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Prumnopitys Andina Fruit Extract Activates Liver X Receptors after In Vitro Digestion

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Scope: 20-Hydroxyecdysone (20E) is the main phytochemical present in the fresh arils of *Prumnopitys andina*. 20E is reported to have anabolic effects by modulation of gene transcription by interaction with nuclear receptors. Our aim is to evaluate the in vitro bioaccessibility, transepithelial transport of 20E, and the capacity of *P. andina* fruit extract and 20E to activate selected mammalian nuclear receptors in transiently transfected human cells after simulated gastrointestinal digestion.

Results: 20E shows good stability, solubility, and micellization after in vitro digestion. 20E is taken up by Caco-2 cells, but poorly transported through the epithelial cell membrane, possibly due to P-glycoprotein-mediated efflux. In transiently transfected HepG2 cells, the fruit extract significantly induces the signal intensity for the liver X receptor (LXR)-α and -β by 1.6 and 1.4-fold, respectively. In contrast, the treatment with 20E, irrespective of its concentration, did not change the activity of both LXR receptors. No effects are observed for the pregnane X receptor or the constitutive androstane receptor.

Conclusion: Our findings show that components of the digested *P. andina* extract other than 20E are responsible for the effects on LXR-α and -β. Our findings open new perspectives on the potential role of *P. andina* fruits in cholesterol metabolism and inflammatory diseases.

1. Introduction

Phytoecdysteroids are a large group of steroids that are synthesized by plants as defense against insects and are present in several plant species.[1,2] Numerous phytoecdysteroids have been identified in nature, and among them, 20-hydroxyecdysone (20E) is the most common one in plants.[3] 20E has been recently isolated from the fruits of the *Prumnopitys andina*, showing dose-dependent cytoprotective effects against oxidative stress in gastric epithelial cells.[4]

Food bioactives undergo chemical changes during gastrointestinal digestion, which alters their stability, solubility, bioaccessibility, intestinal uptake, and ultimately their bioavailability and bioactivity.[5] Under alkaline conditions, 20E is oxidized to structurally related compounds with altered potency to induce Akt phosphorylation in murine skeletal muscle cells.[3] Hence, the biological effects of phytoecdysteroids from foods may differ from those obtained in vitro. In mammals, phytoecdysteroids have anabolic, hypolipidemic and gastroprotective activities,[6] and therefore are sold as dietary supplements, with marketing claims such as "green anabolics" or "all-natural muscle building" compounds.[7] It has been suggested that phytoecdysteroids may modulate gene transcription by interaction with nuclear receptors, in particular, the farnesoid X receptor (FXR), and the liver X receptors (LXR).[1,8] An in silico study predicted that phytoecdysteroids can bind with high affinity to the glucocorticoid receptor, with medium affinity to the liver X receptor β, and with low/very low affinity to androgen/estrogen receptors.[9] However, none of the tested ecdysteroids displayed estrogenic, glucocorticoid or androgenic effects in radioligand binding assays, indicating that they do not bind to the vertebrate steroid receptors.[10] In transiently transfected HEK293 cells, ecdysterone activated a reporter gene, and the anabolic effects were associated to the estrogen receptor (ER)-β.[11] However, the molecular mechanisms explaining the bioactivity of phytoecdysteroids have not been well characterized.[6,12]

The pharmacokinetics of phytoecdysteroids have been studied in vivo,[13] and we still know little regarding the absorption and

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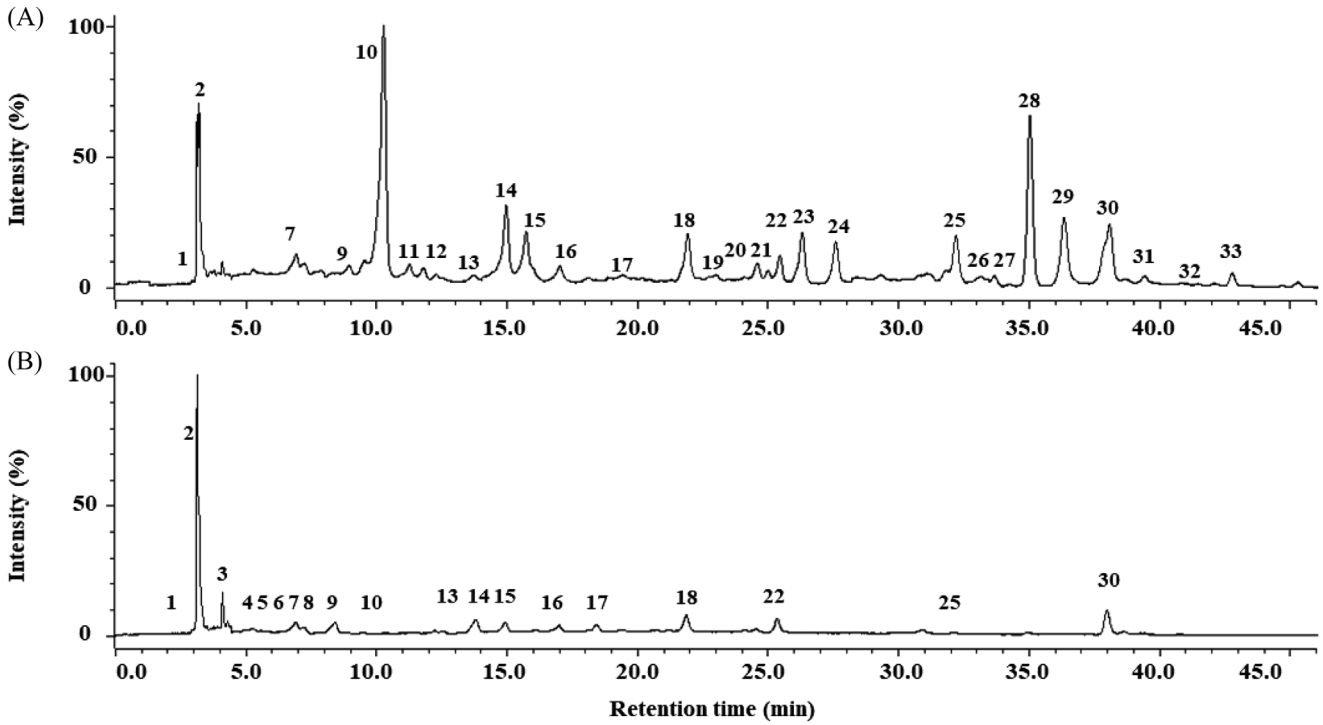

Figure 1. Chromatograms of *Prumnopitys andina* before (A) and after (B) in vitro digestion (280 nm).

transport of phytoecdysteroids through the epithelial barrier and their interactions with nuclear receptors. We therefore aimed to fill these gaps by performing simulated in vitro digestion experiments, transepithelial transport studies with Caco-2 cells and reporter gene assays with digested 20E.

2. Results and Discussion

2.1. Characterization of Secondary Metabolites from *Prumnopitys andina* Fruits before and after In Vitro Digestion

Studies on the biological activity of extracts obtained from edible plants are traditionally carried out in vitro, offering valuable information regarding the chemical composition and potential uses of them. However, these studies do not consider the changes induced by the gastrointestinal process in the content and composition of the different phytochemicals, as well as the effect of this changes in the reported bioactivity. The COST-INFOGEST network has developed a standardized method to mimic in vitro the gastrointestinal digestion, allowing researchers to understand the impact of the digestion process and to analyze the resulting digestion products.[5] The profiles of the *P. andina* fruit extract before and after digestion were compared by HPLC-DAD (**Figure 1**). Compounds were tentatively identified based on the UV-max and mass fragmentation by comparison with commercial standards and with the literature (**Table 1**). A total of 33 compounds were identified, including three amino acids and two disaccharides, two phenolic acids, five glycosylated phenolic acids, seven hydroxycinnamic acids, ten flavonols, three flavones and one phytoecdysteroid, of which, 16 compounds withstood the in vitro digestion, 13 compounds were degraded during the simulated digestion process, and five new compounds appeared as digestion products.

The compounds quinic acid (**1**) and shikimic acid (**2**) were detected at the solvent front and identified by the [M–H]– signal at m/z 191 and 173 amu, respectively. Compound **7** was previously isolated from *P. andina* fruit extracts and identified as caffeic acid β-glucoside.[4] Compounds **9** and **14** presented a common $[M–H]^-$ signal at m/z 337 amu and MS^2 peaks at m/z 163 and 173 amu, respectively, and thus were identified as 3- and 4-*p*-coumaroylquinic acids. Compound **10** was previously isolated from *P. andina* fruit extracts and identified as 3-caffeoylquinic acid.[4] Compound **15** presented at $[M-H]^-$ ion at m/z 335 amu, followed by the neutral loss of 156 amu to the caffeoyl moiety at m/z 179 and was identified as 5-*O*-caffeoylshikimic acid. Compounds **16**, **18**, **20**, and **21** were identified as the dihexoside, hexoside, quinic and pentosylhexoside derivatives of ferulic acid, respectively. In addition, the characteristic signal of sinapic acid at m/z 223 amu was essential to tentatively identify compound **12** as sinapoyl hexoside. Finally, compounds **31** and **32** showed an UV_{max} at 320 nm and the MS^2 fragment at m/z 179 amu, indicative of the caffeic acid core. The compounds differed by 16 amu, indicating the presence of an alcohol substituent. The compounds were identified as caffeoylcalleryanin and gastrodin-*O*-caffeoylester, in agreement with the literature.[14] Compound **6** was identified at the end of the in vitro digestion, but it was not present in the non-digested sample. The compound presented an $[M–H]^-$ ion at m/z 315 amu, followed by the neutral loss of a hexose (–162 amu) to the MS^2 ion at m/z 153 amu, in agreement with protocatechuic acid hexoside. Protocatechuic acid is a common metabolite found as a digestion product of flavonols, anthocyanins, and simple phenolic acids.[15,16]

Table 1. HPLC-DAD-APCI-MS data obtained from *Prumnopitys andina* fruit extracts before and after in vitro gastrointestinal digestion.

Peak	RT [min]	Molecular mass [*m*/*z*]	Mass error [ppm]	MS [*m*/*z*]	Tentative identification	Before	After
1	2.12	191.0557	0.665	173.0445	Quinic acid	x	x
2	3.11	173.0449	0.410	137.0235	Shikimic acid	x	x
3	4.32	341.1994	1.542	179.0556	Dissacharide 1	–	x
4	4.76	341.1093	1.472	179.0556	Dissacharide 2	–	x
5	5.44	166.0865	0.235	120.0812	Phenylalanine	–	x
6	6.01	315.0729	1.852	153.0186	Protocatechuic acid hexoside	–	x
7	6.91	341.1089	1.022	179.0343, 135.0442	Caffeic acid glucoside	x	x
8	7.92	205.0973	0.186	188.0708	Tryptophan	–	x
9	9.52	337.0933	1.526	163.0395	3-*p*-coumaroylquinic acid	x	x
10	10.26	353.0881	1.421	191.0556, 179.0343	3-*O*-caffeoylquinic acid	x	x
11	11.22	449.1093	1.502	287.0565	Dihydrokampferol hexoside 1	x	–
12	11.78	385.1873	1.626	223.1341	Sinapoyl hexoside	x	–
13	13.92	449.1102	2.182	287.0570	Dihydrokaempferol hexoside 2	x	x
14	14.95	337.0928	1.036	173.0448, 163.0395	4-*p*-coumaroylquinic acid	x	x
15	15.72	335.0777	1.556	179.0343, 135.0443	5-*O*-caffeoylshikimic acid	x	–
16	16.98	517.1568	1.618	193.0503	Feruloyl dihexoside	x	x
17	19.11	431.0989	1.627	311.0564, 283.0612	Apigenin-*C*-glucoside	x	x
18	21.89	355.1036	1.311	193.0502	Feruloyl hexoside	x	x
19	23.01	447.0939	1.712	327.0515	Luteolin-8-C-glucoside	x	–
20	23.44	367.1041	1.701	173.0450	Feruloyl quinic acid	x	–
21	24.52	487.1457	1.103	193.0502	Feruoyl pentosyl-hexoside	x	x
22	25.42	481.3159	0.000	445.2953, 371.2221	*β*-ecdysone	x	x
23	26.28	609.1467	1.719	301.0355	Quercetin rutinoside	x	–
24	27.56	465.1030	0.278	303.0500	Quercetin hexoside	x	–
25	32.16	593.1518	1.694	285.0406	Kaempferol rutinoside	x	x
26	33.11	505.0989	1.273	301.0355	Quercetin acetylhexoside	x	–
27	33.65	447.0922	0.002	285.0407	Kaempferol hexoside 2	x	–
28	34.95	623.1620	1.389	315.0514	Isorhamnetin rutinoside	x	x
29	36.37	431.0981	0.837	269.0458	Apigenin-*O*-glucoside	x	x
30	38.05	477.1042	1.438	315.0436	Isorhamnetin hexoside	x	x
31	39.39	463.1253	1.852	179.0343	Caffeloylcalleryanin	x	–
32	41.22	447.1302	1.647	179.0343	Gastrodin-O-caffeoyl ester	x	–
33	42.74	519.1148	1.493	315.0514	Isorhamnetin acetylhexoside	x	–

The changes in the content of the main hydroxycinnamic acids (HCA) in *P. andina* fruits during in vitro digestion are presented in **Table 2**. A total of seven phenolic acids and seven HCA were identified before the digestion process, but only six phenolic acids and three HCA withstood the digestion process. The total content of HCA was 21.3 mg/100 fresh fruit weight (fw), and 3-caffeoylquinic acid (**10**) was the main HCA, with a total content of 12.9 mg/100 g fw. However, the content of HCA was significantly reduced at the end of the in vitro digestion, with an overall stability of only 14.2 ± 0.2%. 3-caffeoylquinic acid (**10**) was the compound with lowest stability (0.34 ± 0.14%), while the caffeic acid glucoside was the most stable compound (82.5 ± 4.85%) (Table 2). The same trend was observed in terms of solubility and micellization, with overall values of 13.5 ± 0.03% and 12.6 ± 0.2%, respectively. The low stability of HCA during the digestion process has been widely reported, with values ranging from 2 to 45%, depending on the food source.[17,18]

Ten different flavonols were detected before the digestion process, but only three flavonols withstood the digestion process. The compounds **11** and **13** were identified as dihydrokaempferol hexosides 1 and 2, respectively, while compounds **25** and **27** were identified as kaempferol rutinoside and kaempferol hexoside, respectively. Compounds **23**, **24**, and **26** presented a common MS^2 ion at *m*/*z* 301 amu and were identified as quercetin rutinoside, quercetin hexoside and quercetin acetylhexoside, respectively. Similarly, compounds **28**, **30**, and **33** were identified as the rutinoside, hexoside and acetylhexoside of isorhamnetin. Compounds **17** and **19** presented the same neutral loss of 120 amu, in agreement with the presence of a C-glycosides of flavonoids. Compound **17** presented a $[M\text{-}H]^-$ ion at *m*/*z* 431 and a main MS^2 ion at *m*/*z* 311 amu, in agreement with apigenin-C-hexoside. Compound **19** was previously isolated from *P. andina* fruits and characterized as luteolin-8-C-glucoside (orientin).[4] In addition, compound **29** presented the characteristic UV profile of flavones

Table 2. Quantification of main compounds in *P. andina* fruit extracts and their stability, solubility, and micellization at the end of the gastrointestinal digestion.

Compound	Content [mg/100 g fresh fruit]	%Stability	%Solubility	%Micellization
Hydroxycinnamic acids				
Caffeic acid glucoside	0.38 ± 0.01	82.53 ± 4.85	81.66 ± 3.27	78.71 ± 2.02
3-*p*-coumaroylquinic acid	0.24 ± 0.01	12.37 ± 1.60	16.46 ± 0.98	14.69 ± 2.36
3-*O*-caffeoylquinic acid	12.93 ± 0.22	0.34 ± 0.14	0.26 ± 0.48	0.30 ± 0.03
Sinapoyl hexoside	0.41 ± 0.01	24.08 ± 2.77	27.36 ± 1.48	24.87 ± 6.28
4-*p*-coumaroylquinic acid	2.81 ± 0.07	26.21 ± 0.40	23.22 ± 0.48	21.81 ± 0.28
5-*O*-caffeoylquinic acid	1.97 ± 0.05	19.44 ± 1.15	18.23 ± 2.53	18.36 ± 1.44
Feruloyl-*O*-dihexoside	0.73 ± 0.03	54.45 ± 0.97	51.34 ± 0.00	48.22 ± 1.40
Feruloyl hexoside	2.01 ± 0.00	54.69 ± 0.18	53.17 ± 0.56	47.42 ± 0.86
Caffeoylcalleryanin	0.23 ± 0.02	–	–	–
Total	21.31 ± 0.40	14.18 ± 0.22	13.57 ± 0.03	12.66 ± 0.27
Phytoecdysteroids				
β-ecdysone	28.50 ± 0.60	84.76 ± 0.75	84.64 ± 0.58	76.97 ± 1.75
Flavonoids				
Quercetin-3-rutinoside	4.26 ± 0.06	0.65 ± 0.07	–	–
Quercetin hexoside	3.47 ± 0.03	0.76 ± 0.21	0.15 ± 0.00	–
Kaempferol rutinoside	1.95 ± 0.11	7.59 ± 0.31	1.83 ± 0.20	2.27 ± 0.07
Kaempferol hexoside	0.51 ± 0.06	3.20 ± 0.19	–	–
Isorhamnetin rutinoside	13.97 ± 0.09	6.80 ± 0.15	1.58 ± 0.03	1.80 ± 0.04
Apigenin-O-glucoside	2.48 ± 0.02	2.05 ± 0.09	0.31 ± 0.00	–
Isorhamnetin hexoside	2.77 ± 0.06	71.59 ± 0.08	66.33 ± 1.91	59.47 ± 0.49
Isorhamnetin acetylhexoside	1.11 ± 0.05	2.60 ± 0.68	–	–
Total	30.52 ± 0.47	10.64 ± 0.05	6.90 ± 0.17	6.37 ± 0.03

with UV max around 330 nm. The compound was identified as apigenin-O-hexoside given its MS^2 ion at *m/z* 269 amu and the neutral loss of 162 amu.

The total content of flavonoids in the *P. andina* fruit extract was 30.5 mg/100 g fw, with isorhamnetin rutinoside (**28**) being the main compound. The stability of flavonoids at the end of the in vitro digestion was low, reaching only 10.6 ± 0.05% of their initial concentration (Table 2), similar to the overall low stability of flavonoids after in vitro gastrointestinal digestion.[19] The most affected compounds were quercetin rutinoside (**23**) and quercetin hexoside (**24**), with losses of 99 ± 0.2%. At the end of a simulated intestinal digestion, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and quercetin from *Capparis spinosa* and *Crithmum maritimum* were below detection limits.[20] The low concentration of flavonols in the non-digested sample impeded us to detect other potential metabolites/degradation products derived from the in vitro digestion. Of the identified compounds, protocatechuic acid hexoside (**6**) could be attributed to the degradation of flavonoids.

In terms of solubility, most flavonoids were poorly soluble, with the exception of isorhamnetin hexoside (66 ± 1.9%). After filtration, the percent of flavonoids that where embedded in micelles with size <0.2 μm was 6.4 ± 0.03% of the total content of flavonoids, and the main representative was isorhamnetin hexoside. In literature, a similar observation was reported, with high recoveries between 74.3% and 81.0% for different glycosides of isorhamnetin.[21]

Compound 22 was identified as 20-hydroxyecdysone (20E), a compound that was previously isolated from *P. andina*.[4] The content of 20E in the non-digested sample was 28.5 ± 0.6 mg/100 g fw (Table 2). Although spread throughout the plant kingdom, it is estimated that only 6% of plants contain detectable amounts of phytoecdysteroids.[2] Common food sources of phytoecdysteroids include fresh spinach (*Spinacia oleracea*, 18.5 mg 20E g^{-1} fw) and quinoa (*Chenopodium quinoa*, Amaranthaceae, 31.6 mg 20E g^{-1} fw).[2] However, the content of phytoecdysteroids in these foods decreases by 20–30% during the cooking process. [2] Since *P. andina* fruits are normally consumed raw, they can be considered an interesting source of 20E. Moreover, after in vitro digestion, the stability of the compound was about 85 ± 0.7%, with a solubility of 85 ± 0.6% and micellization of 77 ± 1.8% (Table 2). To confirm these observations, a standard of 20E was submitted to the same digestion process, resulting a stability of 112 ± 6%, a solubility of 101 ± 3% and a micellization efficiency of 103 ± 2%. In a recent study, 20E was not metabolized in the stomach or intestine of rats after oral ingestion, but was structurally modified by the colonic microbiota, and in a minor proportion by intestinal and hepatic cells.[11] The COST-INFOGEST network does not include the colonic fermentation on the standardized protocol and thus was not included in our study.[5] However, using the data available in literature,[13,22] we carried out an extracted ion chromatogram of the digested sample and standard, to search for the reported metabolites of 20E, namely 14-deoxy-20-hydroxyecdysone

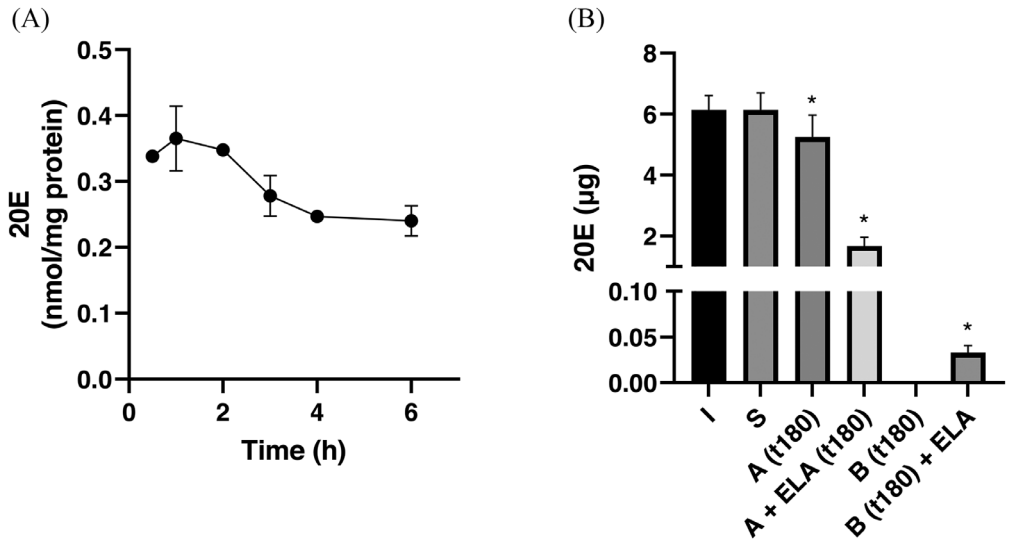

Figure 2. a) Uptake of 20E in Caco-2 cells; b) Total 20E at 0 min (I), after 180 min of incubation at 37°C (S); leftover 20E in the apical (A) chamber after 180 min of incubation, and (B) accumulated amount of 20E in the basolateral compartments, in the presence or absence of elacridar (ELA).

($C_{27}H_{44}O_6$, 464.3138 g mol^{-1}), poststerone ($C_{21}H_{30}O_5$, 362.2093 g mol^{-1}), 14-deoxypoststerone ($C_{21}H_{30}O_4$, 346.2144 g mol^{-1}) and 25-pentahydroxy-5-cholest-8,14-diene ($C_{27}H_{46}O_6$, 466.3294 g mol^{-1}). In addition, the oxidized metabolites of 20E, namely 9,20-dihydroxyecdysone ($C_{27}H_{44}O_8$, 496.3036 g mol^{-1}), 25-hydroxy dacryhainansterone ($C_{27}H_{42}O_7$, 478.2931 g mol^{-1}), 11α-hydroxycalonysterone ($C_{27}H_{42}O_8$, 492.2723 g mol^{-1}), calonysterone/isocalonysterone ($C_{27}H_{40}O_7$, 476.2774 g mol^{-1}) and 4,15-dihydro-14α-hydroxycalonysterone ($C_{27}H_{42}O_8$, 494.2880 g mol^{-1})[3] were searched in the mass spectra of digested 20E and *P. andina* fruit extract. Neither related ecdysteroids, nor 20E metabolites could be identified in the obtained mass spectrometric analyses, confirming that 20E is not modified during the simulated physiological digestion process to any of these derivatives.

Finally, compounds **3**, **4**, **5**, and **8** were present after the in vitro digestion process and identified as disaccharides (**3**-**4**) and amino acids (**5** and **8**). The analysis of the chyme solution indicated that these compounds derive from the digestive solutions and are not metabolites generated from the in vitro digestion of 20E or the *P. andina* fruit extract.

2.2. 20-Hydroxyecdysone Is Taken up by Caco-2 Cells, but Poorly Transported through the Cell Monolayer

Differentiated Caco-2 cells were first exposed to micelles generated from the in vitro digestion of the *P. andina* fruit extract, the 20E standard and the chyme solution as control to evaluate possible cytotoxic effects. No cytotoxicity was observed when cells were treated with dilutions higher than 1:10 v/v (Figure S1, Supporting Information). At lower dilutions, cell death was observed for the chyme solution and the samples, indicating that cell death was due to the composition of the chyme. In literature, the low toxicity of phytoecdysteroids has been experimentally proven in mice and rabbits, showing no effects after oral doses of up to 9 g kg^{-1} in male mice.[23] Consequently, and in agreement with the calculated LOQ, maximum working concentrations of 200 µmol L^{-1} for 20E and 200 µg mL^{-1} for the *P. andina* fruit extract were established.

The total cellular uptake of 20E, normalized to the total protein content obtained in the recovery process, is depicted in **Figure 2**A. Under our assay conditions, 20E is taken up by the cells with a Tmax close to 1 h and a Cmax of 0.355 ± 0.045 nmol mg^{-1} protein. Afterwards, the content inside the cells started to decrease, reaching a concentration of 0.24 nmol mg^{-1} protein at 6 h incubation. In humans, ingested ecdysteroids reach plasma with a Tmax of 30 min, followed by a clearance from the blood with an effective elimination half-life of 9 h.[13,24]

Prior to assessing the transport of 20E, we tested the stability of the compound under the experimental conditions. 20E was diluted to the working concentrations in HBSS (pH 6.5) and incubated at 37°C for 3 h. At the end of the incubation, no significant changes were observed in the concentrations of 20E (Figure 2B, compare t0 vs. t180 min). In the transport experiment, the content of 20E at the apical side decreased by 19 ± 2% at the end of the 180 min incubation. However, 20E was below the detection limit at the basolateral side of the chamber. In the same way, 20E was not detected in the cell lysates recovered at the end of the incubation. This is in accordance with the literature, where a low bioavailability (<2%) and mainly fecal elimination of 20E was observed after oral administration in mice.[25] Interestingly, the total amount of 20E decreased by 70 ± 7% at the apical side when 3.5 µmol L^{-1} elacridar was added. Moreover, 20E was found at the basolateral chamber, with a permeability coefficient (Papp) of 0.53 ± 0.16 × 10^{-6} cm s^{-1}; which can be considered low permeability.[26] Elacridar is a potent inhibitor of P-glycoprotein (P-gp),[27] which is located on the apical membrane of small intestinal cells, in the canalicular apical membrane of hepatocytes, and on the brush border of proximal tube cells, allowing cells to excrete exogenous substrates, toxins, and endogenous metabolites.[28] Elacridar also inhibits ABCG2 proteins, which are expressed in the apical membrane

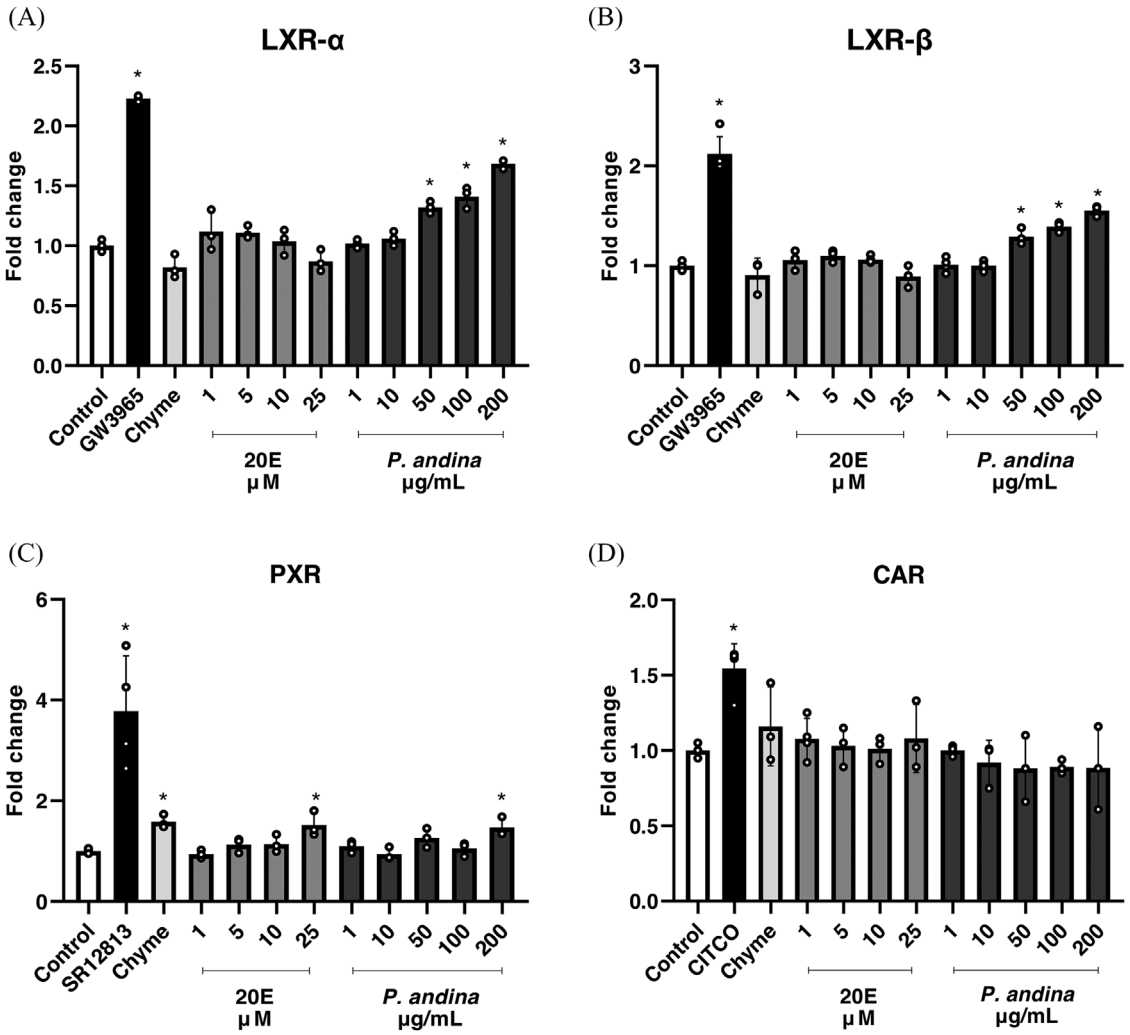

Figure 3. Effect of the *Prumnopitys andina* fruit extract and 20E on the luciferase activity regulated by A) liver X receptors (LXR)-α, B) LXR-β, C) constitutive androstane receptor (CAR), and D) pregnane X receptor (PXR). Results are expressed as fold-change compared to untreated control group in each experiment and are presented as mean values ± SD ($n = 3$; three cell passages). *$p < 0.05$.

of epithelial cells of the duodenum, playing a key role in limiting the absorption of xenobiotics from the diet.[29] Our results indicate that, in the presence of elacridar, the disappearance of 20E from the apical side of the cell culture is enhanced. In the literature, the cellular uptake of ecdysteroids in insects has been described as facilitated diffusion through a solute carrier organic anion transporter (OATP),[30] while there is no information regarding specific transporters in mammals. In an in silico/in vitro study using the Parallel Artificial Membrane Permeability Assay for blood brain barrier, 20E was classified as a non-permeable and relatively hydrophilic compound.[7] However, the assay only considers membrane diffusion, not considering membrane transporters. On the other hand, antagonistic effects of ecdysteroids towards P-gp have been reported.[31] Thus, 20E could be a substrate of P-gp and ABCG2; however, the role of OATP, ABCG2, and P-gp in the uptake and efflux of ecdysteroids in the intestinal epithelium needs to be experimentally confirmed in future studies.

2.3. *Prumnopitys andina* Fruit Extract, but not 20-Hydroxyecdysone, Cctivates Liver-X Receptors in HepG2 Cells

Phytoecdysteroids can affect metabolic pathways in mammals, such as protein synthesis, lipid and carbohydrate metabolism, acting as hepatoprotective, immunostimulants, and hypoglycemic agents.[11,12] The anabolic properties of phytoecdysteroids in mammals have supported the idea that these compounds might have effects in the endocrine system.[9] However, studies show contradictory results on the binding of 20E and ecdysone to some mammalian nuclear receptors.[9,10] To evaluate if 20E could act as agonist of nuclear receptors and eventually affect their transcriptional activity, HepG2 cells were transiently transfected with specific reporter plasmids. The agonists CITCO, GW3965 and SR12813 significantly activated CAR, LXR and PXR, respectively, with 1.5- to 4.8-fold induction compared to the basal controls (**Figure 3**A–D). Cells treated with chyme showed no effects on CAR or LXR-α and -β (Figure 3A,

B, and D); however, a 2.0-fold induction was observed for PXR (Figure 3C). Bile acids, such as litocholic, chenodeoxycholic, deoxycholic, and cholic acid can act as endogenous ligands of PXR, playing key roles in the regulation of drug metabolism.[32] For PXR, the highest concentration of the *P. andina* fruit extract and 20E significantly induced the transcriptional activity. However, this induction was not statistically significant when compared to the activity induced by the chyme (Figure 3C). Hence, the activating effects observed eventually could be attributed to bile acids present in the dilutions of the digested 20E and the *P. andina* fruit extract. Since 20E was also a substrate for P-gp, a product of the activation of PXR,[33] it cannot be discarded that 20E could have some effects in the PXR pathway. When cells were transfected with CAR and treated with different concentrations of *P. andina* fruit extract or 20E, no significant changes in the transcriptional activity were observed (Figure 3D). In contrast, a dose-dependent increasing effect of *P. andina* fruit extract was observed in cells transiently transfected with LXR-α and LXR-β (Figure 3A and B). At 200 µg mL^{-1}, the fruit extract significantly induced the signal intensity for LXR-α and LXR-β by 1.6 and 1.4-fold, respectively. In contrast, the treatment with 20E, irrespective of its concentration, did not change the activity of both LXR receptors. This indicates that components of the digested *P. andina* extract other than 20E are responsible for this activating effect on LXR-α and LXR-β (Figure 3A and B). Of the flavonoids present in the *P. andina* fruit extract, only apigenin glucoside was found at detectable levels in the UHPLC-MS analysis of the digested extract, while all the quercetin glycosides present in the non-digested fruit did not withstand the digestion process (Tables 1 and 2). In the literature, the non-anthocyanin phenolic compounds from cherry (*Prunus avium*) have been shown to modulate the activity of LXR in mice.[34] Apigenin was reported to selectively activates LXR-α, but not LXR-β.[35] Hence, it is possible that the increase in the LXR-α and -β transcriptional activity observed by us is due to the apigenin glycoside content of the digested *P. andina* fruit extract. However, future experiments are warranted to test this hypothesis.

LXR is a class II nuclear receptor associated to the thyroid hormone receptor-like subfamily and is expressed in two isoforms: LXR-α and LXR-β. LXR-α is mainly expressed in metabolically active tissues, such as the liver, the intestinal epithelia and the adipose tissue, while LXR-β is more ubiquitously expressed.[36] LXRs form heterodimers with the retinoid X receptor alpha and bind to specific regions of the DNA known as the LXR response element.[36] Both receptors have pivotal roles in the transcriptional control of lipid metabolism and cholesterol homeostasis, including the upregulation of the ATP-binding cassette transporter 1 (ABCA1), the sterol regulator element binding protein SREBP-1c, and lipogenic genes such as the fatty acid synthase (FAS).[41] In order to validate the results obtained from the gene-reporter assays, the effects of digested *P. andina* fruit extract on the expression of LXR-target genes was assessed in human hepatic HepG2 cells (non-transfected). Relative mRNA expression of the LXR target genes *ABCA1, SREPB-1c*, and *FAS* were significantly increased 24 h after treatment with the LXR agonist GW3965 (**Figure 4**A–C). When cells were treated with the chyme solution, no significant increase in the expression of these genes was observed. However, upon treatment with the digested *P. andina* fruit extract, a significant dose-dependent in-

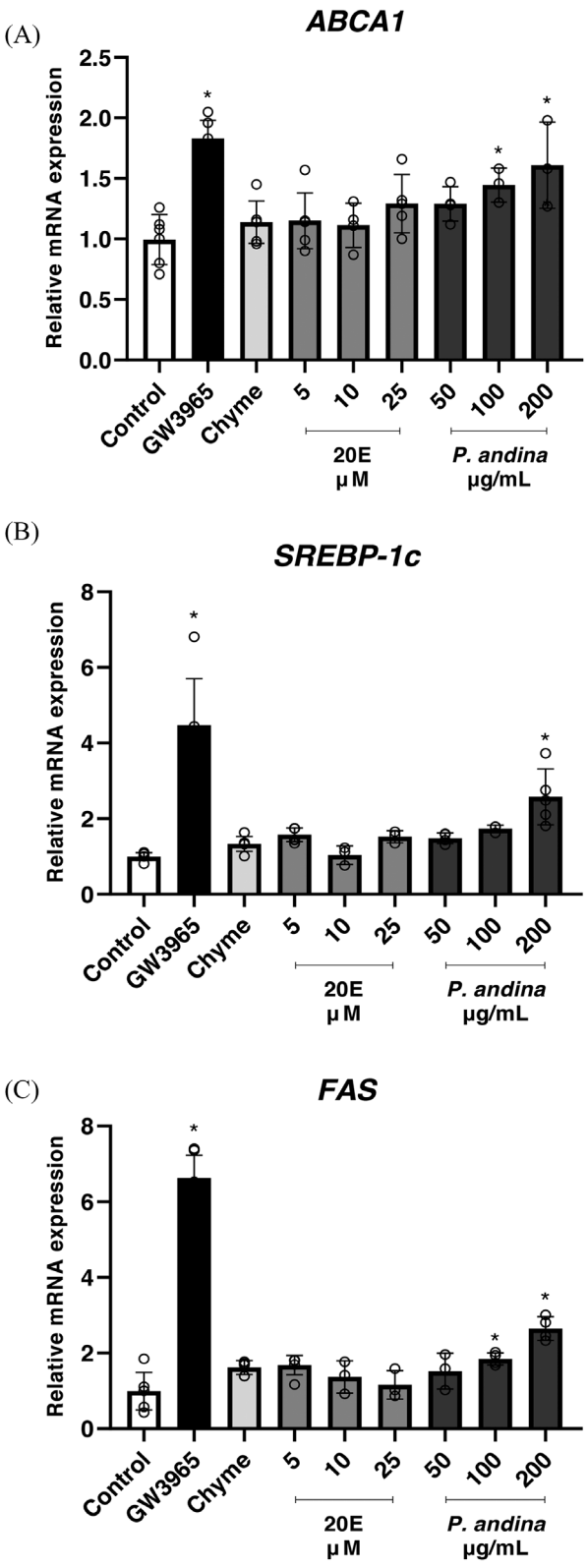

Figure 4. Effect of *Prumnopitys andina* fruit extract and 20E on the relative mRNA expression of A) *ABCA-1*; B) *SREBP1c* and C) *FAS* of HepG2 cells. Results are expressed as fold-change compared to untreated control group in each experiment and are presented as mean values ± SD ($n = 3$; three cell passages). *$p < 0.05$.

crease in the relative mRNA expression of *ABCA-1* was observed (Figure 4A), ranging from 1.28 to 1.59-fold, compared to untreated cells. Similarly, there was an increase of 1.60 to 2.49-fold in the relative mRNA of *SREBP-1c* (Figure 4B) and of 1.75 to 2.54-fold for *FAS* (Figure 4C). In the literature, apigenin dose-dependently increased *ABCA1* mRNA expression in cells and mice.[37] In HepG2 cells, on the other hand, apigenin reduced the mRNA expression of *SREBP-1c* and *FAS*,[38] and in human osteosarcoma cells that of *FAS*.[39] Hence, further experiments will be required to elucidate the effects of *P. andina* fruit extract on the LXR-pathway and to identify the responsible compounds and/or their active metabolites.

3. Concluding Remarks

In conclusion, our hypothesis that in vitro digestion may degrade some of the phytochemicals present in *P. andina* fruit extracts was partially confirmed, since the hydroxycinnamic acids and flavonoids were significantly decreased, whereas 20E showed good stability, solubility, and micellization. Digested *P. andina* fruit extract, but not digested 20E, activated LXR-α and LXR-β and its target genes. Therefore, compounds other than 20E, perhaps apigenin, present in the extract could be responsible for the activation of LXR. Further experiments are necessary to investigate the interactions of phytochemicals from *P. andina* fruits with nuclear receptors and their impact on health and disease, in particular, on lipid metabolism and inflammation.

4. Experimental Section

Materials: Common salts and solvents were from Carl Roth GmbH (Karlsruhe, Germany). Enzymes, cell culture medium, and specific receptor agonists were from Sigma-Aldrich (Schnelldorf, Germany). Cell lines were from the American Type Culture Collection (ATCC, Rockville, MD, USA). Standards were from PhytoLab (Vestenbergsgreuth, Germany). The *P. andina* fruit extract was prepared as previously described.[4]

Simulated Gastrointestinal Digestion: In vitro digestion was performed following the INFOGEST protocol.[5] Briefly, samples were dissolved in simulated saliva fluid,[5] supplemented with $CaCl_2$. The mixture was incubated in the dark for 2 min at 37°C. After this, simulated gastric fluid[5] was added along with $CaCl_2$ and porcine pepsin (2000 U mL^{-1}). The pH was adjusted to 3.0 ± 0.1 and the mixture was incubated at 180 rpm for 2 h in the dark at 37°C. To the resulting mixture, a simulated intestinal fluid[5] containing pancreatin (2.4 mg mL^{-1}), lipase (2000 U mL^{-1}), and bile extract (10 mM) along with $CaCl_2$ were added.[5] The pH was adjusted to 7.0 ± 0.1, overlaid with nitrogen gas and incubated at 180 rpm for 2 h and 37°C in the dark. At the end of the incubation, the stability of compounds was determined using Equation (1):

$$\%\text{Stability} = \left(\frac{\text{mol of compound in digested samples}}{\text{mol of compounds in non digested samples}}\right) \times 100 \quad (1)$$

The remaining solution was centrifuged for 1 h at 5000 × *g* and 4°C, and the supernatant was collected and analyzed to determine the solubility of compounds according to Equation (2)

$$\%\text{Solubility} = \left(\frac{\text{mol of compound in supernatant}}{\text{mol of compound in non digested samples}}\right) \times 100 \quad (2)$$

The supernatant was then filtered (Filtropur S 0.2 µm disc, Sarstedt, Nümbrecht, Germany), to separate bile-salt-lipid mixed micelles from the larger lipid emulsions. The micellization of compounds was determined according to Equation (3).

$$\%\text{Micellization} = \left(\frac{\text{mol of compound in filtered fraction}}{\text{mol of compound in non digested samples}}\right) \times 100 \quad (3)$$

The remaining filtered solution was aliquoted and stored at –80°C.

Chromatographic Analyses: The spectrophotometric analyses were carried out using a JASCO HPLC system (Jasco, Groβ-Umstadt, Germany). The spectrometric analyses were carried out using an Agilent 1290 Infinity Ultra Performance Liquid Chromatography (Thermo Fischer Scientific, Waltham, MA, USA). Samples were run through a Kinetex C18 column (4.6 × 150 mm; 2.6 µm) maintained at 30°C. Mobile phases were 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) in a gradient elution previously described.[4] Quantification was performed by external calibration curves of the commercial standards of 20-hydroxyecdysone (0.035–2.5 µmol mL^{-1}, r^2 = 0.9987, LOD: 0.038 µmol mL^{-1}; LOQ: 0.114 µmol mL^{-1}); quercetin-3-glucuronide (0.01–0.105 µmol mL^{-1}, r^2 = 0.9968, LOD: 0.018 µmol mL^{-1}; LOQ: 0.054 µmol mL^{-1}) and 3-caffeoylquinic acid (0.014–1.412 µmol mL^{-1}, r^2 = 0.9985, LOD: 0.003 µmol mL^{-1}; LOQ: 0.009 µmol mL^{-1}).

Cell Viability Assay: Caco-2 cells were cultured in high-glucose DMEM supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential-amino acids, and 1% penicillin/streptomycin. HepG2 cells were cultivated in high-glucose DMEM supplemented with 10% FBS and antibiotics. Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 (Hera Cell 150, Thermo Electron Corporation, Langenselbold, Germany). Cells were split and seeded at appropriate density according to the experimental model. Medium was changed every 2 days and Caco-2 cells were maintained for 21 days post-confluence for differentiation.

For the cell viability assay, differentiated Caco-2 cells were incubated for 24 h with different concentrations of the in vitro digested *P. andina* and 20E. Untreated cells were used as 100% viability control. At the end of the incubation, cell viability was determined by means of the MTT assay (0.5 mg mL^{-1} MTT, 2.5 h).

Caco-2 Cellular Uptake of 20-Hydroxyecdysone (20E): The uptake was simulated using differentiated Caco-2 cells (18–21 days post-confluence), following the methodology already described.[40] Briefly, cells were incubated for 0.5, 1, 2, 3, 4, and 6 h in medium containing digested 20E (100 µmol L^{-1}). After incubation, the supernatant was removed and cells were washed and scraped off using H_2O:ACN:formic acid (40:50:10, v/v/v). To recover metabolites, cells were lysed, centrifuged (8000 x *g*, 10 min, 4°C) and extracted with ethyl acetate. The organic phase was recovered and evaporated to dryness, redissolved in MeOH and chromatographically analyzed. All experiments were conducted between passages 12–25 with three replicates and three different passages. Results were normalized to the total protein content recovered after cell lysis, quantified by means of the Bradford assay (Roti®Quant reagent, Carl-Roth GmBH).

Caco-2 Transepithelial Transport of 20-Hydroxyecdysone (20E): Permeability experiments were carried out with differentiated Caco-2 cells using Transwell® inserts (1.2 cm^2 polycarbonate membrane, 0.4 µm pore size, Corning, NY, USA), seeded at a density of 1 × 10^5 cells per Transwell. Permeation of 20E was investigated in the apical-to-basolateral direction at 37°C (n = 5, three different cell passages). The Caco-2 cell monolayer was washed and incubated for 1 h with HBSS pH 7.4 supplemented with 4% BSA for initial equilibration. Then, medium was replaced on the apical side with HBSS pH 6.5 containing 200 µg mL^{-1} of digested *P. andina* fruit extract, 150 µmol L^{-1} of digested 20E, or 150 µmol L^{-1} of digested 20E + 3.5 µM of Elacridar, a dual inhibitor of P-glycoprotein and transporters of the ATP-binding cassette superfamily G (ABCG2). Lucifer yellow (100 µmol L^{-1}) was also added to the apical chamber, and its concentration was measured in the basolateral fluid after 3 h incubation.

The integrity of the differentiated epithelia was confirmed by measuring the transepithelial electrical resistance (TEER) and lucifer yellow permeability. The TEER was measured with an EVOM-G Ohm Meter (World

Precision Instruments, FL, USA), every 30 min for 3 h, and the validity of the Caco-2 system in the present study was ensured by TEER values greater than 250 Ω cm^2. Inserts with lucifer yellow permeability above 3% were excluded from analysis. The concentration of 20E in the apical and basolateral chamber was measured after 3 h by HPLC analysis.

The apparent permeability coefficient (P_{app}) was calculated as:

$$P_{app}(cms^{-1}) = \frac{C_f \times V}{t \times C_i \times A} \quad (4)$$

where C_f is the final concentration in the basolateral chamber (µmol mL^{-1}), V is the volume of the BL chamber (mL), t is the duration of the experiment (s), C_i is the initial concentration in the apical chamber (µmol mL^{-1}) and A is the surface area of the cell monolayer (cm^2).[41]

Transient Transfection and Luciferase Reporter Assay in HepG2 Cells: The effects of digested *P. andina* fruit extract and 20E on the constitutive androstane receptor (CAR), liver X receptor (LXR)-α and LXR-β, and pregnane X receptor (PXR) were studied in transiently transfected HepG2 cells. Cells were seeded at 25 000 cells per well in 96-well plates and incubated for 24 h at 37°C and 5% CO_2. All transfection plasmids were kindly provided by Dr. Oliver Burk (Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology IKP, Stuttgart, Germany). The reporter gene vector pGL3-basic was from Promega (Madison, WI, USA). For LXR overexpression, cells were transfected with pGL3-TK(SMPDL3A_LXRE) (50 ng per well) and pcDhuLXRα or pcDhuLXRβ (5 ng per well).[42] For PXR and CAR activation assays, cells were transfected with pGL3-CYP3A4(-7830Δ7208-364) (50 ng per well),[43] and pcDhuPXR (5 ng per well) or pcDhuCAR1 (10 ng per well).[44] For normalization on transfection efficiency, cells were transfected with pCMV-LacZ (8.6 ng per well) (Clontech, Takara Bio Company, Mountain View, CA, USA). Total DNA was adjusted to 113.6 ng per well using pGL3-basic-pcDNA3 (Invitrogen, Waltham, MA, USA). XtremeGENE 9 DNA Transfection reagent was used (Roche Life Science, Penzberg, Germany). Plasmids and transfection reagent were diluted in OptiMem. After 1 h of incubation at 37°C, the following treatments were added: a) OptiMem as basal control; b) the chyme mixture (1:10, v/v); c) the positive control (1 µM GW3965 for LXRα and LXRβ; 1 µM SR-12813 for PXR, and 5 µM CITCO for CAR); d) digested 20E (1-25 µM); or e) digested *P. andina* fruit extract (1-200 µg mL^{-1}). After 24 h incubation, the medium was removed and 100 µL of lysis buffer was added (25 mM Tris pH 8.0, 8 mM $MgCl_2$, 1% Triton-X-100 v/v and 15% glycerol, v/v) and kept on shaker at 600 rpm for 15 min. Next, 50 µL of the lysates were transferred to a white 96-well plate and mixed with 100 µL of the reporter reading buffer (2.8 µmol L^{-1} D-luciferin and 1.3 mM ATP in lysis buffer). Luciferase activity was measured using a microplate reader (Synergy MX, Biotech Instruments, VT, USA). The remaining 50 µL of cell lysate were mixed with 100 µL of 3.32 mM 2-nitrophenyl-β-galactopyranoside in development buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0), incubated for 2 h at 37°C and read at 405 nm using the same microplate reader. Results of the luciferase activity were divided by the β-galactosidase activity for normalization. Results are presented as fold induction in treated cells in comparison to the basal control and are presented as mean values ± S.D. from three independent experiments with four different cell passages.

Real-Time Quantitative PCR: HepG2 cells were seeded at 500 000 cells per well in 6-well plates and incubated for 24 h. Then, cells were treated with digested *P. andina* fruit extract (50–200 µg mL^{-1}), 20E (1–50 µmol L^{-1}) or GW3965 (1 µM) for 24 h. Controls with culture medium and chyme were included. After incubation, total RNA was isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany).

Purified RNA (1 µg) was used to generate cDNA (iScript cDNA synthesis kit, Bio-Rad Medical Diagnostics GmbH, Dreieich, Germany) using a MJ Research PTC-200 thermocycler (Bio-Rad). RT-PCR was carried out using 100 ng of cDNA in a final reaction volume of 10 µL (iQ SYBR Green Supermix, Bio-Rad), in a CFX96™ Real-Time PCR Detection System (Bio-Rad). The primers were selected based on literature data, reviewed with the Nucleotide Blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and are described in Table S1, Supporting Information. All primer pairs were from biomers.net GmbH (Ulm, Germany) (biomers.net GmbH, Ulm, Germany). Annealing temperatures were experimentally determined by PCR product size in agarose gels. The thermal conditions were set at 95°C during 3 min for enzyme activation, followed by 40 PCR cycles of 15 s at 95°C for denaturation, 30 s at optimized annealing temperature (Table S1, Supporting Information), and 30 s at 72°C for elongation. The relative mRNA expression was normalized against the geometric mean of the reference genes β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA box binding protein (*TBP*) and the ribosomal protein P0 (*RPLP0*). Data are expressed as fold-change relative to untreated control and are presented as mean values ± S.D. from three independent experiments with three different cell passages.

Statistical Analyses: One-way analysis of variance (ANOVA) with Dunnet's multiple comparison post hoc test was applied to compare results from treatments against the control treatment. All statistical analyses were carried out using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA) at a confidence level of 95%.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

F.J.A. designed the study, conducted the experiments, collected data and wrote the manuscript. Authors N.S., J.P. S.B., and T.A.K. provided methods and conceptional discussion. Author J.F. was involved in the study design, data analysis, and manuscript edition. All authors read and approved the submission of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

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