university of groningen

University Medical Center Groningen

University of Groningen

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

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

Published in:
Food Research International

DOI:
10.1016/j.foodres.2020.109461

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
González-Ponce, H. A., Martínez-Saldaña, M. C., Tepper, P. G., Quax, W. J., Buist-Homan, M., Faber, K. N., & Moshage, H. (2020). Betacyanins, major components in Opuntia red-purple fruits, protect against acetaminophen-induced acute liver failure. *Food Research International*, *137*, [109461]. https://doi.org/10.1016/j.foodres.2020.109461

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

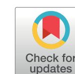
Food Research International 137 (2020) 109461

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

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



Herson Antonio González-Ponce[a,*], Ma. Consolación Martínez-Saldaña[b], Pieter G. Tepper[c], Wim J. Quax[c], Manon Buist-Homan[a,d], Klaas Nico Faber[a,d], Han Moshage[a,d]

[a] Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, the Netherlands
[b] Department of Morphology, Basic Sciences Centre, Universidad Autónoma de Aguascalientes, Mexico
[c] Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, the Netherlands
[d] Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, the Netherlands

ARTICLE INFO

Keywords:
Acetaminophen
Acute liver failure
Oxidative stress
Opuntia fruit extracts
Betacyanins
Nutraceutical compounds

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 major 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*-aminophenol (APAP) is a safe and effective analgesic and antipyretic OTC drug when used as recommended (Wang et al., 2017). However, APAP misuse or overdose can lead to ALF and APAP overdose is currently the leading cause of ALF in adults in Western countries (Fontana, 2008; Kim et al., 2015; Larson et al., 2005). At therapeutic doses, APAP is conjugated by glucuronidation or sulphation in the liver and excreted into the urine ($>90\%$). A small amount is excreted unchanged and $<10\%$ is biotransformed by cytochrome P450 enzymes into the reactive intermediate *N*-acetyl-*p*-benzoquinone-imine (NAPQI), which

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; *Sod2*, superoxide dismutase 2; *Hmox1*, heme oxygenase 1; *Gclc*, glutamate-cysteine ligase, catalytic subunit; *Gadd45*, growth arrest and DNA-damage-inducible; NFkb, nuclear factor kappa B; Sp1, Sp1 transcription factor; GCDCA, glycochenodeoxycholic 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).

https://doi.org/10.1016/j.foodres.2020.109461
Received 27 December 2019; Received in revised form 5 April 2020; Accepted 16 June 2020
Available online 18 June 2020
0963-9969/ © 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license
(http://creativecommons.org/licenses/by/4.0/).

under normal conditions is inactivated by reduced glutathione (GSH) (Eugenio-Pérez, Montes de Oca-Solano, & Pedraza-Chaverri, 2016; Lancaster, Hiatt, & Zarrinpar, 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, Héliés-Toussaint, Guéraud, & Négre-Salvayre, 2017) such as antioxidant (Coria Cayupán, Ochoa, & Nazareno, 2011), anti-inflammatory (Antunes-Ricardo, Gutiérrez-Uribe, López-Pacheco, Alvarez, & Serna-Saldívar, 2015), hepatoprotective (González-Ponce et al., 2016), hypoglycemic (Leem, Kim, Hahm, & Kim, 2016), neuroprotective (Dok-Go et al., 2003), anti-carcinogenic (Sreekanth et al., 2007), anti-atherogenic (Keller et al., 2015), and anti-genotoxic (Brahmi et al., 2011). These effects are in part due to the presence of natural pigments (e.g. betalains, carotenoids and flavonoids) and other phenolic compounds. Betalain pigments are particularly abundant in the Caryophyllales order and can be found in roots, flowers, fruits and some vegetative tissues of plants (González-Ponce et al., 2018). They provide protection against UV radiation and pathogens and act as optical attractants to pollinators. Betalains can be classified into betacyanins (red-violet) or betaxanthins (yellow-orange). The active cyclic amine group of betalains functions as hydrogen donor and confers reducing properties to these compounds (Kanner, Harel, & Granit, 2001). The betacyanins such as betanin and betanidin have enhanced antioxidant capacity compared to betaxanthins due to the presence of a phenolic ring which increases their electron transfer capability (Stintzing et al., 2005).

The aim of this study was to investigate the therapeutic effect of fruit extracts of two *Opuntia* species, *Opuntia robusta* and *Opuntia streptacantha* on APAP-induced hepatotoxicity both *in vivo* and *in vitro*, and to identify the main component(s) possibly related to their protective properties.

2. Materials and methods

2.1. Plant materials and preparation of extracts

Ripe fruits of *Opuntia robusta* and *Opuntia streptacantha* were collected from randomly selected plants in a semi-arid region of Aguascalientes, México (21°46′55.86″ N, 102°6′16.08″ O, and 1994 m above sea level). The juice extraction of each *Opuntia* fruit species was carried out by using a Braun J500 juice extractor (Braun, GmbH, Taunus, Germany) and juice was collected into 50 ml dark tubes to remove non-soluble fibers by centrifugation at 5000 rpm for 15 min at 4 °C. After that, the juice extracts were filtered through an 8-μm pore size Whatman filter paper, frozen at −80 °C and lyophilized as described previously (González-Ponce et al., 2016).

2.2. Betacyanins content

The betacyanins content was performed as described by (Sumaya-Martínez et al., 2011). Juice extracts were reconstituted in 50 ml of deionized water and clarified by centrifugation at 12,000*g* for 15 min at 15 °C. Determination was carried out spectrophotometrically at 535 nm and the concentration was calculated using the follow equation:

$$\text{Betacyanins [mg/L]} = [(A * DF * MW * 1000) / (\in * 1)]$$

where: A = absorbance 535 nm, DF = dilution factor, MW = molecular weight (550 g/mol), ∈ = 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, Poejo, Matias, Bronze, & Duarte, 2013), with some modifications. It was performed at 35 °C in an Atlantis dC_{18} (5 μm, 150 mm × 4 mm i.d.) column from Waters (Milford, MA, USA) with a security guard column C_{18} AJ0-4287 (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-033-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, Verviers, 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% CO_2. 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 homogenates from experimental animals was determined according to (Hissin & Hilf, 1976), using *o*-phtaldehyde (OPT) as the fluorescent reagent. The fluorescence intensity was measured at 420 nm using 350 nm as the excitation wavelength using a luminescence spectrophotometer (Model LS-50B, PerkinElmer Inc., Waltham, MA, USA). The values represent the mean of six samples ± SEM and are expressed as µg/g. Determination of malondialdehyde (MDA), a product of lipid peroxidation, was performed using the thiobarbituric acid reactive substance (TBARS) method according to (Uchiyama & Mihara, 1978) with some modifications. Samples were measured spectrophotometrically (Varian UV visible spectrophotometer, model DMS80) at 530 nm. The values represent the mean of six samples ± SEM and are expressed as nmol/100 mg. Histological analysis was performed by collecting liver tissue from the experimental animals 24 h after APAP intoxication. Animals were anesthetized with sodium pentobarbital and systemically perfused with saline solution (sodium chloride 0.9%), containing 0.5% heparin and 0.1% procaine and fixed *in situ* with neutral formalin (10%). The hepatic tissue was embedded in paraffin blocks and sections of 5 µm were prepared with a microtome RM2125RT (Leica Biosystems, USA). The sections were stained with hematoxylin/eosin (H&E). Liver tissue images were obtained using a slide scanner NanoZoomer 2.0 HT (Hamamatsu Photonics, Japan) and Aperio ImageScope Pathology slide viewer software (Leica Biosystems).

2.6.2. In vitro experiments

Stock solutions of acetaminophen (APAP, 2 mol/L in DMSO) and N-acetylcysteine (NAC, 1 mol/L in PBS) were prepared for all the *in vitro* experiments. *Opuntia* cactus fruit extracts were sterilized through filtration (0.2 µm pore size) before use.

Hepatocyte cultures were divided into seven groups following the same set up as in the *in vivo* experiments. Cells from Groups 2, 5, 6 and 7 were treated with 10 mmol/L APAP for biochemical and molecular assays and 20 mmol/L for cell death assays (González-Ponce et al., 2016). After 0.5 h, cells were therapeutically treated with a single dose of each *Opuntia* extract (16.5 mg of lyophilized extract ≈ 10 mg/mL) or NAC (5 mmol/L) (Odewumi et al., 2011). Cells were harvested at 24 h after APAP intoxication for biochemical assays and RNA isolation. LDH assay was used to determine necrotic cell death and performed 24 h after APAP intoxication as described by (Verhaag et al., 2016). Percentage of LDH released was calculated by measuring the LDH activity in both the medium and cell lysates. Determination of LDH in each group was performed in triplo *per* experiment and values represent the mean of three different experiments ± SEM.

After 24 h of APAP intoxication, SYTOX® Green nuclei acid stain (Invitrogen) was added to the cells for 15 min at 37 °C (1:40,000, diluted in William's E medium) to determine necrotic cell death by fluorescence microscopy (DMI6000B, Leica Microsystems, Germany) at 450–490 nm as reported by (Conde de la Rosa et al., 2006).

2.7. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA from *in vivo* and *in vitro* samples was isolated using Tri-reagent (Sigma-Aldrich), following manufacturer's protocol. RNA quantity and quality were determined using the Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription PCR (RT-PCR) was performed with 2.5 µg of RNA using the Moloney murine leukemia virus (M – MLV) reverse transcriptase system and random nanomers from Life Technologies (Breda, The Netherlands). RT-PCR was performed in 3 steps: 10 min at 25 °C, 1 h at 37 °C and 5 min at 95 °C with the GeneAmp PCR system (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands). Quantitative real-time PCR (qPCR) was performed using 4 µl 20-fold diluted cDNA in combination with 2x master mix (Eurogentec, Maastricht, The Netherlands) in a total volume of 20 µl. *18S* mRNA levels were used as housekeeping gene. Fluorescence was measured using the 7900HT Fast Real-Time System, and SDS 2.3 software (Applied Biosystems) (Verhaag et al., 2016). Results are expressed as fold induction and each value represents the mean of four samples (*in vivo*) and three different experiments (*in vitro*) ± SEM. Primers and probes are listed in Supplemental Table 1.

2.8. Statistical analysis

Data acquired from the experiments were statistically analyzed using GraphPad Prism 5 software (La Jolla, CA, USA). Considering a normal distribution of the values, a one-way analysis of variance (ANOVA) and a post-hoc Dunnett's multiple comparison test were used to compare the experimental groups and to determine significant differences with a confidence interval of 95%. For the betacyanins determination and time-response curves of the *in vitro* studies a two-tail unpaired T-test was performed to compare the control and treated groups at each time point with a confidence interval of 99%.

3. Results

3.1. Betacyanins content

The amount of betacyanins present in the *Opuntia robusta* and *Opuntia streptacantha* fruit extracts are shown in Table 1. *Opuntia robusta* fruit extract had a significantly higher concentration of betacyanins (2.21 fold; $P < 0.01$) compared to *Opuntia streptacantha* fruit extract suggesting a more potent biological activity of *Opuntia robusta*

Table 1
Quantification of betacyanins content in the *Opuntia* fruit extracts.

Fruit extract	Betacyanins (mg equivalents/L)
Opuntia robusta	464.974 ± 10.87¶
Opuntia streptacanta	148.941 ± 5.49

Values represent the mean of three different measurements ± SD. ¶ $P < 0.01$ vs *Opuntia streptacantha*.

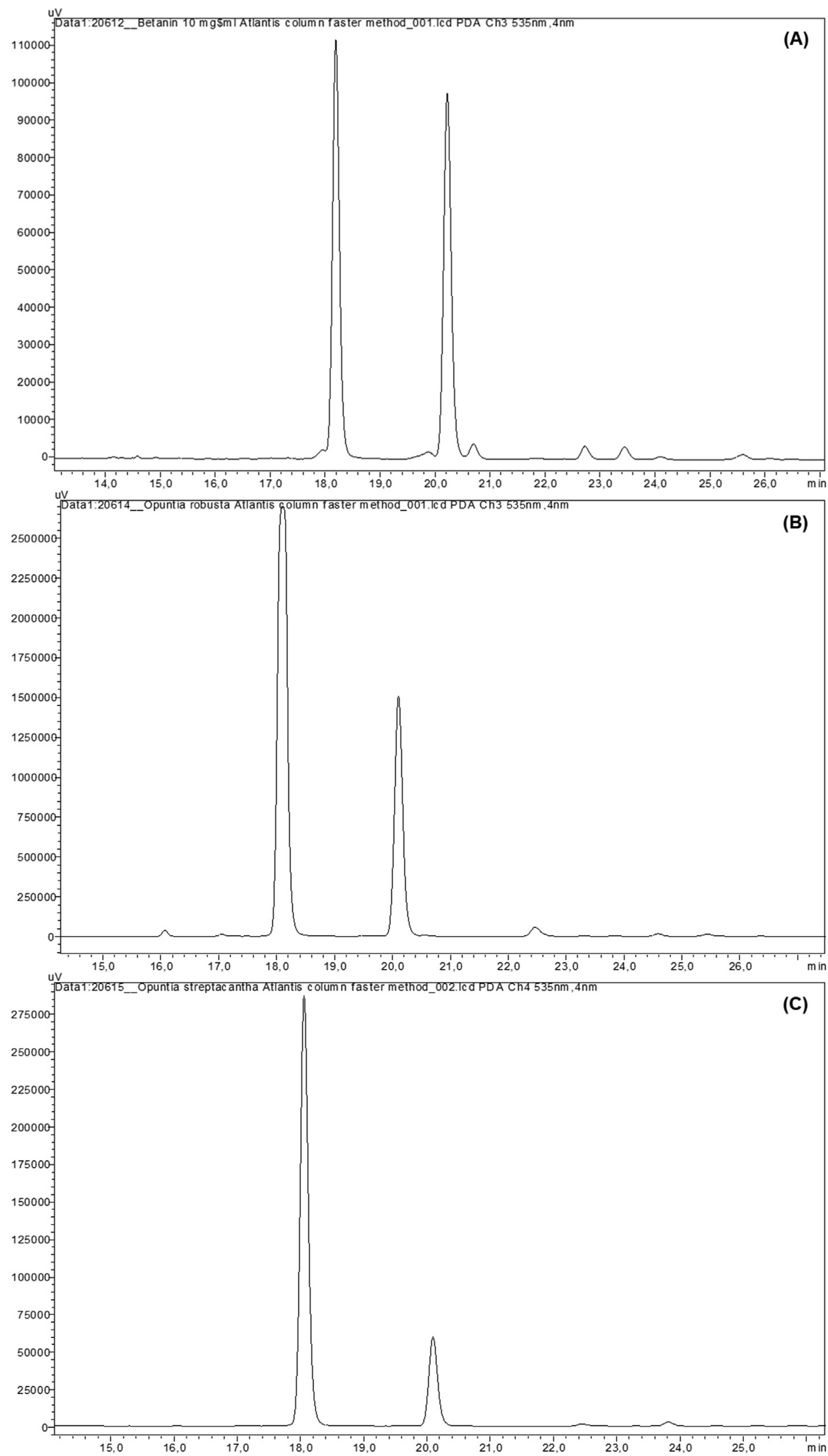


Fig. 1. HPLC chromatograms obtained at 535 nm from the betanin standard (A), *Opuntia robusta* extract (B), and *Opuntia streptacantha* extract (C). Betanin and isobetanin were detected after 18 and 20 min, respectively.

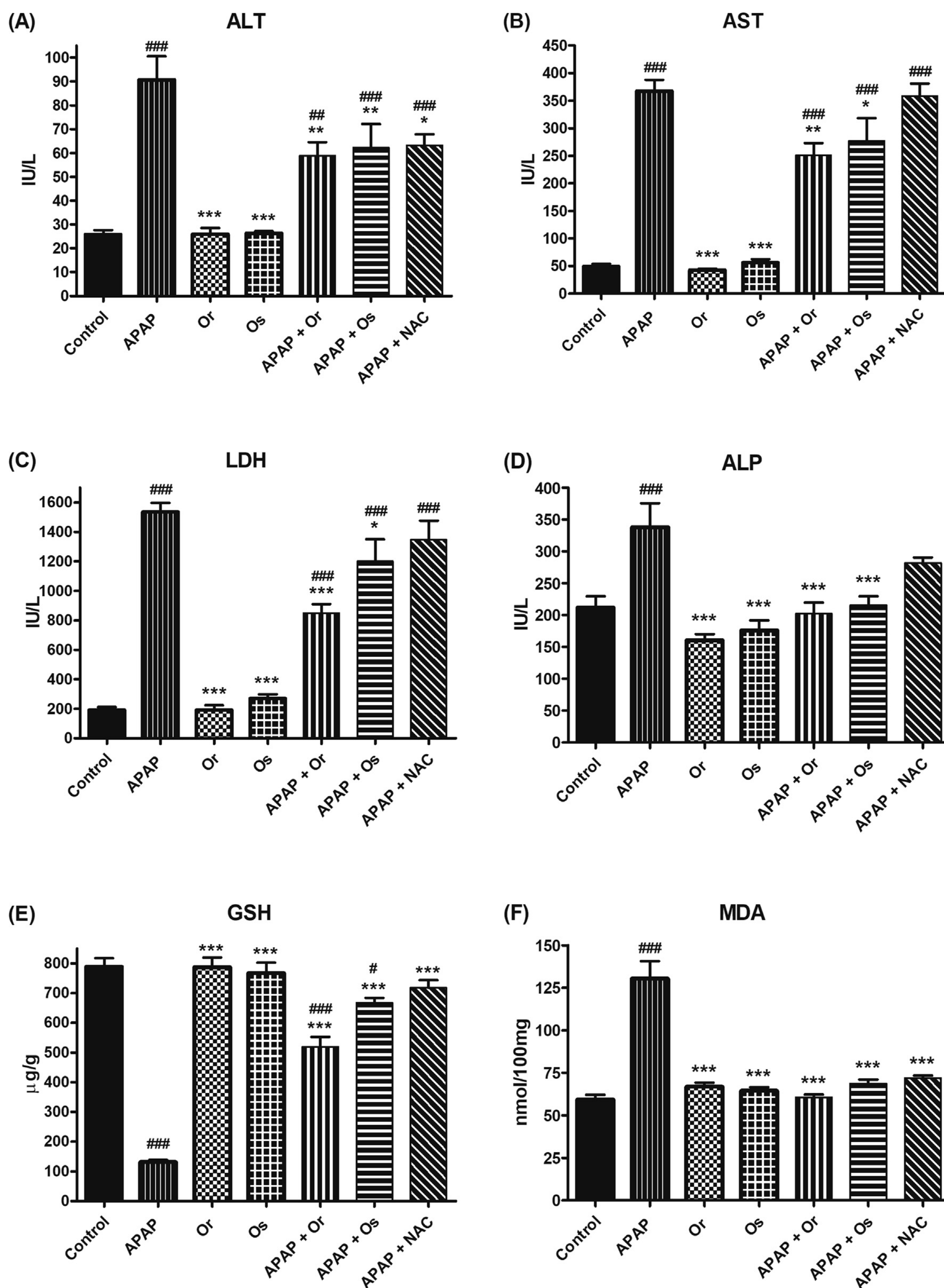


Fig. 2. Biochemical markers of liver damage in plasma (A) ALT, (B) AST, (C) LDH, (D) ALP and liver tissue (E) GSH and (F) MDA of the experimental groups after 6 h of acetaminophen intoxication and/or different treatments. Each bar represents the mean of six samples ± SEM. * $P < 0.05$ compared to APAP group. # $P < 0.05$ compared to control group.

fruit extract treatment against APAP-induced hepatotoxicity in comparison to *Opuntia streptacantha* fruit extract.

3.2. Characterization 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

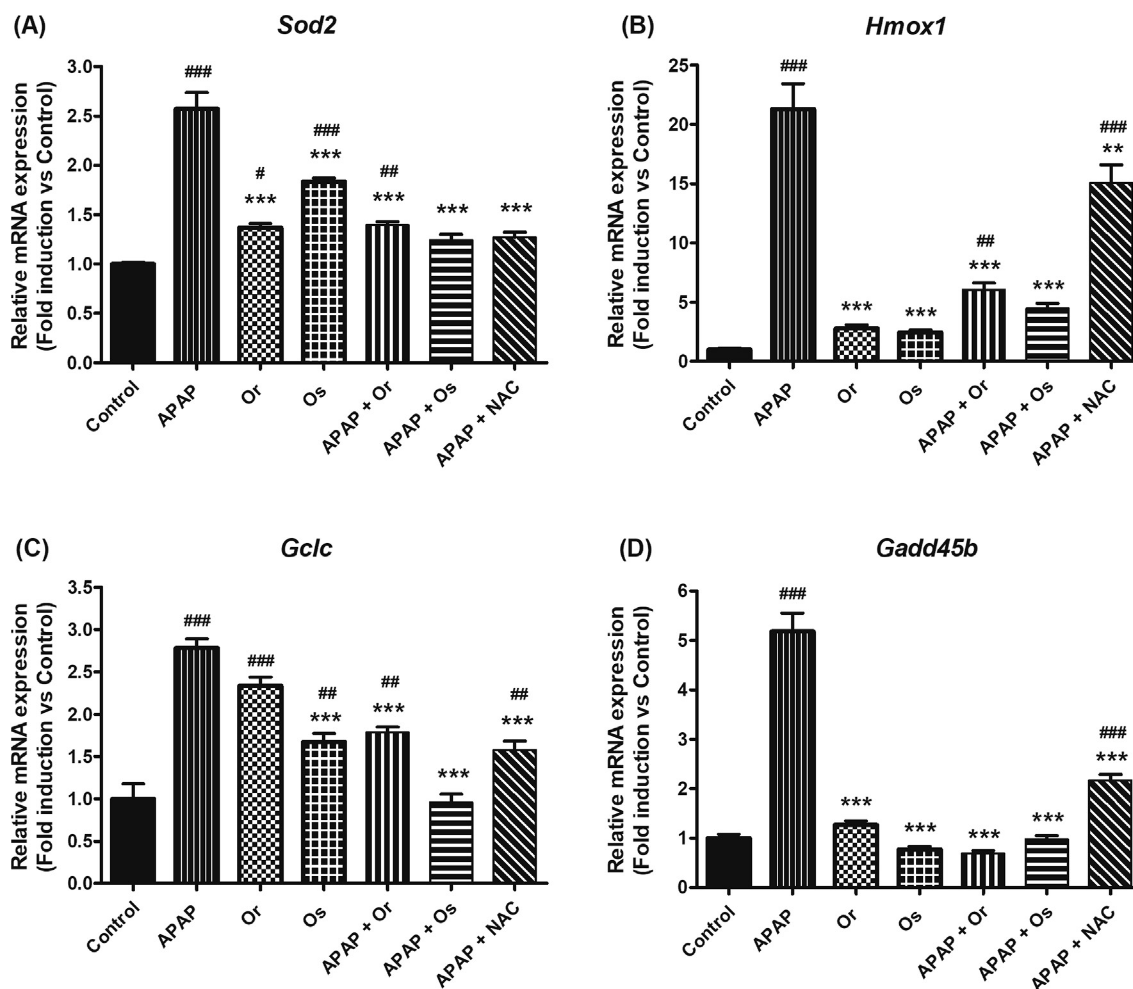


Fig. 3. Relative gene expression of oxidative stress-related genes, (A) *Sod2*, (B) *Hmox1*, (C) *Gclc*, and the cell survival promotor (D) *Gadd45b*, 6 h after APAP intoxication and/or different treatments in rats. Each bar represents the mean of four samples ± SEM. * $P < 0.05$ compared to APAP group. $^{\#}$ $P < 0.05$ 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%,

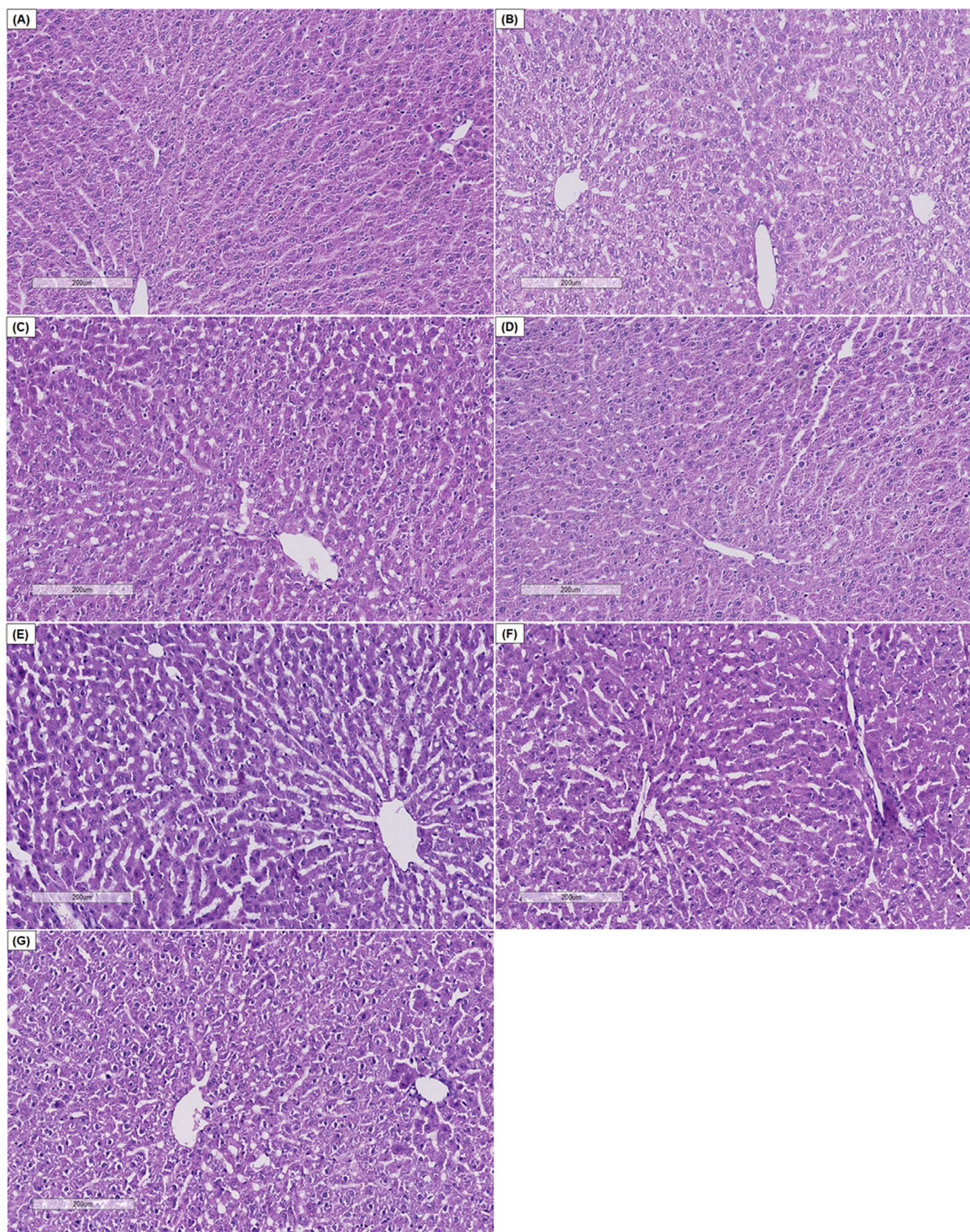


Fig. 4. Micrographs of hepatic parenchyma of the central area from liver sections of the experimental animals after hematoxylin-eosin staining, magnification 200×. (A) Control, (B) Acetaminophen – APAP, (C) *Opuntia robusta* – Or, (D) *Opuntia streptacantha* – Os, (E) APAP + Or, (F) APAP + Os, and (G) APAP + NAC.

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.

Table 2
Levels of LDH released after 24 h of exposure to APAP and therapeutic treatments with *Opuntia* extracts and NAC.

Group	LDH leakage (%)
Control	14.69 ± 2.38 †
APAP	70.32 ± 5.75 ¶
Or	14.05 ± 4.00 †
Os	10.76 ± 1.75 †
APAP + *Or*	18.23 ± 2.12 †
APAP + *Os*	13.28 ± 2.33 †
APAP + NAC	16.89 ± 0.36 †

Values represent the mean of three different experiments ± SEM. † $P < 0.05$ vs APAP. ¶ $P < 0.05$ vs Control. *Or, Opuntia robusta; Os, Opuntia streptacantha;* APAP, acetaminophen; NAC, N-acetylcysteine.

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 328%, 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, betanidin 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 vivo* and *in vitro*. 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 centrilobular 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 ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Two types of SOD enzymes (Sod1 and Sod2) are distinguished: cytoplasmic Sod1 and mitochondrial Sod2 (Wang, Branicky, Noë, & Hekimi, 2018). (Chen et al., 2015), described that increasing the

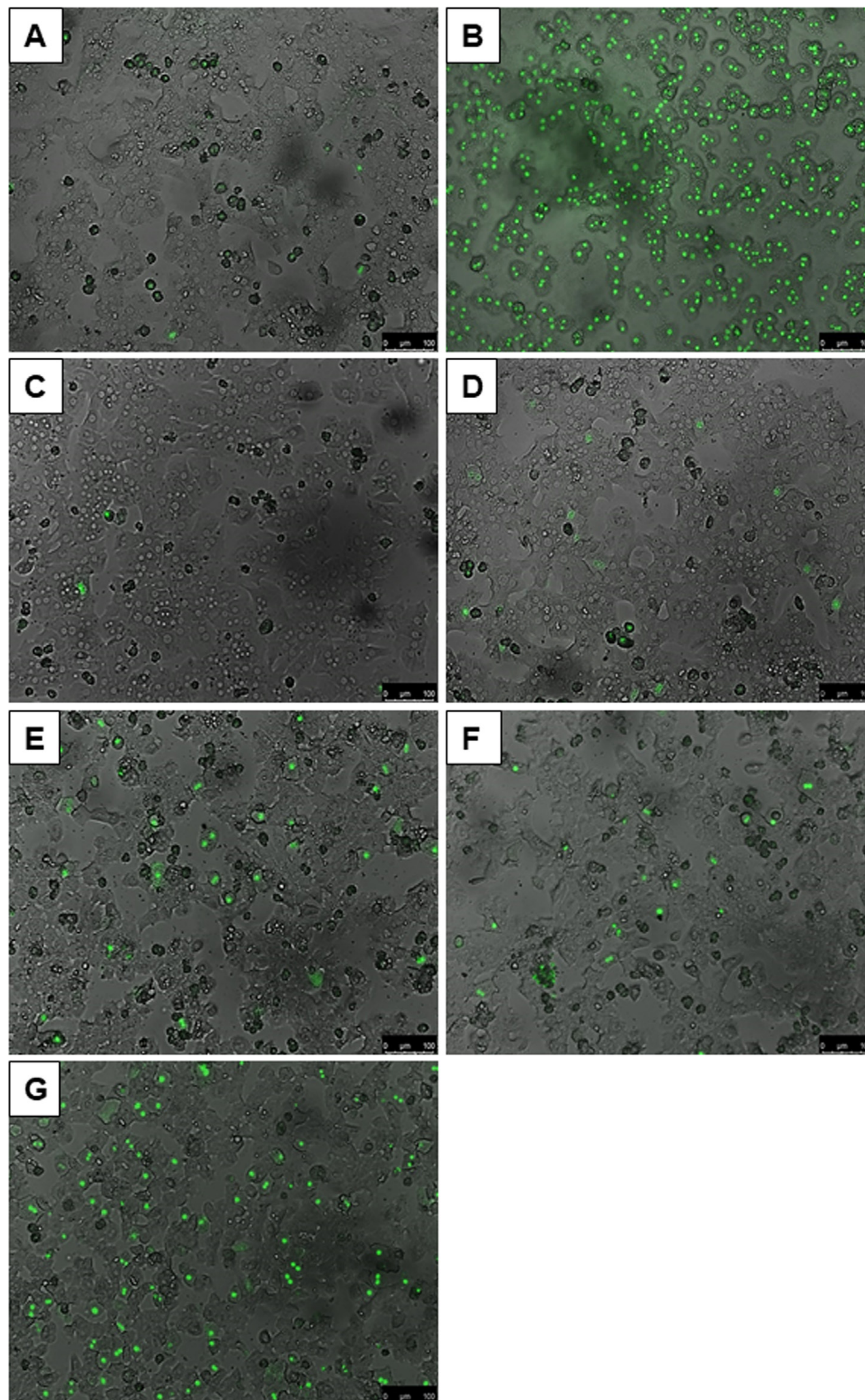


Fig. 5. Necrotic cell death determined by Sytox green fluorescent dye in primary rat hepatocytes after 24 h of exposure to acetaminophen and/or treatments. (A) Control, (B) Acetaminophen – APAP, (C) *Opuntia robusta* – Or, (D) *Opuntia streptacantha* – Os, (E) APAP + Or, (F) APAP + Os, and (G) APAP + NAC.

activity of Sod2 reduced glycochenodeoxycholic acid (GCDCA)-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-

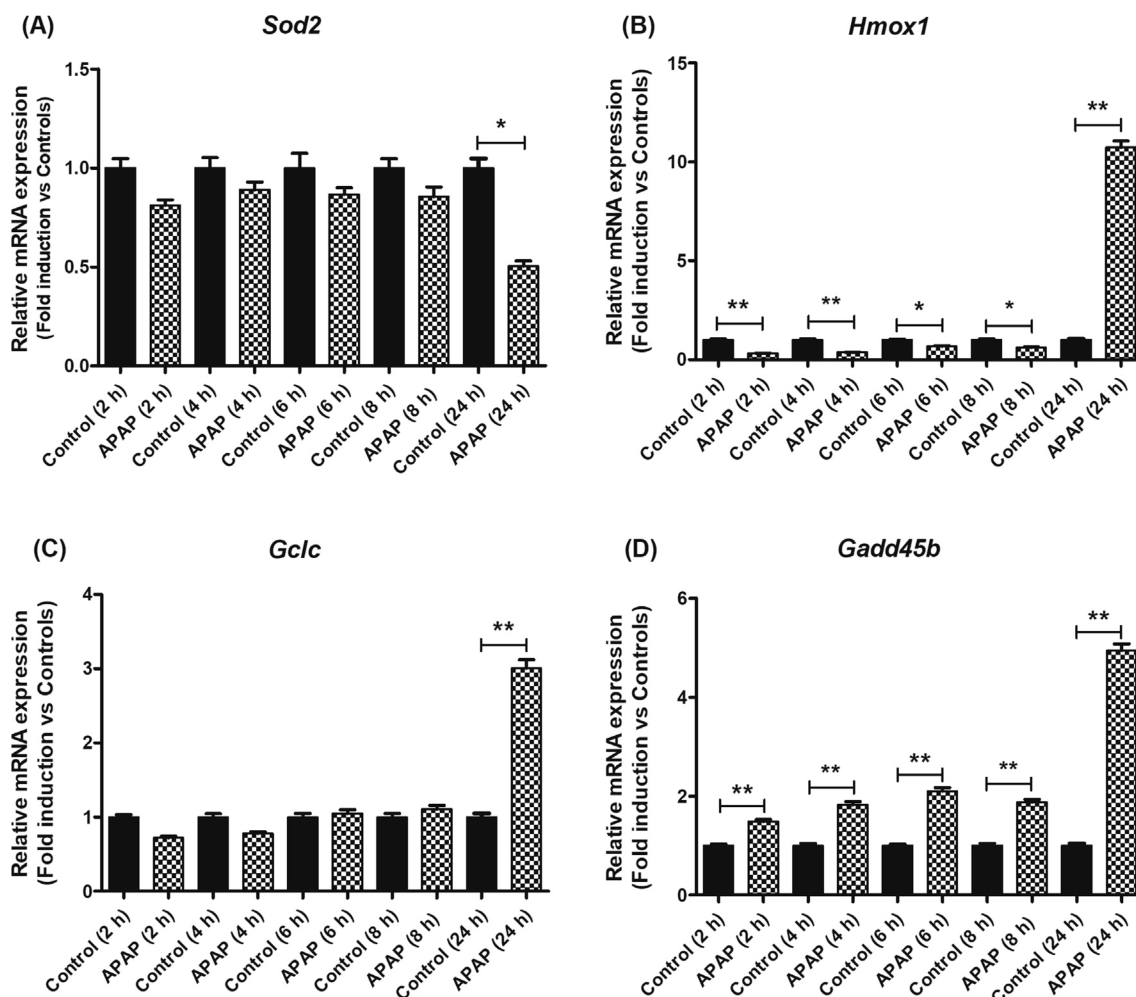


Fig. 6. Relative gene expression of oxidative stress-related genes at different time points after APAP intoxication. Results are shown for (A) *Sod2*, (B) *Hmox1*, (C) *Gclc*, and (D) *Gadd45b* in primary hepatocyte cultures. Each bar represents the mean of three independent experiments ± SEM. * $P < 0.001$ compared to the respective time control.

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 hepatotoxicity. 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

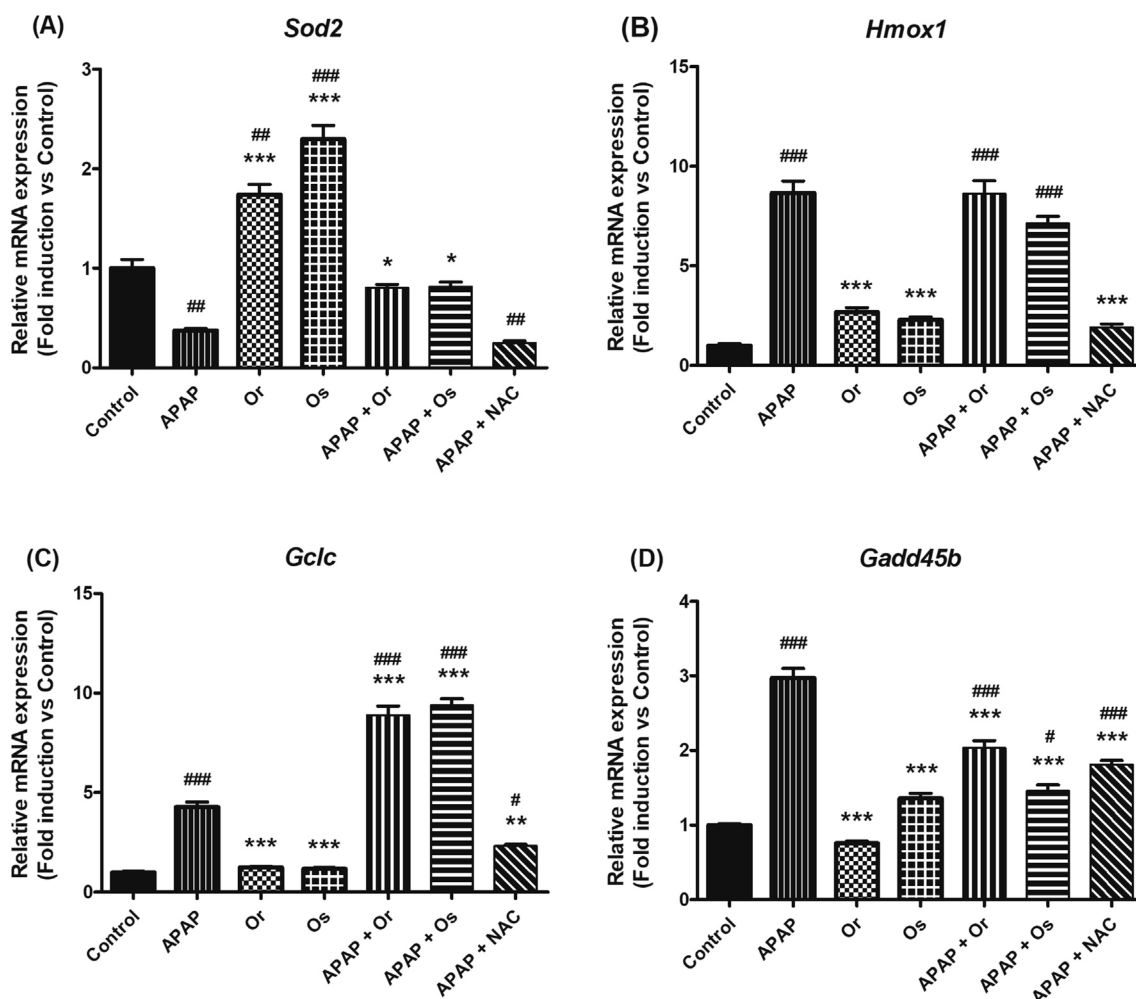


Fig. 7. Relative gene expression of the (A) *Sod2*, (B) *Hmox1*, (C) *Gclc*, and (D) *Gadd45b* in primary hepatocyte cultures after 24 h of acetaminophen exposure and/or the treatments. Each bar represents the mean of three independent experiments ± SEM. * $P < 0.05$ compared to APAP group. # $P < 0.05$ compared to control group.

(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 hepatoxicity 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.

Acknowledgments

The authors like to thank the Universidad Autónoma de

Aguascalientes and the University of Groningen for supporting this study.

Funding

This work was supported by the Graduate School of Medical Sciences (GSMS) of University of Groningen; and National Council of Science and Technology Mexico (CONACYT) [grant number 336940].

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109461.

References

Abdelmegeed, M. A., Moon, K.-H., Chen, C., Gonzalez, F. J., & Song, B.-J. (2010). Role of cytochrome P450 2E1 in protein nitration and ubiquitin-mediated degradation during acetaminophen toxicity. *Biochemical Pharmacology, 79*(1), 57–66. https://doi.org/10.1016/j.bcp.2009.07.016.

Antunes-Ricardo, M., Gutiérrez-Uribe, J. A., López-Pacheco, F., Alvarez, M. M., & Serna-Saldívar, S. O. (2015). In vivo anti-inflammatory effects of isorhamnetin glycosides isolated from Opuntia ficus-indica (L.) Mill cladodes. *Industrial Crops and Products, 76*, 803–808. https://doi.org/10.1016/j.indcrop.2015.05.089.

Botta, D., Shi, S., White, C. C., Dabrowski, M. J., Keener, C. L., Srinouanprachanh, S. L., ... Kavanagh, T. J. (2006). Acetaminophen-induced Liver Injury Is Attenuated in Male Glutamate-cysteine Ligase Transgenic Mice. *Journal of Biological Chemistry, 281*(39), 28865–28875. https://doi.org/10.1074/jbc.M605143200.

Brahmi, D., Bouaziz, C., Ayed, Y., Ben Mansour, H., Zourgui, L., & Bacha, H. (2011). Chemopreventive effect of cactus Opuntia ficus indica on oxidative stress and genotoxicity of aflatoxin B1. *Nutrition & Metabolism, 8*(1), 73. https://doi.org/10.1186/1743-7075-8-73.

Chen, Y., Qing, W., Sun, M., Lv, L., Guo, D., & Jiang, Y. (2015). Melatonin protects hepatocytes against bile acid-induced mitochondrial oxidative stress via the AMPK-SIRT3-SOD2 pathway. *Free Radical Research, 49*(10), 1275–1284. https://doi.org/10.3109/10715762.2015.1067806.

Chiu, H., Brittingham, J. A., & Laskin, D. L. (2002). Differential Induction of Heme Oxygenase-1 in Macrophages and Hepatocytes during Acetaminophen-Induced Hepatotoxicity in the Rat: Effects of Hemin and Biliverdin. *Toxicology and Applied Pharmacology, 181*(2), 106–115. https://doi.org/10.1006/taap.2002.9409.

Conde de la Rosa, L., Schoemaker, M. H., Vrenken, T. E., Buist-Homan, M., Havinga, R., Jansen, P. L. M., & Moshage, H. (2006). Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: Involvement of JNK and ERK MAP kinases. *Journal of Hepatology, 44*(5), 918–929. https://doi.org/10.1016/j.jhep.2005.07.034.

Conde de la Rosa, L., Vrenken, T. E., Hannivoort, R. A., Buist-Homan, M., Havinga, R., Slebos, D.-J., ... Moshage, H. (2008). Carbon monoxide blocks oxidative stress-induced hepatocyte apoptosis via inhibition of the p54 JNK isoform. *Free Radical Biology and Medicine, 44*(7), 1323–1333. https://doi.org/10.1016/j.freeradbiomed.2007.12.011.

Coria Cayupán, Y. S., Ochoa, M. J., & Nazareno, M. A. (2011). Health-promoting substances and antioxidant properties of Opuntia sp. Fruits. Changes in bioactive-compound contents during ripening process. *Food Chemistry, 126*(2), 514–519. https://doi.org/10.1016/j.foodchem.2010.11.033.

Dhar, S. K., Xu, Y., & St. Clair, D. K. (2010). Nuclear Factor κB- and Specificity Protein 1-dependent p53-mediated Bi-directional Regulation of the Human Manganese Superoxide Dismutase Gene. Journal of Biological Chemistry, 285(13), 9835-9846. https://doi.org/10.1074/jbc.M109.060715.

Dok-Go, H., Lee, K. H., Kim, H. J., Lee, E. H., Lee, J., Song, Y. S., ... Cho, J. (2003). Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from Opuntia ficus-indica var. Saboten. *Brain Research, 965*(1), 130–136. https://doi.org/10.1016/S0006-8993(02)04150-1.

Eugenio-Pérez, D., Montes de Oca-Solano, H. A., & Pedraza-Chaverri, J. (2016). Role of food-derived antioxidant agents against acetaminophen-induced hepatotoxicity. *Pharmaceutical Biology, 54*(10), 2340–2352. https://doi.org/10.3109/13880209.2016.1150302.

Ferner, R. E., Dear, J. W., & Bateman, D. N. (2011). Management of paracetamol poisoning. *BMJ, 342*, Article d2218. https://doi.org/10.1136/bmj.d2218.

Fontana, R. J. (2008). Acute Liver Failure Including Acetaminophen Overdose. *Common Hepatic Emergencies, 92*(4), 761–794. https://doi.org/10.1016/j.mcna.2008.03.005.

Geng, Y., Ma, Q., Liu, Y.-N., Peng, N., Yuan, F.-F., Li, X.-G., ... Song, W. (2015). Heatstroke induces liver injury via IL-1β and HMGB1-induced pyroptosis. *Journal of Hepatology, 63*(3), 622–633.

González-Ponce, H., Martínez-Saldaña, M., Rincón-Sánchez, A., Sumaya-Martínez, M., Buist-Homan, M., Faber, K., ... Jaramillo-Juárez, F. (2016). Hepatoprotective Effect of Opuntia robusta and Opuntia streptacantha Fruits against Acetaminophen-Induced Acute Liver Damage. *Nutrients, 8*(10), 607. https://doi.org/10.3390/nu8100607.

González-Ponce, H., Rincón-Sánchez, A., Jaramillo-Juárez, F., & Moshage, H. (2018). Natural Dietary Pigments: Potential Mediators against Hepatic Damage Induced by Over-The-Counter Non-Steroidal Anti-Inflammatory and Analgesic Drugs. *Nutrients, 10*(2), 117. https://doi.org/10.3390/nu10020117.

Hempel, N., M. Carrico, P., & Melendez, J. A. (2011). Manganese Superoxide Dismutase (Sod2) and Redox-Control of Signaling Events That Drive Metastasis. Anti-Cancer Agents in Medicinal Chemistry, 11(2), 191-201. https://doi.org/10.2174/187152011795255911.

Hissin, P. J., & Hilf, R. (1976). A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Analytical Biochemistry, 74*(1), 214–226. https://doi.org/10.1016/0003-2697(76)90326-2.

Kanner, J., Harel, S., & Granit, R. (2001). Betalains. A New Class of Dietary Cationized Antioxidants. *Journal of Agricultural and Food Chemistry, 49*(11), 5178–5185. https://doi.org/10.1021/jf010456f.

Kay, H. Y., Won Yang, J., Kim, T. H., Lee, D. Y., Kang, B., Ryu, J.-H., ... Kim, S. G. (2010). Ajoene, a Stable Garlic By-Product, Has an Antioxidant Effect through Nrf2-Mediated Glutamate-Cysteine Ligase Induction in HepG2 Cells and Primary Hepatocytes. *The Journal of Nutrition, 140*(7), 1211–1219. https://doi.org/10.3945/jn.110.121277.

Keller, J., Camaré, C., Bernis, C., Astello-García, M., de la Rosa, A.-P. B., Rossignol, M., del Socorro Santos Díaz, M., Salvayre, R., Negre-Salvayre, A., & Guéraud, F. (2015). Antiatherogenic and antitumoral properties of Opuntia cladodes: Inhibition of low density lipoprotein oxidation by vascular cells, and protection against the cytotoxicity of lipid oxidation product 4-hydroxynonenal in a colorectal cancer cellular model. Journal of Physiology and Biochemistry, 71(3), 577-587. https://doi.org/10.1007/s13105-015-0408-x.

Khandelwal, N., James, L. P., Sanders, C., Larson, A. M., Lee, W. M., & and the Acute Liver Failure Study Group. (2011). Unrecognized acetaminophen toxicity as a cause of indeterminate acute liver failure. Hepatology, 53(2), 567-576. https://doi.org/10.1002/hep.24060.

Kim, H. P., Pae, H.-O., Back, S. H., Chung, S. W., Woo, J. M., Son, Y., & Chung, H.-T. (2011). Heme oxygenase-1 comes back to endoplasmic reticulum. *Biochemical and Biophysical Research Communications, 404*(1), 1–5. https://doi.org/10.1016/j.bbrc.2010.11.067.

Kim, Y.-H., Hwang, J. H., Kim, K.-S., Noh, J.-R., Choi, D.-H., Kim, D.-K., ... Lee, C.-H. (2015). Metformin ameliorates acetaminophen hepatotoxicity via Gadd45β-dependent regulation of JNK signaling in mice. *Journal of Hepatology, 63*(1), 75–82. https://doi.org/10.1016/j.jhep.2015.02.008.

Lancaster, E. M., Hiatt, J. R., & Zarrinpar, A. (2015). Acetaminophen hepatotoxicity: An updated review. *Archives of Toxicology, 89*(2), 193–199. https://doi.org/10.1007/s00204-014-1432-2.

Larson, A. M., Polson, J., Fontana, R. J., Davern, T. J., Lalani, E., Hynan, L. S., Reisch, J. S., Schiødt, F. V., Ostapowicz, G., Shakil, A. O., Lee, W. M., & the Acute Liver Failure Study Group. (2005). Acetaminophen-induced acute liver failure: Results of a United States multicenter, prospective study. Hepatology, 42(6), 1364-1372. https://doi.org/10.1002/hep.20948.

Leem, K.-H., Kim, M.-G., Hahm, Y.-T., & Kim, H. (2016). Hypoglycemic Effect of Opuntia ficus-indica var. Saboten Is Due to Enhanced Peripheral Glucose Uptake through Activation of AMPK/p38 MAPK Pathway. *Nutrients, 8*(12), 800. https://doi.org/10.3390/nu8120800.

McGill, M. R., & Jaeschke, H. (2013). Metabolism and Disposition of Acetaminophen: Recent Advances in Relation to Hepatotoxicity and Diagnosis. *Pharmaceutical Research, 30*(9), 2174–2187. https://doi.org/10.1007/s11095-013-1007-6.

Odewumi, C., Badisa, V., Le, U., Latinwo, L., Ikediobi, C., Badisa, R., & Darling-Reed, S. (2011). Protective effects of N-acetylcysteine against cadmium-induced damage in cultured rat normal liver cells. *International Journal of Molecular Medicine, 27*(2), https://doi.org/10.3892/ijmm.2010.564.

Papa, S., Zazzeroni, F., Bubici, C., Jayawardena, S., Alvarez, K., Matsuda, S., ... Franzoso, G. (2004). Gadd45β mediates the NF-κB suppression of JNK signalling by targeting MKK7/JNKK2. *Nature Cell Biology, 6*(2), 146–153. https://doi.org/10.1038/ncb1093.

Papa, S., Zazzeroni, F., Fu, Y.-X., Bubici, C., Alvarez, K., Dean, K., ... Franzoso, G. (2008). Gadd45β promotes hepatocyte survival during liver regeneration in mice by modulating JNK signaling. *Journal of Clinical Investigation, 118*(5), 1911–1923. https://doi.org/10.1172/JCI33913.

Saenz, C. (2000). Processing technologies: An alternative for cactus pear (Opuntia spp.) fruits and cladodes. *Journal of Arid Environments, 46*(3), 209–225. https://doi.org/10.1006/jare.2000.0676.

Santos Díaz, M. del S., Barba de la Rosa, A.-P., Héliés-Toussaint, C., Guéraud, F., & Négre-Salvayre, A. (2017). Opuntia spp.: Characterization and benefits in chronic diseases. *Oxidative Medicine and Cellular Longevity, 2017*, 1–17. https://doi.org/10.1155/2017/8634249.

Seki, E., Brenner, D. A., & Karin, M. (2012). A Liver Full of JNK: Signaling in Regulation of Cell Function and Disease Pathogenesis, and Clinical Approaches. *Gastroenterology, 143*(2), 307–320. https://doi.org/10.1053/j.gastro.2012.06.004.

Serra, A. T., Poejo, J., Matias, A. A., Bronze, M. R., & Duarte, C. M. M. (2013). Evaluation of Opuntia spp. Derived products as antiproliferative agents in human colon cancer cell line (HT29). *Food Research International, 54*(1), 892–901. https://doi.org/10.1016/j.foodres.2013.08.043.

Sreekanth, D., Arunasree, M. K., Roy, K. R., Chandramohan Reddy, T., Reddy, G. V., & Reddanna, P. (2007). Betanin a betacyanin pigment purified from fruits of Opuntia ficus-indica induces apoptosis in human chronic myeloid leukemia Cell line-K562. *Phytomedicine, 14*(11), 739–746. https://doi.org/10.1016/j.phymed.2007.03.017.

Stintzing, F. C., Herbach, K. M., Mosshammer, M. R., Carle, R., Yi, W., Sellappan, S., ... Felker, P. (2005). Color, Betalain Pattern, and Antioxidant Properties of Cactus Pear (*Opuntia* spp.) Clones. *Journal of Agricultural and Food Chemistry, 53*(2), 442–451. https://doi.org/10.1021/jf048751y.

Sumaya-Martínez, M. T., Cruz-Jaime, S., Madrigal-Santillán, E., García-Paredes, J. D., Cariño-Cortés, R., Cruz-Cansino, N., ... Alanís-García, E. (2011). Betalain, Acid Ascorbic, Phenolic Contents and Antioxidant Properties of Purple, Red, Yellow and White Cactus Pears. *International Journal of Molecular Sciences, 12*(10), 6452–6468. https://doi.org/10.3390/ijms12106452.

Tian, J., Huang, H., Hoffman, B., Liebermann, D. A., Ledda-Columbano, G. M., Columbano, A., & Locker, J. (2011). Gadd45β is an inducible coactivator of transcription that facilitates rapid liver growth in mice. *Journal of Clinical Investigation, 121*(11), 4491–4502. https://doi.org/10.1172/JCI38760.

Uchiyama, M., & Mihara, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analytical Biochemistry, 86*(1), 271–278. https://doi.org/10.1016/0003-2697(78)90342-1.

Ueda, T., Kohama, Y., Kuge, A., Kido, E., & Sakurai, H. (2017). GADD45 family proteins suppress JNK signaling by targeting MKK7. *Archives of Biochemistry and Biophysics, 635*, 1–7. https://doi.org/10.1016/j.abb.2017.10.005.

Verhaag, E. M., Buist-Homan, M., Koehorst, M., Groen, A. K., Moshage, H., & Faber, K. N. (2016). Hormesis in Cholestatic Liver Disease; Preconditioning with Low Bile Acid Concentrations Protects against Bile Acid-Induced Toxicity. *PLOS ONE, 11*(3), Article e0149782. https://doi.org/10.1371/journal.pone.0149782.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature, 408*(6810), 307–310. https://doi.org/10.1038/35042675.

Wang, X., Wu, Q., Liu, A., Anadón, A., Rodríguez, J.-L., Martínez-Larrañaga, M.-R., ... Martínez, M.-A. (2017). Paracetamol: Overdose-induced oxidative stress toxicity, metabolism, and protective effects of various compounds *in vivo and in vitro*. *Drug Metabolism Reviews, 49*(4), 395–437. https://doi.org/10.1080/03602532.2017.1354014.

Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *The Journal of Cell Biology, 217*(6), 1915. https://doi.org/10.1083/jcb.201708007.

Wolf, M. S., King, J., Jacobson, K., Di Francesco, L., Bailey, S. C., Mullen, R., ... Parker, R. M. (2012). Risk of Unintentional Overdose with Non-Prescription Acetaminophen Products. *Journal of General Internal Medicine, 27*(12), 1587–1593. https://doi.org/10.1007/s11606-012-2096-3.

Woudenberg-Vrenken, T. E., Buist-Homan, M., Conde de la Rosa, L., Faber, K. N., & Moshage, H. (2010). Anti-oxidants do not prevent bile acid-induced cell death in rat hepatocytes: Anti-oxidants do not prevent bile acid-induced cell death. *Liver International, 30*(10), 1511–1521. https://doi.org/10.1111/j.1478-3231.2010.02325.x.