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Betacyanins, major components in Opuntia red-purple fruits, protect against acetaminophen-induced acute liver failure

Herson Antonio González-Ponce, Ma. Consolación Martínez-Saldaña, Pieter G. Tepper, Wim J. Quax, Manon Buist-Homan, Klaas Nico Faber, Han Moshage

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ABSTRACT

Acetaminophen (APAP) misuse or overdose is the most important cause of drug-induced acute liver failure. Overdoses of acetaminophen induce oxidative stress and liver injury by the electrophilic metabolite N-acetyl-p-benzoquinone imine (NAPQI). Plant-based medicine has been used for centuries against diseases or intoxications due to their biological activities. The aim of this study was to evaluate the therapeutic value of Opuntia robusta and Opuntia streptacantha fruit extracts against acetaminophen-induced liver damage and to identify the main biocomponents on them. Opuntia fruit extracts were obtained by peeling and squeezing each specie, followed by lyophilization. HPLC was used to characterize the extracts. The effect of the extracts against acetaminophen-induced acute liver injury was evaluated both in vivo and in vitro using biochemical, molecular and histological determinations. The results showed that betacyanins are the main components in the analyzed Opuntia fruit extracts, with betanin as the highest concentration. Therapeutic treatments with Opuntia extracts reduced biochemical, molecular and histological markers of liver (in vivo) and hepatocyte (in vitro) injury. Opuntia extracts reduced the APAP-increased expression of the stress-related gene Gadd45b. Furthermore, Opuntia extracts exerted diverse effects on the antioxidant related genes Sod2, Gclc and Hmox1, independent of their ROS-scavenging ability. Therefore, betacyanins as betanin from Opuntia robusta and Opuntia streptacantha fruits are promising nutraceutical compounds against oxidative liver damage.

1. Introduction

Acute liver failure (ALF) is a rare and unpredictable clinical syndrome, characterized by sudden, severe liver dysfunction associated with coagulopathy and hepatic encephalopathy (Khandelwal et al., 2011). An important cause of ALF is unintentional misuse of over-the-counter (OTC) pain medication, in particular acetaminophen, the most commonly used OTC product in the United States (Wolf et al., 2012). Acetaminophen, or paracetamol, 4-hydroxy-acetanilide, N-acetyl-p-benzoquinone imine (NAPQI). Plant-based medicine has been used for centuries against diseases or intoxications due to their biological activities. The aim of this study was to evaluate the therapeutic value of Opuntia robusta and Opuntia streptacantha fruit extracts against acetaminophen-induced liver damage and to identify the main biocomponents on them. Opuntia fruit extracts were obtained by peeling and squeezing each specie, followed by lyophilization. HPLC was used to characterize the extracts. The effect of the extracts against acetaminophen-induced acute liver injury was evaluated both in vivo and in vitro using biochemical, molecular and histological determinations. The results showed that betacyanins are the main components in the analyzed Opuntia fruit extracts, with betanin as the highest concentration. Therapeutic treatments with Opuntia extracts reduced biochemical, molecular and histological markers of liver (in vivo) and hepatocyte (in vitro) injury. Opuntia extracts reduced the APAP-increased expression of the stress-related gene Gadd45b. Furthermore, Opuntia extracts exerted diverse effects on the antioxidant related genes Sod2, Gclc and Hmox1, independent of their ROS-scavenging ability. Therefore, betacyanins as betanin from Opuntia robusta and Opuntia streptacantha fruits are promising nutraceutical compounds against oxidative liver damage.

Abbreviations: ALF, acute liver failure; OTC, over-the-counter; APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone-imine; GSH, reduced glutathione; GSSG, glutathione disulfide; NAC, N-acetylcysteine; ROS, reactive oxygen species; RNS, reactive nitrogen species; JNK, c-Jun-N-terminal kinase; MPT, mitochondrial permeability transition; ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; MDA, malondialdehyde; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; Nfe2l2, nuclear factor, erythroid 2-like 2; Keap1, Kelch-like ECH-associated protein 1; Gsh2, glutathione reductase; Hmox1, heme oxygenase 1; Gclc, glutamate-cysteine ligase, catalytic subunit; Gadd45, growth arrest and DNA-damage-inducible; Nrf2, nuclear factor kappa B; Sp1, Sp1 transcription factor; GCDCA, glycochenodesoxycholic acid; Bax, BCL2 associated X; Fas, Fas cell surface death receptor; NASH, non-alcoholic steatohepatitis

⁎ Corresponding author at: Hanzeplein 1, 9713 GZ Groningen, the Netherlands.

E-mail addresses: herson_qfbd@hotmail.com (H.A. González-Ponce), mcmtzsal@correo.uaa.mx (M.C. Martínez-Saldaña), m.buist-homan@umcg.nl (M. Buist-Homan), k.n.faber@umcg.nl (K.N. Faber), a.j.moshage@umcg.nl (H. Moshage).

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under normal conditions is inactivated by reduced glutathione (GSH) (Eugenio-Pérez, Montes de Oca-Solano, & Pedraza-Chaverri, 2016; Lan caster, Hiatt, & Zarrinar, 2015). At high doses of APAP, the glucuronidation and sulphation pathways are saturated resulting in excessive production of NAPQI causing depletion of liver GSH. NAPQI then forms covalent bonds (adducts) with proteins and non-protein thiols, initiating alkylation of proteins, lipid peroxidation of membranes, imbalance of intracellular calcium homeostasis, production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), ATP depletion and eventually cell death (McGill & Jaeschke, 2013; Seki, Brenner, & Karin, 2012). The only approved treatment for APAP overdose is N-acetylcysteine (NAC), a precursor of GSH. This reduces oxidative stress and liver injury (Ferner, Dear, & Bateman, 2011). However, NAC is not always effective and liver transplantation is the last therapeutic option. Therefore, there is an urgent need for novel and effective interventions to improve the prognosis of APAP-induced ALF.

Plants and their derivatives have been part of traditional medicine due to the presence of bioactive components and they play an important role in the treatment and prevention of diseases (González-Ponce, Rincón-Sánchez, Jaramillo-Juárez, & Moshage, 2018). In Mexico, cactus species (Opuntia spp.) are an important dietary component (Saenz, 2000) and have been used because of their beneficial effects (Santos Díaz, Barba de la Rosa, Hélíes-Toussaint, Guéraud, & Negré-Salvayre, 2017) such as antioxidant (Coria Cayupán, Ochoa, & Nazareno, 2011), anti-inflammatory (Antunes-Ricardo, Gutiérrez-Uribé, López-Pacheco, Alvarez, & Serna-Saldivar, 2015), hepatoprotective (González-Ponce et al., 2016), hypoglycemic (Leem, Kim, Hahm, Taunus, Germany) and juice was collected into 50 ml dark tubes to carry out by using a Braun J500 juice extractor (Braun, GmbH, München). Juice extract was reconstituted in 50 ml of deionized water and clarified by centrifugation at 12,000 rpm for 15 min at 15 °C. Determination was carried out spectrophotometrically at 535 nm and the concentration was calculated using the follow equation:

\[
\text{Betacyanins [mg/L]} = \left( \frac{A \times \text{DF} \times \text{MW} \times 1000}{(\epsilon \times 1)} \right)
\]

where: \( A \) = absorbance 535 nm, \( \text{DF} \) = dilution factor, \( \text{MW} \) = molecular weight (550 g/mol), \( \epsilon \) = extinction coefficient (60,000 L/mol cm), and \( 1 \) = width of the spectrophotometer cell (1 cm). The quantification was performed in triplicate on a Biotek PowerWave XS microplate reader and the results were expressed as mg of betacyanins equivalents/L.

2.3. High-performance liquid chromatography (HPLC) characterization

HPLC analysis was carried out using a Shimadzu-VP system, consisting of an LC-10AT pump, SIL-20A autosampler, and diode array detector SPD-M10A (Shimadzu corporation, Kyoto, Japan). Separation set up was based on the method described by (Serra, Poi, Matias, Bronze, & Duarte, 2013), with some modifications. It was performed at 35 °C in an Atlantis dC18 (5 µm, 150 mm × 4 mm i.d.) column from Waters (Milford, MA, USA) with a security guard column C4A 300-4.287 (8 mm × 3.2 mm i.d.) from Phenomenex (Torrance, CA, USA). The injected volume of standard and samples was 20 µL. Separation flow rate was 800 µL/min and the mobile phase consisted of a gradient mixture of eluent A (water + 0.1% formic acid) and eluent B (acetonitrile + 0.1% formic acid). The eluent gradient used was: 0–5 min eluent A; 5–8 min from 0 to 7% eluent B; 8–18 min from 7 to 10% eluent B; 18–21 min 10% eluent B; 21–28 min from 10 to 20% eluent B; 28–35 min from 20 to 50% eluent B; 35–40 min from 50 to 100% eluent B; 40–45 min 100% eluent B; 45–50 min from 100 to 0% eluent B; 50–55 min 100% eluent A.

A known concentration of betanin (10 mg/ml), gallic acid (0.5 mg/ml) and quercetin (0.5 mg/ml) standards from Sigma-Aldrich (St. Louis, MO, USA) were used to identify the main biocomponents in the Opuntia extracts by comparing retention time and spectra at 535, 280 and 360 nm, respectively.

2.4. Animals

Adult male Wistar rats (200–250 g) were used for the in vivo and in vitro studies. The animals were obtained from the animal facility of the Universidad Autónoma de Aguascalientes (for the in vivo experiments) and University Medical Center Groningen (for the in vitro experiments) and kept in polypropylene cages at room temperature (25 ± 2 °C) with food and water ad libitum. Experiments were approved by and performed according to the guidelines of the local committee for care and use of laboratory animals (permission No. 6415A of the committee for care and use of laboratory animals of the University of Groningen and Mexican governmental guideline NOM-003-ZOO-1995).

2.5. Rat hepatocyte isolation

Hepatocytes were isolated from albino male Wistar rats (Charles River Laboratories Inc. Wilmington, MA, USA) by two-step collagenase perfusion as described by (Woudenberg-Vrenken, Buist-Homan, Conde de la Rosa, Faber, & Moshage, 2010). Only isolations with a viability higher than 85% determined by Trypan blue exclusion assay, were used. Cells were allowed to attach for 4 h on 6-well plates in William’s E medium (Invitrogen, Breda, The Netherlands) supplemented with 50 µg/mL gentamycin (Invitrogen), 1% penicillin-streptomycin-fungizone (PSF) (Lonza, Lavern, Belgium), 5% fetal calf serum (FCS) (Invitrogen) and 50 nmol/L dexamethasone (Department of Pharmacy,
UMCG, Groningen, The Netherlands). Cells were cultured in a humidified incubator at 37 °C and 5% CO₂. Before the start of the experiments, medium was changed to medium without FCS and dexamethasone.

2.6. Experimental design

2.6.1. In vivo experiment

Albino male Wistar rats (200 – 250 gr) were randomly divided into seven groups (n = 10): Group 1 – Control; Group 2 – APAP; Group 3 – Opuntia robusta (Or) treated; Group 4 – Opuntia streptacantha (Os) treated; Group 5 – APAP + Or treated; Group 6 – APAP + Os treated; and Group 7 – APAP + NAC. Rats (groups 2, 5, 6 and 7) were intoxicated with a single dose of APAP (500 mg/kg, intraperitoneally, Sigma-Aldrich). After 0.5 h, rats in the appropriate groups were therapeutically treated with a single dose of Opuntia extract (800 mg/kg, orally) (González-Ponce et al., 2016) or NAC (300 mg/kg, intraperitoneally, Sigma-Aldrich) (Geng et al., 2015). After 6 h of APAP intoxication samples of blood and liver tissue were collected from six animals of each group for the assessment of biochemical markers of hepatic damage and for RNA isolation. Liver tissue from the other animals was collected 24 h after APAP intoxication for histological evaluation.

Biochemical markers of liver damage, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) were measured spectrophotometrically (Varian UV visible spectrophotometer, model DMS80, Varian, Inc., CA, USA) in plasma using commercial kits (SPINREACT, Girona, Spain). The values represent the mean of six samples ± standard error of the mean (SEM) and are expressed as IU/L. Hepatic GSH content in tissue evaluation. Liver tissue from the other animals was collected 24 h after APAP intoxication for histological evaluation.

Biochemical markers of liver damage, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) were measured spectrophotometrically (Varian UV visible spectrophotometer, model DMS80, Varian, Inc., CA, USA) in plasma using commercial kits (SPINREACT, Girona, Spain). The values represent the mean of six samples ± standard error of the mean (SEM) and are expressed as IU/L. Hepatic GSH content in tissue homogenates from experimental animals was determined according to (Hissin & Hilf, 1976), using homogenates from experimental animals was determined according to (Uchiyama & Mihara, 1978) for the betacyanins de-
Fig. 1. HPLC chromatograms obtained at 535 nm from the betanin standard (A), *Opuntia robusta* extract (B), and *Opuntia streptacantha* extract (C). Betanin and isobetanin were detected after 18 and 20 min, respectively.
fruit extract treatment against APAP-induced hepatotoxicity in comparison to *Opuntia streptacantha* fruit extract.

### 3.2. Characterisation of *Opuntia* extracts by HPLC

Betacyanins, specifically betanin and its isomer (isobetanin) are the most abundant antioxidant-related components in the *Opuntia* cactus fruit extracts. No other phenolic acids (280 nm) or flavonoids (360 nm) with comparable intensity were identified (Supplemental Figs. 1 and 2). The chromatograms from *Opuntia robusta* (Fig. 1B) and *Opuntia streptacantha* (Fig. 1C) at 535 nm were compared to the chromatogram of the betanin standard (Fig. 1A). The standard showed a retention time of

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**Fig. 2.** Biochemical markers of liver damage in plasma (A) ALT, (B) AST, (C) LDH, (D) ALP and liver tissue (E) GSH and (F) MDA of the experimental groups after 6 h of acetaminophen intoxication and/or different treatments. Each bar represents the mean of six samples ± SEM. *P* < 0.05 compared to APAP group. **P** < 0.05 compared to control group.
18 and 20 min for betanin and isobetanin, respectively. Both Opuntia extracts showed two peaks at the same retention time as the betanin standard confirming the presence of betanin and isobetanin. In both extracts there were no additional peaks in the whole chromatogram (55 min) ensuring that betanin and isobetanin are the major components in these extracts. The amount of both betanin and isobetanin appeared to be higher in the Opuntia robusta extract as compared to the Opuntia streptacantha extract.

3.3. In vivo experiments

3.3.1. Biochemical markers of liver damage

The levels of the biochemical markers of hepatic injury in plasma and tissue homogenates are shown in Fig. 2.

APAP significantly increased (P < 0.05) the levels of ALT (90.77 ± 9.76 U/I), AST (367.40 ± 8.50 U/I), LDH (1572.22 ± 57.95 U/I) and ALP (338.06 ± 37.58 U/I) which represent an increase of 252, 648, 729 and 67%, respectively, compared to the control group where the results were 25.76 ± 1.85 U/I for ALT, 49.09 ± 4.50 U/I for AST, 189.61 ± 22.44 U/I for LDH and 202.17 ± 18.65 U/I for ALP. A therapeutic single dose of Opuntia robusta or Opuntia streptacantha significantly reduced (P < 0.05) all markers of liver injury in plasma (35.2% and 31.5% for ALT; 31.8% and 24.6% for AST; 45.9% and 23.6% for LDH; 40.2% and 36.3% for ALP, respectively) compared to the APAP group. NAC was only effective in decreasing ALT levels (30.3%), the main marker of liver damage, compared to the APAP group (P < 0.05). Opuntia cactus fruits alone did not induce significant alterations in the biochemical markers.

There was a significant decrease of 83.3% of GSH content in liver tissue of the APAP group (131.72 ± 6.25 µg/g) compared to the control group (788.59 ± 28.75 µg/g) (P < 0.05) (Fig. 2-E). Treatment with Opuntia robusta and streptacantha fruit extracts preserved the GSH content in liver tissue of APAP-intoxicated rats with a non-significant reduction of 34.1% and 15.4% compared to the control group (Fig. 2-E). Treatment with Opuntia extracts alone did not induce alterations in the total GSH content. NAC was also effective in maintaining the levels of hepatic GSH in the APAP-treated group with a non-significant reduction of 8.9% compared to the control group (Fig. 2-E).

APAP intoxication induced a significant increase of MDA levels of 119.4% (130.35 ± 10.34 nmol/100 mg) in liver tissue as compared to the control group (59.40 ± 2.75 nmol/100 mg) (P < 0.05) (Fig. 2-F). Treatment with Opuntia fruit extracts and NAC after APAP intoxication significantly reduced (P < 0.05) levels of MDA (53.6% for APAP + Or, 47.3% for APAP + Os, and 44.9% for APAP + NAC groups) to control levels (Fig. 2-F). Opuntia extracts alone did not change the levels of MDA compared to the control group.

3.3.2. Relative mRNA expression of oxidative stress-related genes

After 6 h of APAP intoxication, liver tissue was collected to quantify the relative mRNA expression of the main antioxidant enzymes (Sod2, Hmox1, Gclc) and the cell survival promotor Gadd45b (Fig. 3).

APAP significantly increased the expression of Sod2 in 157%,...
Hmox1 in 2029%, Gclc in 178% and Gadd45b in 418% compared to the control group ($P < 0.05$). Treatment with a single dose of *Opuntia robusta* and *Opuntia streptacantha* fruit extracts also induced a significant increase ($P < 0.05$) in the gene expression of the antioxidant enzymes Sod2 (36.9% and 83.6%, respectively) and Gclc (133.6% and 67.2%, respectively) but not for Hmox1 and the cell stress sensor Gadd45b compared to the control group. For the APAP-intoxicated groups treated with *Opuntia robusta*, *Opuntia streptacantha* or NAC, the relative mRNA expression levels of the enzymes Sod2 (45.5%, 51.8% and 50.6%, respectively), Hmox1 (71.7%, 79.1% and 29.2%, respectively), Gclc (35.6%, 65.5% and 43.2%, respectively) and Gadd45b (86.8%, 81.1% and 58.3%, respectively) were significantly reduced compared to the APAP group ($P < 0.05$) (Fig. 3).

### 3.3.3. Histopathology

APAP intoxication induced significant hydropic degeneration (cellular edema) and focal necrosis in the hepatocytes near the central vein (centrilobular) (Fig. 4-B). In addition, the normal structure of hepatic parenchyma (polygonal form of the cells and hepatic sinusoids) was disrupted in the APAP group (Fig. 4-B) compared to the control group which showed a normal architecture of liver (Fig. 4-A). Treatment with *Opuntia* extracts (Fig. 4-E,F) or NAC (Fig. 4-G) after APAP intoxication reduced focal necrosis and ballooning degeneration of the central hepatocytes (centrilobular) of the hepatic acinus (zone III). *Opuntia* extracts alone did not induce alterations in the morphology of the hepatic lobule (central area) (Fig. 4-C,D). *Opuntia robusta* treatment appeared to be more protective than *Opuntia streptacantha* and NAC with respect to the histopathological changes induced by APAP.
3.4. In vitro experiments

3.4.1. LDH leakage

Primary hepatocytes exposed to a single dose of APAP showed significant LDH release (3.8 fold increase) into the medium after 24 h compared to the control group (Table 2). Therapeutic treatment with Opuntia robusta or Opuntia streptacantha after APAP intoxication significantly reduced LDH release to control levels indicating improved survival compared to the APAP group (P < 0.05) (Table 2). Opuntia extracts alone did not induce liver cell death after 24 h of exposure (P > 0.05 vs control). NAC treatment was also effective in protecting the hepatocytes against APAP-induced cell death and significantly reduced LDH release compared to the APAP group (P < 0.05) (Table 2).

3.4.2. Sytox green stain

Cell membrane disruption and necrotic cell death induced by APAP was confirmed using the cell-impermeable fluorescent dye SYTOX® Green. As shown in Fig. 5, necrotic cell death was dramatically increased 24 h after APAP intoxication compared to the control group (Fig. 5-A). Therapeutic treatment with Opuntia extracts (Fig. 5-E,F) or NAC (Fig. 5-G) considerably reduced necrotic cell death in primary hepatocytes exposed to APAP compared to the APAP group (Fig. 5-B). Treatment with Opuntia extracts alone did not alter membrane permeability of the primary hepatocytes (Fig. 5-C,D).

3.4.3. Relative mRNA expression of oxidative stress-related genes

The mRNA level of Sod2 did not change up to 24 h after APAP exposure but was significantly reduced (62.2%) after 24 h of intoxication compared to the control (P < 0.05) (Fig. 6-A). The mRNA levels of antioxidant enzymes Hmox1 and Gclc were significantly increased (766 and 3288%, respectively) after 24 h of APAP intoxication (P < 0.05) (Fig. 6-B,C). mRNA level of the cell stress sensor Gadd45B gradually increased after exposure to APAP and peaked (197%) at 24 h after APAP exposure (Fig. 6-D).

Opuntia extracts and NAC displayed diverse effects on the APAP-induced changes in oxidative stress-related genes: therapeutic treatment with Opuntia robusta and Opuntia streptacantha fruit extracts of APAP-intoxicated hepatocytes restored Sod2 expression (42.2 and 43.6% vs APAP group), whereas therapeutic treatment with NAC did not restore Sod2 expression. Interestingly, Opuntia robusta and Opuntia streptacantha fruit extracts alone induced Sod2 expression compared to controls (74.2 and 130%, respectively). With regard to Hmox1, Opuntia extracts, in contrast to NAC (91.1% vs control group), did not attenuate the APAP-induced increase of Hmox1 (762% for APAP + Or; and 613% for APAP + Os vs control group). Opuntia extracts alone moderately, but not significantly, increased Hmox1 expression compared to control. Yet another effect was observed for Gclc: Opuntia robusta and Opuntia streptacantha fruit extracts further increased the APAP-induced increase of Gclc (789 and 939% vs control group, respectively; or, 107.7 and 119.6% vs APAP group, respectively), whereas NAC attenuated 196% the APAP-induced increase of Gclc expression. Opuntia extracts alone did not significantly change (P > 0.05) Gclc expression compared to the control group. Finally, both Opuntia robusta and Opuntia streptacantha fruit extracts, and NAC tended to significantly attenuate (94, 151 and 116%, respectively) the APAP-induced increase of Gadd45B expression (Fig. 7).

4. Discussion

Opuntia spp. fruits contain many bioactive components with potential health benefits but the exact composition is dependent on physical, chemical, geographical and environmental factors. Thus, it is important to identify the main bioactive compounds that are responsible for the potential protective mechanisms.

In this study we quantified spectrophotometrically the betacyanin content and determined by HPLC analysis that betalains, specifically betacyanins, are the most important components in extracts of Opuntia robusta and Opuntia streptacantha fruits. In our previous study, we quantified betalains, flavonoids, ascorbic acid and total phenolics in Opuntia robusta and Opuntia streptacantha fruit extracts by spectrophotometry and reported that betalains are the second major component after total phenolics (González-Ponce et al., 2016). In support, (Stintzing et al., 2005), reported that betacyanins are the second major group of components after total phenolics in the fruits of Opuntia ficus-indica clones, although it is important to remark that betacyanins such as betanin and its isomer might be detected as phenolic compounds due to the presence of a phenolic ring in their structure. They identified betanin and isobetanin as the most abundant betacyanins in these clones, although they also identified additional betacyanins such as gomphrenin I, betadin and neobetanin. (Serra et al., 2013), showed that betacyanins are the major components in hydroalcoholic extracts obtained from Opuntia ficus-indica and Opuntia robusta.

Our results demonstrate the hepatoprotective effect of therapeutic treatment with betacyanin-rich Opuntia purple fruit extracts against APAP-intoxication both in vitro and in vivo. The protective effect is mainly due to the reduction of oxidative stress induced by the free radical NAPQI. In vivo, Opuntia extracts reduced the biochemical markers of liver damage; diminished the hepatic levels of malondialdehyde and restored the levels of glutathione, indicating diminished oxidative stress; and improved the hepatic architecture, specifically at the centrolobular region (zone III of the hepatic acinus) where the expression of the CYP2E1 isoform is highest and APAP is biotransformed into the electrophilic metabolite NAPQI causing most damage in this region (Abdelmegeed, Moon, Chen, Gonzalez, & Song, 2010). In vitro, treatment with Opuntia extracts reduced LDH leakage into the medium and Sytox green nuclear staining, indicating reduced necrotic cell death. Of note, our results indicate that the treatment with Opuntia extracts may have therapeutic value, since the protective effect of the extracts was observed when administered after APAP intoxication, both in vivo and in vitro. We have previously demonstrated the protective effect of the prophylactic consumption of both extracts (González-Ponce et al., 2016). In addition, the protective effect appeared to be at least as effective as observed with NAC, the currently used treatment for APAP-induced acute liver failure, with Opuntia robusta being slightly more protective than Opuntia streptacantha.

The antioxidant status of cells is dependent on many factors, including several oxidative stress-related enzymes like mitochondrial superoxide dismutase 2, heme oxygenase 1 and the rate-limiting enzyme in glutathione synthesis, glutamate-cystein ligase.

Superoxide dismutases (SOD) play a key role in the protection against reactive oxygen species (ROS). They catalyze the conversion of superoxide anions (O2•–) into hydrogen peroxide (H2O2) and oxygen (O2). Two types of SOD enzymes (Sod1 and Sod2) are distinguished: cytoplasmic Sod1 and mitochondrial Sod2 (Wang, Brancicky, Noé, & Hekimi, 2018). (Chen et al., 2015), described that increasing the
activity of Sod2 reduced glycochenodeoxycholic acid (GCDC)-induced mitochondrial oxidative stress in rat hepatocytes. On the other hand, Sod2 has also been related to tumorigenicity, both as a tumor suppressor and as tumor promotor (Hempel et al., 2011). Both Hmox1 and Gclc are inducible target genes of the oxidative stress-responsive transcription factor Nfe2l2. Gclc plays an important role in the synthesis of GSH. (Botta et al., 2006), demonstrated that overexpression of Gclc in transgenic animals protects the liver against APAP-induced liver injury. (Kay et al., 2010), reported that the treatment with ajoene, a component of garlic, increased GSH content through Nfe2l2 activation and induction of Gclc, protecting HepG2 cells and hepatocytes against oxidative stress. Hmox1 is another Nfe2l2-regulated antioxidant enzyme. It is an ubiquitous stress-responsive enzyme with several functions in tissue homeostasis (Kim et al., 2011). We have previously shown that overexpression of the oxidative stress-responsive enzyme Hmox1 protects hepatocytes against apoptosis via inhibition of superoxide anion-

Fig. 5. Necrotic cell death determined by Sytox green fluorescent dye in primary rat hepatocytes after 24 h of exposure to acetaminophen and/or treatments. (A) Control, (B) Acetaminophen – APAP, (C) Opuntia robusta – Or, (D) Opuntia streptacantha – Os, (E) APAP + Or, (F) APAP + Os, and (G) APAP + NAC.
induced JNK activity (Conde de la Rosa et al., 2008). (Chiu, Brittingham, & Laskin, 2002), described that Hmox1 is an important antioxidant enzyme in the protection against APAP-induced hepato-toxicity. Although these oxidative stress-related genes are important, little is known about their role and regulation during APAP intoxication and their regulation by natural products.

In the present study, APAP alone increased the gene expression of Hmox1, Gclc and Gadd45b in vivo and in vitro. Expression of Sod2 was increased in vivo and decreased in vitro by APAP. The induction of Hmox1 and Gclc is in accordance with exposure to oxidative stress and their regulation by the oxidative stress responsive transcription factor Nfe2l2. In contrast, Sod2 is not an exclusive target gene of Nfe2l2 since it has been described that its expression is also modulated by the transcription factors NFkb and Sp1-dependent p53. p53 is a tumor suppressor protein and is known to modulate cell survival and apoptotic pathways. p53 target genes are involved in cell proliferation (e.g. Gadd45) and apoptotic cell death (e.g. Fas, Bax) (Vogelstein, Lane, & Levine, 2000). (Dhar et al., 2010) observed that gene expression of Sod2 is regulated in a dose-dependent manner by p53 via the transcription factors NFkb and Sp1. They propose that p53 has bi-directional effects leading to either cell survival or cell death by suppressing or activating target genes like Sod2. At present, the explanation for the opposite regulation in this study of Sod2 in vivo and in vitro is not clear, although it is very likely that the presence of other liver cell types in the in vivo situation, including inflammatory cells with activated NFkb (cytokine release) and abundant ROS production, lead to a different response in the regulation of Sod2. It should also be noted that for the mRNA expression studies, RNA was isolated under non-lethal conditions, both in vivo and in vitro.

Opuntia extracts alone enhanced the cytoprotective defenses by significantly increasing the expression of Sod2 in vivo and in vitro. These results indicate that the Opuntia extracts not only contain compounds that scavenge reactive oxygen species, but also contain factors that actually increase the expression of antioxidant genes.

Therapeutic treatment with Opuntia extracts prevented the APAP-induced increase of Sod2, Hmox1 and Gclc mRNA expression in vivo. However, Opuntia extracts exerted divergent effects in vitro: although they normalized Sod2 expression, they did not attenuate the APAP-induced increase in Hmox1 expression and even further increased the APAP-increased expression of Gclc. The reason for these divergent effects may be that oxidative stress induces the expression of oxidative stress-related genes and therefore, antioxidants attenuate these changes, but that in this case components in the Opuntia extracts modulate the expression of these genes independent of their ROS scavenging effects.

Finally, Gadd45 is a family of genes which are induced in response to (patho)physiological stresses. Gadd45 proteins have important functions as regulators of the cell cycle, cell survival or apoptosis, DNA repair and genomic stability (Ueda, Kohama, Kuge, Kido, & Sakurai, 2017). Gadd45b is an early predictor of liver dysfunction and stress
(Tian et al., 2011). (Papa et al., 2004), demonstrated the cytoprotective effect of Gadd45b via the activation of NFκB and the capacity to bind and block MKK7, an essential activator of pro-apoptotic JNK signaling. In addition, Gadd45b knock-out mice show decreased hepatocyte proliferation and increased programmed cell death after partial hepatectomy compared to wildtype mice (Papa et al., 2008). A recent study showed that APAP toxicity induced Gadd45b expression, which was further increased by the protective agent metformin and reduced JNK phosphorylation. Finally, increased cell death and sustained JNK phosphorylation was detected in primary hepatocytes with Gadd45b deficiency after sub-toxic doses of APAP (Y.-H.Kim et al., 2015). Together, these data indicate that Gadd45b is not only a sensor for cellular stress but also protects against cellular stress. In our study, we observed that APAP induced Gadd45b expression both in vivo and in vitro and that Opuntia extracts alone did not modulate Gadd45b expression. These results are in line with Gadd45b being a sensor of cellular stress. In addition, both in vivo and in vitro, Opuntia extracts reduced APAP-induced Gadd45b expression, again in line with Gadd45b being a sensor of cellular stress and Opuntia extracts relieving APAP-induced stress.

5. Conclusion

In conclusion, we observed a therapeutic effect of Opuntia robusta and Opuntia streptacantha against APAP-induced hepatotoxicity. Opuntia robusta appeared to be slightly more protective, probably due to the higher amount of betacyanin compounds than Opuntia streptacantha. In addition, the Opuntia extracts were at least as potent as NAC in the protection against APAP-induced hepatotoxicity. Furthermore, in addition to scavenging reactive oxygen species, we show that Opuntia extracts modulate the expression of important oxidative stress-related genes at the transcriptional level. In the current study, the therapeutic action of Opuntia extracts was investigated 30 min after APAP intoxication. Further studies are required to investigate whether more delayed administration of the extracts is effective as well. In addition, it will be interesting to investigate whether Opuntia extracts protect against other hepatotoxic drugs (e.g. diclofenac), non-drug hepatotoxicity like bile acids (cholestatic liver diseases) or fatty acid-induced lipotoxicity (non-alcoholic steatohepatitis). Finally, studies are required using purified components of the extracts to confirm the identity of the protective agents in order to facilitate clinical application.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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