kit (Promega) (Chapter 11). Similar results were observed by Horiuchi et al. (1987), wherein methanol extract from Jatropha oil and one purified Jatropha PE (DHPB (12-deoxy-16-hydroxyphorbol-4'-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0]hexane-(13-O)-2'-[carboxylate]-(16-O)-3 '- [8'-butenoic-10']ate);which was later re-characterized as factor C_1 by Haas et al. (2002)) produced tumour promotion in mice following treatment of an initiator compound (7, 12-dimethylbenz[a]anthracene (DMBA)) and activated PKC *in vitro*. Wink et al. (2000) have also reported the hyper activation of PKC by DHPB (or factor C_1). In addition Horiuchi et al. (1987) have reported that DHPB or factor C_1 is more toxic, but has weak tumour promoter activity compared with PMA. In our study, including factor C_1 all the other Jatropha Factors exhibited tumour promotion properties *in vitro*. A number of reports suggest that the effects of PMA are through the mediation of PKC (especially in skin) and the events that best correlate with skin tumour promoting properties of PEs (PMA) are dermal inflammatory response, induction of epidermal ornithine decarboxylase activity followed by increased polyamine levels, induction of epidermal hyperplasia and dark basal keratinocytes (Di Giovanni et al., 1988). Similarly, in our earlier studies (Chapter 10.2) the topical application of purified Jatropha PEs to RHE and HCE *in vitro* increased the release of inflammatory indicator substances such as IL-1α and PGE_2.

All the Jatropha PEs when tested in purified form exhibited tumour promotion activity *in vitro*. The tumour promotion activity was mediated by the activation of PKC. Further validation of these *in vitro* results using *in vivo* models should be conducted. The use of protective gloves to avoid direct contact with Jatropha oil or Jatropha based products and coproducts are suggested.

13.5.7. Synthesis of Prostratin from phorbol esters enriched fraction (PEEF)

The results from the above studies demonstrated that Jatropha PEs are toxic when administered orally or when applied topically to skin or eye tissues (Chapter 10.1, Chapter 10.2 and Chapter 11). Thus, Jatropha PEs cannot be used directly in pharmaceutical formulations. However, the PEs could be used either by, (a) structural modification which could reduce its toxicity, without reducing biological activity or by, (b) converting them into a new compound which is nontoxic and still has high biological activity against the target.

In our study, we carried out preliminary studies wherein Jatropha PEs present in the PEEF was used to synthesize a new compound called prostratin. Prostratin (12-deoxyphorbol-13-acetate) is a tigliane diterpene and is a potent PKC activator; unlike PEs it does not cause tumour formation. Prostratin has been found to be promising adjuvant in antiviral therapy. Many antiviral treatments have been successful in decreasing the active viral pool in AIDS patients (highly active antiretroviral treatment (HAART)), but the persistence of latent viral reservoirs limits the complete viral eradication. Prostratin activates this latent virus pool (Wender et al., 2008) in turn acting as an adjuvant in HIV therapy. Wender et al. (2008) suggested that Jatropha PEs could be a good candidate for producing highly bioactive compound prostratin. They synthesized prostratin from croton oil PEs and postulated that Jatropha PEs could also be used as a feedstock. The main difference between the Jatropha PEs and croton oil PEs is the basic skeleton. The croton oil PEs has "phorbol" (e.g. PMA) and Jatropha PEs has "12-deoxy phorbol" as the basic skeleton. Thus, slightly different approach was used with Jatropha PEs. Wender et al. 2008 postulated that Jatropha PEs could be converted into crotophorbolone, an intermediate compound in the prostratin synthesis reaction. In our preliminary study, Jatropha PEs were converted into crotophorbolone by using the method Cairnes et al. (1981) and the crotophorlone was used to synthesize prostratin using the method of Wender et al. (2008). The prostratin synthesized from Jatropha PEs was analyzed by Nano-LC-ESI-MS/MS. The mass and the retention time of the peak of the test sample resembled to that of reference prostratin obtained from Sigma (St. Louis, USA). As this is the preliminary study, we were not able to determine the material balance. However, the study certainly demonstrated that Jatropha PEs could be a potential feedstock for synthesizing prostratin.

Our study showed that prostratin could be synthesized from Jatropha PEs. However, further optimization studies are required to ascertain the synthesis reactions and yield of prostratin synthesized from Jatropha PEs.

Conclusion

Jatropha seeds are promising feedstock of oil for biodiesel production. In our study, attempt has been made to obtain PEs as a coproduct from the Jatropha biodiesel production chain and to evaluate the potential of isolated phorbol ester enriched fraction (PEEF) in agro-pharmaceutical applications. The main conclusions are:

- The majority of toxic compounds (PEs) are localised in the endosperm portion of the kernel and these PEs could be rapidly identified using newly developed qualitative method which uses the combination of solid phase extraction and UV spectroscopy. The method could be easily adopted in Jatropha biodiesel industry to screen the toxic and nontoxic varieties of Jatropha seeds and to check the presence of PEs in various coproducts and ecosystems surrounding the Jatropha biodiesel production chain.
- The PEs from the Jatropha oil could be easily isolated using methanol to get phorbol ester enriched fraction (PEEF) and the residual oil. The extracted PEEF is biologically active and the biodiesel prepared from the residual oil meets the European and American biodiesel standards. In addition, the residual oil is less toxic (~78−80%) when compared with the untreated Jatropha oil. The PEs extraction process can be easily integrated into the Jatropha biodiesel production chain.
- The PEEF exhibit high biological activity when screened by different bioassays (*Physa fontinalis, Artemia salina, Daphnia magna* and micro organisms). Among the assays tested, the snail bioassay is most sensitive. The PEs in PEEF have strong molluscicial, antibacterial and antifungal activities. The snail bioassays are sensitive (LC_{100}, 1ppm), easy to handle and can be used to monitor the presence of PEs in various Jatropha derived products (e.g. oil, biodiesel, kernel meal, cake and latex), contaminated soil and other matrices in the ecosystem interacting with Jatropha production and use chain.
- The PEs present in the PEEF are most susceptible to oxidation at room temperature and their stability could be increased either by inclusion of antioxidants (butylated

hydroxyl anisole, baynox and α-tocopherol) or by storing at cold temperature (-80 °C).

- The Jatropha PEs are easily biodegradable in soil and the degraded products are innocuous. The PEs present in the PEEF and seed cake are completely degradable within 9−19 days and 15−23 days respectively, when mixed in soil. The PEs degradation increases with increase in temperature and moisture content of the soil.
- The PEEF exhibit contact toxicity and antifeedant activity against *S. frugiperda*, which is a common pest in corn fields suggesting potential agricultural applications of PEs as insecticide.
- The PEs from Jatropha oil could be purified to homogeneity. The ratio of PMA (generally used as a reference for quantification of PEs using HPLC) to factor C_1 was 41.3 to 1, indicating higher sensitivity of Jatropha PEs in the HPLC at 280 nm. As the factor C_1 is predominant among the Jatropha PEs, factor C_1 could be used for expressing the concentration of PEs as equivalent to factor C_1. The purified PEs are highly active as determined by the snail and artemia based bioassays and they exhibit platelet aggregation property *in vitro*.
- The intra gastric exposure of Jatropha PEs to mice elicit toxicity (LD_{50}, 0.66 mg/kg body mass, factor C_1 equivalent or 27.34 mg/kg body mass, PMA equivalent) and the major affected organs are lungs, kidney and digestive system. The oral exposure to PEs either accidentally or in the form of drug should be avoided.
- The PEs present in oil or purified PEs are toxic towards reconstituted human epithelium and human corneal epithelium (both RHE and HCE) with marked cellular alterations and release of inflammatory substances (IL-α and PGE_2).
- All the Jatropha PEs (factor C_1, C_2, $C_{3mixture}$, (C_4+C_5) and PEs-rich extract) exhibit tumour promotion property *in vitro*. The tumour promotion activity was mediated by the activation of PKC.

The toxicity studies demonstrated that use of Jatropha PEs in medicinal formulation could cause adverse effects at tissue and cellular levels. The direct contact with toxic Jatropha oil or phorbol ester containing Jatropha products should be avoided. However, it should be noted that for the past 16 years no negative or harmful effect was observed in any of the vast number of workers in our laboratory. These workers have handled Jatropha oil, seed, kernel meal and other Jatropha products for periods ranging from months to years. Nevertheless, it is advised to use the protective

gloves and glasses when handling PEs-containing Jatropha products. The data obtained from toxicity studies reported in this study would aid in developing safety procedures, guidelines and occupational exposure limits for processing and handling Jatropha oil and various coproducts obtained during biodiesel production.

Alternatively, our preliminary studies indicated that Jatropha PEs could be used as an intermediate for synthesizing prostratin, which is a promising adjuvant in anti-HIV therapy.

For any successful bio-control agent, among others, the preliminary requirement is its high bioactivity on the target organism, increased shelf-life and biodegradability in soil. In addition, the bioactive phytochemical should be available in large quantity with continuous supply and should also be easily extractable. The PEEF potentially satisfies aforesaid requirements. The abundance and novelty of PEs present in Jatropha species could form a new 'stock' for the agro-pharmaceutical industries. The maximum utilization of these biomolecules could only be possible if the agro-pharmaceutical industries get their continuous supplies in the future. In recent years, increased interest in the utilization of non edible Jatropha seed oil as a feedstock for biodiesel production has propelled many developing countries to cultivate Jatropha at industrial scales.

By 2015, approximately 12.8 million hectares of land worldwide is expected to be under Jatropha cultivation and an estimated oil yield of 26 million tons/annum is projected. This would generate a huge amount of raw materials for both biodiesel and pharmaceutical industries indicating the opportunity that would enable to harness huge quantities of PEs (~ >1900 tonnes, equivalent to Jatropha factor C_1). The harnessed PEs in the form of the PEEF could be used either as insecticide in agricultural applications or as a feedstock for synthesizing prostratin in pharmaceutical applications. Thus, integration of our optimized methods during biodiesel production could increase sustainability of the Jatropha biodiesel chain.

Future perspectives

The results reported in this thesis enlarge the possibilities of utilizing Jatropha phorbol esters (PEs) as a value added co-product for agro-pharmaceutical applications. However, to

translate the laboratory knowledge generated through this work to industrial/field applications the following investigations are required:

- The integration of optimized methods for PE isolation from Jatropha oil in the industrial production of biodiesel so that PEs could be isolated before the oil is taken for biodiesel production.
- Screening for effective antioxidants and developing economically viable formulations would facilitate the storage stability of PEs present in the phorbol ester enriched fraction (PEEF) and use of the PEEF for various agro-pharmaceutical applications.
- The standard operating procedures for storage, use and disposal of the PEEF should be established.
- Evaluation of the PEEF in various pest models (e.g. *Spodoptera frugiperda* and *Callosobruchus maculates*) and disease causing vectors (e.g. snail for schistosomiasis, mosquitoes and houseflies) both *in vitro* and in field experiments could increase the applicability of the PEEF as a bio control agent.
- The fate of Jatropha PEs in the environment such as adsorption to various matrices and transfer and degradation in the environment should be evaluated. The transfer processes in the field such as volatilization, runoff, spray drift, absorption, leaching and crop removal that could move the PEs away from the target site should be investigated. The breakdown process such as chemical breakdown, microbial breakdown and photo-degradability of Jatropha PEs should be addressed.
- When used as a bio-control agent, the effect of PEs on non-target micro and macro biota should be evaluated.
- The studies covering genotoxic effect of Jatropha PEs would be useful in preparing industrial safety procedures.
- Studies utilizing the Jatropha PEs as an intermediate in the synthesis of nontoxic phorbol ester analogues (e.g. prostratin) and their utilization in pharmaceutical applications would increase the economic viability of Jatropha biodiesel production chain.
- The regulations and the policies established at the local and regional level would give ample opportunity for the symbiotic existence among agro-pharmaceutical-biofuel industries, which in turn could open new avenues for the sustainable eco-friendly development.

References

1. Acda MN. 2009. Toxicity, tunneling and feeding behavior of the termite, *Coptotermes vastator*, in sand treated with oil of the Physic nut, *Jatropha curcas*. *J.* Insect Sci 6:1–8.
2. Achten WMJ, Mathijs E, Verchot L, Singh VP, Aerts R, Muys B. 2007. *Jatropha* biodiesel fueling sustainability?. Biofuels Bioprod Bior 1:283−291.
3. Achten WMJ, Verchot L, Franken YJ, Mathijs E, Singh VP, Aerts R, Muys B. 2008. Jatropha bio-diesel production and use. Biomass Bioenerg 32:1063−1084.
4. Adam SEI. 1974. Toxic effects of *Jatropha curcas* in mice. Toxicology 2:67–76.
5. Aiyelaagbe OO, Adesogan EK, Ekundayo O, Adeniyi BA. 2000. The antimicrobial activity of roots of *Jatropha podagrica* (Hook). *Phytother Res* 14:60−62.
6. Areces LB, Kazanietz MG, Blumberg PM. Close similarity of baculovirus-expressed n-chimaerin and protein kinase C alpha as phorbol ester receptors. J Biol Chem 269:19553−19558.
7. ASTM (American Society for Testing and Materials). 2008. Standard specification for biodiesel fuel blend stock (B100) for middle distillate fuels, ASTM D6751-09. In: ASTM Annual Book of Standards. American Society for Testing and Materials, West Conshohocken. http://www.astm.org)
8. Augustus GDPS, Jayabalan M, Seiler GJ. 2002. Evaluation and bioinduction of energy components of *Jatropha curcas*. Biomass Bioenerg 23:161–164.
9. Azam MM, Waris A, Nahar NM. 2005. Prospects and potential of fatty acid methyl esters of some nontraditional seed oils for use as biodiesel in India. Biomass and Bioenerg 29:293−302.
10. Berenblum, I. 1975. Sequential aspects of chemical carcinogenesis: Skin. In: Becker, F.F., ed., Cancer: A Comprehensive Treatise, Vol. 1, New York, Plenum Press, pp. 323−344.
11. Berry EW. 1929. An eogene tropical forest in the Peruvian desert. Proc Natl Acad Sci U S. A. 15:345–346.
12. Bertolini TM, Giorgione J, Harvey DF, Newton AC. 2003. Protein kinase C translocation by modified phorbol esters with functionalized lipophilic regions. J Org Chem 68:5028–5036.

13. Betz A, Ashery U, Rickmann M, Augustin I, Neher E, Südhof TC, Rettig J, Brose N. 1998. Munc13–1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. Neuron 21:123–136.
14. Beutler JA, Ada AB, McCloud TG, Cragg GM. 1989. Distribution of phorbol ester bioactivity in the euphorbiaceae. Phytother Res 3:188–192.
15. Bohm R, Flaschentrager B, Lendle L. 1935. Arch Exp Pathol Pharmakol 177:212.
16. Brittaine R, Lutaladio N, 2010. Jatropha; A smallholder bioenergy crop, the potential for pro-poor development. Integrated Crop Management Vol. 8. http://www.fao.org/docrep/012/i1219e/i1219e.pdf
17. Brynes PJ, Schmidt R, Hecker E. 1980. Plasminogen activator induction and platelet aggregation by phorbol and some of its derivatives: Correlation with skin irritancy and tumour-promoting activity. J Cancer Res Clin Oncol 97:257–266.
18. Cairnes DA, Mirvish SS, Wallcave L, Nagel DL, Smith JW. 1981. A rapid method for isolating phorbol from croton oil. Cancer Lett 14:85-91.
19. Caloca MJ, Fernandez N, Lewin NE, Ching D, Modali R, Blumberg PM, Kazanietz MG. 1997. β2-Chimaerin is a high affinity receptor for the phorbol ester tumour promoters. J Biol Chem 272:26488–26496.
20. Carels N. 2009. *Jatropha curcas*: A Review. Adv Bot Res 50:39−86.
21. Chen Dayi, Ling X and Rong Y. 1995. Phytic acid inhibits the production of Aflatoxin B1. J Food Process Pres 19:27−32.
22. Chu DT, Granner DK. 1986. The effect of phorbol esters and diacylglycerol on expression of the phosphoenolpyruvate carboxykinase (GTP) gene in rat hepatoma H4IIE cells. J Biol Chem 261:16848–16853.
23. Clemens MJ, Trayner I, Menaya J. 1992. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci 103:881–887.
24. Devappa RK, Bhagya S. 2008a. Biochemical and nutritional evaluation of Jatropha protein isolate prepared by steam injection heating for reduction of toxic and antinutritional factors. J Sci Food Agri 88:911–919.
25. Devappa RK, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya, S. 2008b. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas*) in rats. Food Chem Toxicol 46:3621−25.
26. Devappa RK, Makkar HPS, Becker K. 2011. Jatropha Diterpenes: a Review. J Am Oil Chem 88:301−22.

27. Devappa RK, Makkar HPS, Becker K. 2010a. Nutritional, biochemical, and pharmaceutical potential of proteins and peptides from Jatropha: review. J Agric Food Chem 58:6543−55.
28. Devappa RK, Makkar HPS, Becker K. 2010b. Jatropha toxicity – A review. J Toxicol Environ Health B Crit Rev 13:476−07.
29. Devappa RK, Rajesh SK, Swamylingappa B. 2009. Antioxidant and antibacterial Properties of Jatropha *(Jatropha curcas)* meal Extracts. J Food Sci Technol Nepal 5:73−81.
30. Devappa RK, Swamylingappa B. 2007. Effect of processing methods on the removal of toxic and antinutritional constituents of Jatropha meal: a potential protein source. J Food Sci Technol Nep 3:88–95.
31. DiGiovanni J, Kruszewski FH, Coombs MM, Bhatt TS, Pezeshk A. 1988. Structure-activity relationships for epidermal ornithine Decarboxylase induction and skin tumour promotion by anthrones. Carcinogen. 9:1437−1443.
32. Duncan RR, Betz A, Shipston MJ, Brose N, Chow RH. 1999. Transient, phorbol ester-induced DOC2-Munc13 interactions *in vivo*. J Biol Chem 274: 27347–27350.
33. Ebinu JO, Bottorff DA, Chan EYW, Stang SL, Dunn RJ, Stone JC. 1998. RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 280:1082–1086.
34. European Committee for Standardization (CEN). 2008. Automotive fuels fatty acid methyl esters (FAME) for diesel engines requirement methods EN 14214:2008. European Committee for Standardization (CEN), Brussels
35. Freire, FCO, Parente GB. 2006. As doenc¸as das Jatrofas (*Jatropha curcas* L. e J. podagrica Hook.) no estado do Ceara´. Comunicado Te´cnico Embrapa 120. http://www.cnpat.embrapa.br/home/down/index.php?pub/cot_120.pdf.
36. Gandhi VM, Cherian KM, Mulky MJ. 1995. Toxicological studies on ratanjyot oil. Food Chem Toxicol 33:39–42.
37. Garnayak DK, Pradhan RC, Naik SN, Bhatnagar N. 2008. Moisture-dependent physical properties of Jatropha seed (*Jatropha curcas* L.). Ind Crop Prod 27:123−129.
38. GEXSI. 2008. Available at http://www.Jatropha-platform.org/documents/GEXSI_Global-Jatropha-Study_FULL-REPORT.pdf
39. Goel G, Makkar HPS, Francis G, Becker K. 2007. Phorbol esters: structure, biological activity and toxicity in animals. Int J Toxicol 26:279−288.

40. Gutleb AC, Ropstad E, Brandt I, Murk AJ. 2004. *In Vitro* bioassays – valuable tools contributing to the conservation of endangered species. IUCN Otter Spec Group Bull 21A
41. Haas W, 2003. Isolation and characterization of the phorbol esters from *Jatropha curcas* seed oil. PhD thesis submitted to karl-Franzens-Universitat graz
42. Haas W, Strerk H, Mittelbach M. 2002. Novel 12 deoxy-16-hydroxyphorbol diesters isolates from the seed oil of *Jatropha curcas*. J Nat Prod 65:1434–1440.
43. Halim AH, Wassom CE, Mitchel HL, Edmunds LK. 1973. Suppression of fungal growth by isolated trypsin inhibitors of corn grain. J Agric Food Chem 21:1118−1119.
44. Halver JE. 1978. Aquaculture development and coordination programme. Fish feed technology. Lectures presented at the FAO/UNDP Training Course in Fish Feed Technology, Seattle, Washington, 9 October - 15 December. Series title: Project reports (not in a Series) - ADCP/REP/80/11 1980 p. 400 pg
45. Hasan MR. 2001. Nutrition and feeding for sustainable aquaculture development in the third millennium. In R.P. Subasinghe, P. Bueno, M.J. Phillips, C. Hough, S.E. McGladdery & J.R. Arthur, eds. Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium, Bangkok, Thailand, 20−25 February 2000. pp. 193-219. NACA, Bangkok and FAO, Rome.
46. Horiuchi T, Fujiki H, Hirota M, Suttajit M, Suganuma M, Yoshioka A, Wongchai V, Hecker E, Sugimura T. 1987. Presence of tumour promoters in the seed oil of *Jatropha curcas* L. from Thailand. Jpn J Cancer Res 78:223–226.
47. Jing L, Fang Y, Ying X, Wenxing H, Meng X, Syed M N, Fang C. 2005. Toxic impact of ingested Jjatropherol-I on selected enzymatic activities and the ultrastructure of midgut cells in silkworm, *Bomboxy mori* L. J Appl Entomol 129:98−104.
48. Jongschaap REE, Corre WJ, Bindraban PS, Brandenburg WA. 2007. Claims and facts on *Jatropha curcas* L. Plant Research International B.V., Wageningen, The Netherlands. http://www.fact-fuels.org/media_en/Claims_and_Facts_on_Jatropha_-WUR?session = isgsklbna58j7grrfst888n5r7.
49. Karaj S, Joachim Mueller. 2008. Physical, Mechanical and Chemical Properties of *Jatropha curcas.* http://www.tropentag.de/2008/abstracts/full/322.pdf
50. Karaj S, Müller J. 2009. Optimization of mechanical extraction of *Jatropha curcas* seeds. Landtechnik 64:164−167.

51. Kazanietz MG, Caloca MJ, Eroles P, Fujii T, García-Bermejo ML, Reilly M, Wang H. 2000. Pharmacology of the receptors for the phorbol ester tumour promoters: multiple receptors with different biochemical properties. Biochem Pharmacol 60:1417−24.
52. Kinzel V, Richards J, Goerttler K, Loehrke H, Furstenberger G, Marks F. 1984. Interaction of phorbol derivatives with replicating cells. IARC Sci Publ 56:253–264.
53. Kochhar S, Singh SP, Kochhar VK. 2008. Effect of auxins and associated biochemical changes during clonal propagation of the biofuel plant – *Jatropha curcas*. Biomass Bioenerg 32:1136–1143.
54. Kumar A, Sharma S. 2008. An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. Ind Crop Prod 28:1–10.
55. Kumar V, Makkar HPS, Becker K. 2009. Detoxified *Jatropha curcas* kernel meal: an excellent fish meal replacer in common carp (*Cyprinus carpio* L.) diet; Tropentag: Hamburg, Germany, 2009.
56. Liberalino AAA, Bambirra EA, Moraes-Santos T, Viera CE. 1988. *Jatropha curcas* L. seeds. Chemical analysis and toxicity. Braz Arch Biol Technol 31:539–550.
57. Liu SY, Sporer F, Wink M, Jourdane J, Henning R, Li YL, Ruppel A. 1997. Anthraquinones in *Rheum palmatum* and *Rumex dentatus* (Polygonaceae), and phorbol esters in *Jatropha curcas* (Euphorbiaceae) with molluscicidal activity against the schistosome vector snails *Oncomelania, Biomphalaria,* and *Bulinus*. Trop Med Int Health 2:179–188.
58. Makkar H, Maes J, De Greyt W, Becker K. 2009a. Removal and degradation of phorbol esters during pre-treatment and transesterification of *Jatropha curcas* oil. J Am Oil Chem Soc 86:173–181.
59. Makkar HPS, Aderibigbe AO, Becker K. 1998a. Comparative evaluation of non-toxic and toxic varieties of *Jatropha curcas* for chemical composition, digestibility, protein degradability and toxic factors. Food Chem 62:207−215.
60. Makkar HPS, Becker K, Schmook B. 1998b. Edible provenances of *Jatropha curcas* from Quintana Roo state of Mexico and effect of roasting on antinutrient and toxic factors in seeds. Plant Foods Hum Nutr 52:31−36.
61. Makkar HPS, Becker K, Sporer F, Wink M. 1997. Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J Agric Food Chem 45:3152−3157.

62. Makkar HPS, Becker K. 2009b. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. Eur J lipid Sci Technol 111:773–87.
63. Makkar HPS, Francis G, Becker K. 2007. Bioactivity of phytochemicals in some lesser known plants and their effects and potential applications in livestock and aquaculture production systems. Animal 1:1371–1391.
64. Makkar HPS, Francis G, Becker K. 2008b. Protein concentrate from *Jatropha curcas* screw-pressed seed cake and toxic and antinutritional factors in protein concentrate. J Sci Food Agric 88:1542–1548.
65. Makkar HPS, Martinez Herrera J, Becker K. 2008a. Variations in seed number per fruit, seed physical parameters and contents of oil, protein and phorbol ester in toxic and nontoxic genotypes of *Jatropha curcas*. J Plant Sci 3:260–265.
66. Maruyama IN, Brenner S. 1991. Phorbol ester/diacylglycerol-binding protein encoded by the *unc-13* gene of *Caenorhabditis elegans*. Proc Natl Acad Sci USA 88:5729–5733.
67. Mosolov VV, Loginova MD, Fedurkina NV, Benken II. 1976. The biological significance of proteinases inhibitors in plants. Plant Sci Lett 7:77−80..
68. Mosolov VV, Loginova MD, Malova EL, Benken II. 1979. A specific inhibitor of *Colletotrichum lindemuthianum* protease from kidney bean (*Phaseolus vulgaris*) seeds. Planta 144:265−269 (1979).
69. Nishizuka Y. 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.
70. Raju AJS, Ezradanam V. 2002. Pollination ecology and fruiting behaviour in a monoecious species, *Jatropha curcas* L. (Euphorbiaceae). Current Sci 83:1395–1398.
71. Rakshit KD, Darukeshwara J, Rathina Raj K, Narasimhamurthy K, Saibaba P, Bhagya S. 2008. Toxicity studies of detoxified Jatropha meal (*Jatropha curcas)* in rats. Food Chem Toxicol 46:3621–3625.
72. Ron D, Kazanietz MG. 1999. New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J 13:1658–1676.
73. Ruane J, Sonnino A, Agostini A. 2010. Bioenergy and the potential contribution of agricultural biotechnologies in developing countries. Biomass Bioenerg 34:1427−1439.
74. Rug M, Ruppel A. 2000. Toxic activities of the plant *Jatropha curcas* against intermediate snails and larvae of schistosomes. Trop Med Int Health 5:423−430.

75. Ryan CA, Protease inhibitors in plants: Genes for improving defence against insects and pathogens. Annu Rev Phytopathol 28:425−49 (1990).
76. Saetae D, Suntornsuk W. 2010. Antifungal activities of ethanolic extract from *Jatropha curcas* seed cake. J Microbiol Biotechnol 20:319–324.
77. Saturnino HM, Pacheco DD, Kakida J, Tominaga N, Gonsalves NP. 2005. Cultura do pinha˜o-manso (*Jatropha curcas* L.). Informe Agropecua´rio 26:44–78.
78. Sauerwein M, Sporer F, Wink M. 1993. Insect-toxicity of phorbol esters from *Jatropha curcas* seed oil. Planta Med 59:A686.
79. Schmidt R, Hecker E. 1975. Autoxidation of phorbol esters under normal storage conditions. Cancer Res 35:1375−1377.
80. Shah R, Mahajan D, Patel S, Ball J, Colantuoni V, Maraj R. 2009. http://www.biodieselmagazine.com/articles/3541/oxidation-stability-in-biodiesel-a-brief-review-of-current-technology
81. Shah S, Sharma A, Gupta MN. 2005. Extraction of oil from *Jatropha curcas* L. seed kernels by combination of ultrasonication and aqueous enzymatic oil extraction. Bioresour Technol 96:121−123.
82. Sharma DK, Pandey AK, Lata. 2008. Use of *Jatropha curcas* hull biomass for bioactive compost production. Biomass Bioenerg 33:159–162.
83. Shelke SS, Jadhav LD, Salunkhe GN. 1985. Ovipositional and adult repellent action of some vegetable oils/extracts against potato tuber moth. J Maharashtra Agric Univ 10:284–286.
84. Shelke SS, Jadhav LD, Salunkhe GN. 1987. Ovicidal action of some vegetable oils and extracts in the storage pest of potato, *Phthorimaea operculella* Zell. Biovigyanam 13:40–41.
85. Silinsky EM, Searl TJ. 2003. Phorbol esters and neurotransmitter release; more than just protein kinase C? Br J Pharmacol 138:1191–1201.
86. Singh RN, Vyas DK, Srivastava NSL, Narra M. 2008. SPRERI experience on holistic approach to utilize all parts of *Jatropha curcas* fruit for energy. Renew Energ 33:1868–1873.
87. Solsoloy AD. 1995. Pesticidal efficacy of the formulated physic nut, *Jatropha curcas* L. oil on pests of selected field crops. Philippine J Sci 124:59–74.

88. Song Y, Ailenberg M, Silverman M. 1999. Human munc13 is a diacylglycerol receptor that induces apoptosis and may contribute to renal cell injury in hyperglycemia. Mol Biol Cell 10:1609–1619.
89. Tognon CE, Kirk HE, Passmore LA, Whitehead FP, Der CJ, Kay RJ. 1998. Regulation of RasGRP via a phorbol ester-responsive C1 domain. Mol Cell Biol 18:6995–7008.
90. Vanden berghe DA, Vlietinck AJ. 1991. Screening for antibacterial and antiviral agents. In: Hostettmann, K., (Ed.), Methods in Plant Biochemistry (Vol. 6 - Assays for Bioactivity). Academic Press, London, pp. 47–69.
91. Vyas DK, Singh RN. 2007. Feasibility study of Jatropha seed husk as an open core gasifier feedstock. Renew Energ 32:512–517.
92. Weinstein IB, Lee LS, Fisher PB, Mufson A, Yamasaki H. 1979. Action of phorbol esters in cell culture: mimicry of transformation, altered differentiation, and effects on cell membranes. J Supramol Struct 12:195–208.
93. Wender PA, Kee JM, Warrington JM. 2008. Practical synthesis of prostratin, DPP, and their analogs, adjuvant leads against latent HIV. Science 320:649-652.
94. Wilson KA. 1980 The release of proteinase inhibitors from legume seeds during germination. Phytochem 19: 2517−2519.
95. Wink M, Grimm C, Koschmieder C, Sporer F, Bergeot O. 2000. Sequestration of phorbolesters by the aposematically coloured bug *Pachycoris klugii* (Heteroptera: Scutelleridae) feeding on *Jatropha curcas* (Euphorbiaceae). Chemoecol 10:179–184.
96. Wink M, Koschmieder C, Sauerweien M, Sporer F. 1997. Phorbol esters of *J. curcas*—Biological activities and potential applications. In *Biofuel and industrial products* from *Jatropha curcas*, eds. G. M. Gubitz, M. Mittelbach, and Trabi, pp. 160–166. Graz:DBV.
97. Wurdack KJ. 2008. Molecular evolution and phylogenetics of Euphorbiaceae:Beyond the model organisms. Plant and Animal Genomes XVI ConferenceSan Diego, CA. http://www.intl-pag.org/16/abstracts/PAG16_W21_155.html.
98. Xu R, Zhao W, Jiang C. 2009. Ester prodrugs of prostratin and related phorbol compounds. United States Patent Application 2009016358.
99. Yamasaki H, Weinstein IB. 1985. Cellular and molecular mechanisms of tumour promotion and their implications for risk assessment. Methods for estimating risk of chemical injury: human and non-human biota and ecosystems. Edited by V.B. Vouk, GC Butler, DG Hoel and DB Peakall.

100. Zayed S. 1977. Experentia 33:1554−1555.
101. Zucker MB, Troll W, Belman S. 1974. The tumour-promoter phorbol ester (12-O-tetradecanoyl-phorbol-13-acetate), a potent aggregating agent for blood platelets. J Cell Biol 60:325−36.

UNIVERSITÄT HOHENHEIM

Rakshit Devappa Kodekalra

Prof. Dr. Klaus Becker

Supervisor

Prof. Dr. Harinder P.S. Makkar

Co-supervisor

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