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Original article

The flesh ethanolic extract of *Hylocereus polyrhizus* exerts anti-inflammatory effects and prevents murine colitis

Dulce C. Macias-Ceja [a], Jesus Cosín-Roger [b], Dolores Ortiz-Masiá [c], Pedro Salvador [b], Carlos Hernández [a], Sara Calatayud [b], Juan V. Esplugues [a, b], Maria D. Barrachina [b, *]

[a] FISABIO, Hospital Dr. Peset, Valencia, Spain
[b] Departamento de Farmacología and CIBERehd, Facultad de Medicina, Universidad de Valencia, Valencia, Spain
[c] Departamento de Medicina, Facultad de Medicina, Universidad de Valencia, Valencia, Spain

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SUMMARY

Background & aims: IBD is a chronic disorder of the gastrointestinal tract characterized by mucosal inflammation and epithelial damage. Biologic therapy has significantly improved the course of the disease but there are still a high percentage of patients that do not respond to current therapies. We aim to determine the effects of the flesh ethanolic extract of *Hylocereus polyrhizus* (EH) in a mice model of colitis induced by TNBS.

Methods: Balb/c mice received TNBS (175 mg/kg, 100 μl, i.r.) and six and thirty hours later were administered with EH (1 g/kg, i.p.). Mice were weighted daily and after sacrificing (2 and 4 days after TNBS) we analyzed mucosal histology, myeloperoxidase activity (MPO), the expression of pro-inflammatory molecules (qPCR) and NF-κB and Iκβ-α protein levels. The chemical characterization of the EH was determined by LC–MS/MS.

Results: The administration of EH to TNBS-treated mice prevented ($P < 0.05$) the loss of body weight and significantly reduced in the colon: a) histological damage score, b) MPO enzymatic activity c) the expression of pro-inflammatory molecules and d) Iκβ-α degradation and nuclear NF-κβ protein levels. The LC–MS analysis detected metabolites such as polyphenols and fatty acids.

Conclusion: Systemic administration of the ethanolic extract of *H. polyrhizus* exerts an anti-inflammatory effect and prevents murine colitis induced by TNBS.

1. Introduction

IBD is a chronic disorder of the gastrointestinal tract associated with alteration in the epithelial barrier function and mucosal inflammation [1]. The main idiopathic forms of IBD are ulcerative colitis and Crohn's disease, and both exhibit apoptosis of epithelial cells, abnormal alteration of pro-inflammatory molecules and increased activation of adhesion molecules, cellular infiltrates and mucosal damage [2]. The pharmacological treatment of IBD has been significantly improved with the addition of biological therapy to conventional therapy. However, there is still a large part of the population that does not respond to current therapies because of their effectiveness, possible side effects and high cost [3]. In last years, a wide range of phytochemicals have demonstrated antiinflammatory effects and the interest in natural compounds and pigments for the treatment of inflammatory diseases has significantly increased [4].

Red-flashed pitahaya or “dragon fruit” belongs to the caryophyllales order of the genus *Hylocereus* and the family of cactaceae. It is grown in tropical and subtropical regions such as Latin America, Malaysia, Thailand, Vietnam or Taiwan [5]. Different compounds have been identified in the pitahaya flesh, peel and seed and several studies analyzed the chemical composition, level

Abbreviations: IBD, inflammatory bowel disease; TNF-α, tumor necrosis factor; EH, ethanolic extract of *Hylocereus*; HETAB, hexa-decyl-trimethyl-ammonium; TMB, tetramethylbenzidine; MOP, myeloperoxidase; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; NF-κBp65, nuclear factor; LC/MS–MS, Liquid chromatography mass–mass spectrometry; iNOS, Inducible nitric oxide synthase; COX2, Cyclooxigenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6; IL10, interleukin-10.

* Corresponding author. Department of Pharmacology, Faculty of Medicine, University of Valencia, Avda. Blasco Ibáñez 15-17, 46010 Valencia, Spain. Tel.: +34 96 398 3834; fax: +34 96 386 4625.
E-mail address: dolores.barrachina@uv.es (M.D. Barrachina).

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of cytotoxicity and antioxidant activity of *Hylocereus polyrhizus in vitro* [6,7]. In addition, the safety evaluation of the methanolic extract of this fruit has been reported *in vivo* [8] and this extract has been related to a reduction risk of cancer, cardiac disease, decreased levels of blood glucose and blood pressure reduction [9,10]. Of interest, a compound present in the dragon fruit pigments has shown antioxidant and anti-inflammatory effects in rat liver damage [11]. In the actual study we evaluate the effects of the ethanolic extract of *H. polyrhizus* in an experimental murine model of IBD.

2. Materials and methods

2.1. The flesh ethanolic extract of H. polyrhizus

Fruits were bought from local "Boqueria Market" (Barcelona, Spain), and carefully washed with tap water. The flesh was cut into small pieces and mingled in a blender with ethanol 50% (w/v) at room temperature. Afterwards, it was decanted and filtered through a nylon cloth. The filtered extract was lyophilized to obtain the ethanolic extract (EH) concentrate which was stored at −20 °C.

2.2. Animals

Balb/c mice (Jackson Labs.) were accommodated in stainless steel cages in a room held at 22 ± 1 °C with a photoperiod of 12-h light and12-h dark. All protocols were approved by the bioethic committees in the University of Valencia.

2.3. Inducement of experimental colitis

Colitis was induced by a single administration of 100 μl of 2,4,6-trinitrobenzenesulphonic (TNBS, 175 mg/kg, i.r., Sigma) dissolved in ethanol 40%. Mice (7–8 weeks old) were anesthetized and a 16G catheter was then carefully inserted 3 cm through the anus into the colon; 100 μL of NaCl 0.9% dissolved in ethanol 40% was administrated to control animals [12]. Six and thirty hours after TNBS administration mice received an i.p. injection of EH (1 g/kg dissolved in DMEM) [8]. Mice were weighted daily (results are expressed as percentage vs. weight at day 0) and were sacrificed by cervical dislocation on day 2 and 4 after TNBS administration.

2.4. Experimental groups

Animals were randomly assigned to four different experimental groups (n = 11, each group): 1. Vehicle + Vehicle group (Ethanol i.r. + DMEM i.p.) 2. Vehicle + EH group (Ethanol i.r. + EH), 3. TNBS + Vehicle group (TNBS, i.r. + DMEM i.p.) and 4. TNBS + EH group (TNBS, i.r. + EH i.p.).

2.5. Evaluation of colon and histological examination

Colon length was measured and colon samples were sectioned (5 μm), embedded in paraffin, fixed in 4% paraformaldehyde acid and stained with haematoxylin for histological analysis. Damage was measured on a scale 0–10 taking into account the presence of erosion, surface extension of lesion, degree of inflammation, ulceration or necrosis, as previously described [13].

2.6. Myeloperoxidase activity

The determination of MPO activity as a marker of neutrophil infiltration was performed as previously published [14]. Colon samples were frozen and stored at −80 °C. Each piece of tissue was crushed in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide (HETAB) re-suspended in PBS 50 mM pH 6 at a 1:20 (w/v) dilution using a gentle MACS Dissociator. The homogenate was centrifuged at 20,000 × *g*, 30 min, 4 °C. The supernatant obtained was diluted in 50 volumes of 50 mM PBS pH 6. Then it was added consecutively 50 mL of 3,3′,5,5′-tetramethylbenzidine (TMB), HETAB (0.5%), and hydrogen peroxide (0.052%). When the mixture was completed, it was incubated 5 min in the dark at 37 °C. The measured of the plate was carried at 450 nm with a microplate reader (Infinite M2000, TECAN), the results were expressed in MPO U/mg of tissue.

2.7. RNA extraction and qPCR

RNA extraction was performed by using Tripure isolation reagent (Roche Diagnostics, Barcelona, Spain) following manufacturer instructions. Briefly, tissues were gently homogenized by means of a MACS Dissociator (Miltenyi Biotec). cDNA was obtained (Prime Script RT reagent Kit, Takara Biotechnology, Dalian, China) and RT-PCR was performed (Prime Script Reagent Kit Perfect Real Time, Takara, Biotechnology) in a thermo cycler LightCycler (Roche Diagnostics, Mannheim, Germany). Specific oligonucleotides are shown in Table 1. Relative gene expression level of different target was calculated by 2-ΔΔCt. β-actin was used as the housekeeping gene [15].

2.8. Western blot analysis for IKBα, NF-κBp65

The terminal colon tissue samples were homogenized and nuclear and cytoplasmatic proteins were extracted as previously described [12]. Proteins (40 μg) were separated in 10% (w/v) acrylamide gel by SDS-PAGE. IκB-α and NF-κBp65 were detected by western blot using a primary antibody against IκB-α (1:1000 rabbit antibody; Santa Cruz Biotechnology) and NF-κBp65 (1:250 mouse antibody; Invitrogen, Novex by Life Technologies) at 4 °C overnight, followed by HRP-conjugated anti-mouse IgG (1:2500; Thermo Scientific, Rockford, IL, U.S.A.) or anti-rabbit IgG (1:5000 Thermo Scientific) and reveled by femto chemiluminescent substrate (Thermo Scientific). Protein bands were detected by a LAS-3000 (Fujifilm, Barcelona, Spain) and protein levels were quantified by means of densitometry using Image Gauge Version 4.0 software (Fujifilm). Data were normalized to β-actin for cytoplasmic proteins or to nucleolin in the case of nuclear proteins [16].

Table 1
Primer sequences of the genes analyzed by qRT-PCR.

Mouse gene	Sense	Antisense	Length (bp)
iNOS	CGCTTGGGTCTTGTTCACTC	GGTCATCTTGTATTGTTGGGCTG	222
ArgI	GTGGGGAAAGCCAATGAAGAG	TCAGGAGAAAGGACACAGGTTG	232
COX2	CCCGGACTGGATTCTATGGTG	TTCGCAGGAAGGGGATGTTG	153
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	61
IL-1β	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG	136
IL6	GAGTCCTTCAGAGAGATACAGAAAC	TGGTCTTGGTCCTTAGCCAC	150
IL-10	GGACAACATACTGCTAACCGAC	CCTGGGGCATCACTTCTACC	110
β-actin	GCCAACCGTGAAAAGATGACC	GAGGCATACAGGGACAGCAC	95

2.9. LC–MS/MS analysis

The analysis of LC–MS/MS (Agilent1290, Agilent Technologies) for EH was performed using an EC-C18 column (50 × 2.1 mm × 1.7 μm particle size, WatersCorp) [17]. The injection volume was set at 5 μL, the flow rate at 0.4 mL/min, the column temperature was 30 °C and elution conditions consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The initial condition was 5% solvent B for 1 min, gradually increasing to 100% over 13 min, maintained at 100% B for 1 min and then decreased to 5% over 6 min. The total runtime was 20 min.

Mass Spectrometry conditions: Q-TOF-MS analysis was performed using on a TripleTOF 5600 mass spectrometer system with a DuoSpray™ Source (electrospray ionization) (AB Sciex). Vaporizer temperature was 450 °C and voltage was maintained at 4.5 kV. Wrapper gas was at a pressure of 50 psi. Samples were ionized in negative and positive mode. Ion monitoring mode was full scan in the range *m*/*z* 100–1000. Automated calibration was performed using an external calibrant delivery system (CDS) which infused calibration solution prior to sample introduction.

The MS was performed using an IDA acquisition method with two experiments: the survey scan type (TOF-MS) and the dependent scan type (production) using −30 V of collision energy. Data was evaluated using the qualitatively evaluated using the XIC manager in the PeakView™ software where it was filtered based on mass error, retention time, isotope ratio %, difference and library hit purity score.

2.10. Statistical analysis

The statistical analyses were performed using the GraphPad Prism 5.0® software. All data was expressed as mean ± SEM. Data were compared by using the analysis of one way-ANOVA with a Newman–Keuls post hoc correction for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. The ethanolic extract of H. polyrhizus protects against colitis induced by TNBS

The administration of TNBS to mice induced a loss of body weight that peaked 2 days after treatment. Subsequently, mice began to recover and four days after treatment, body weight reached values that were non-significantly different to those of control animals. The administration of EH of *H. polyrhizus* significantly ($P < 0.05$) prevented the loss of body weight (91.9 ± 1.7%) compared with the injection of vehicle (86.4 ± 2.1%), when analyzed two days after TNBS (Fig. 1A). The colon length was not significantly modified by TNBS, at any time analyzed (Fig. 1B).

The cecum, colon and rectum in macroscopic analysis (Fig. 1C) demonstrated hemorrhagic ulceration in TNBS-treated mice which was significantly prevented in mice that had received the EH. The histological analysis of the colon two days after treatment revealed a significant increase in damage score caused by TNBS compared with vehicle administration. The injection of EH significantly prevented mucosal damage induced by TNBS (Fig. 2).

3.2. The ethanolic extract of H. polyrhizus decreases MPO activity in colonic tissue of TNBS-treated mice

Colitis was characterized by an increase in MPO activity, an established marker of neutrophil infiltration. As shown in Fig. 3, two days after TNBS we detected a significant rise in MPO activity in the colon tissue, compared with vehicle. The administration of EH

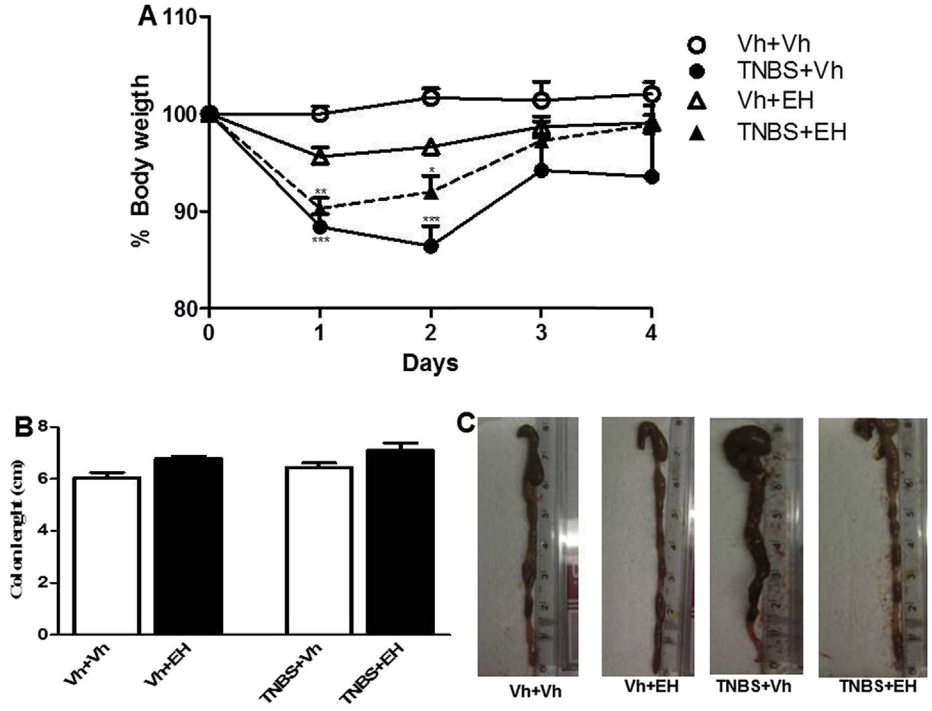

Fig. 1. Effects of the ethanolic extract of *H. polyrhizus* on acute colitis induced by TNBS in mice. Administration of EH (1 g/kg i.p.) six and thirty hours after TNBS: A) prevented the body weight loss, two days after TNBS. Points in the graph represent mean ± s.e.m.,*P < 0.05, **P < 0.01 and ***P < 0.001 vs the respective vehicle-treated group. B) did not significantly modify the colon length. Bars in graph represent mean ± s.e.m.; C) reduced the severity of colon inflammation and hemorrhagic ulceration induced by TNBS. Representative photographs showing rectum, colon and cecum of mice receiving different treatments. The EH administration to vehicle-treated mice did not significantly modify any of the parameters analyzed.

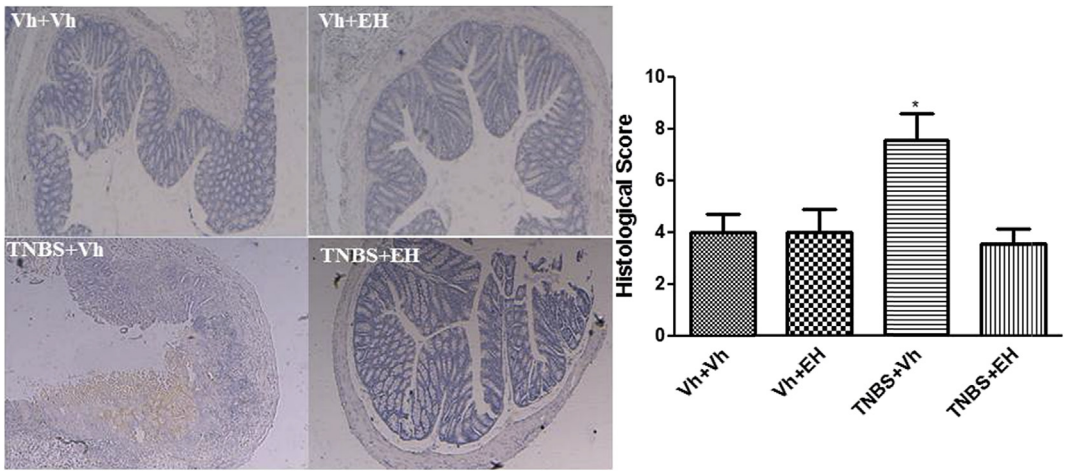

Fig. 2. Effects of the ethanolic extract of *H. polyrhizus* on histological damage induced by TNBS in mice. Administration of EH (1 g/kg i.p.) six and thirty hours after TNBS significantly prevented histological damage induced by TNBS, two days after treatment. Representative photographs showing mucosal histology. Graph shows the histological score in the colonic mucosa of mice. Bars in graph represent mean ± s.e.m. *P < 0.05, vs all experimental groups in the graph.

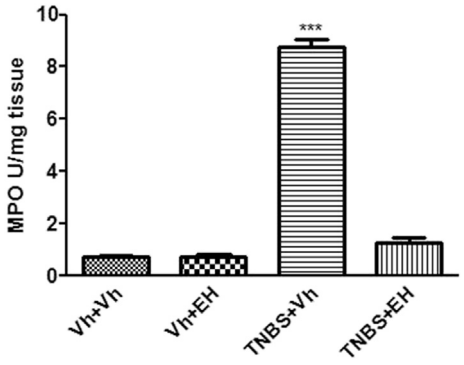

Fig. 3. Effects of the ethanolic extract of *H. polyrhizus* on MPO activity in colonic mucosa of mice treated with TNBS. Administration of EH (1 g/kg i.p.) six and thirty hours after TNBS significantly prevented the increase in MPO activity induced two days after TNBS. Bars in graph represent mean ± s.e.m. ***P < 0.001, vs all experimental groups in the graph.

significantly prevented the increase in MPO in TNBS-treated mice while it did not significantly modify MPO activity in vehicle-treated mice.

3.3. The ethanolic extract of H. polyrhizus inhibits the gene expression of pro-inflammatory cytokines in colonic tissue of TNBS-treated mice

Analysis of the mRNA expression of *iNOS*, *ArgI*, *COX-2*, *TNF-α* and *IL-6* in colonic tissue revealed a significant increase of the expression of these genes, two and four days after TNBS-treatment compared with levels detected in vehicle-treated mice (Fig. 4). In all cases, the administration of EH significantly prevented the increase of expression detected in these genes, 2 and 4 days after TNBS. The mRNA expression of the anti-inflammatory molecule, *Il-10*, was not significantly increased two days after TNBS but it was induced four days after treatment. The administration of EH did not

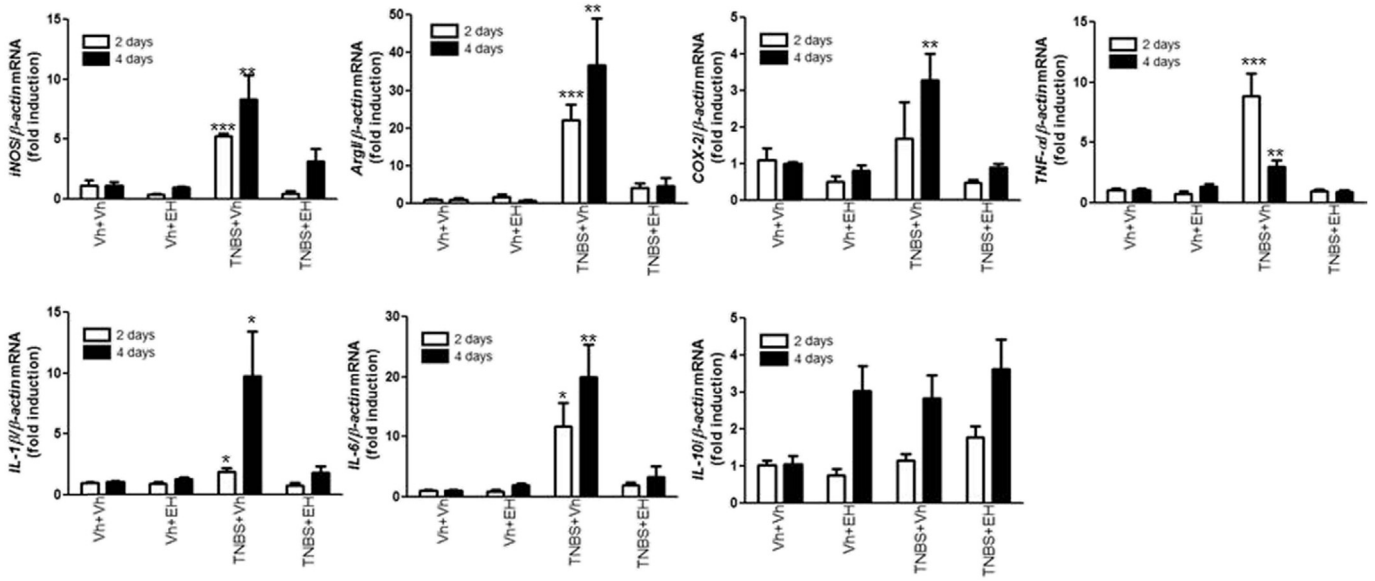

Fig. 4. Effects of the ethanolic extract of *H. polyrhizus* on the mRNA expression of pro-inflammatory mediators in colonic mucosa of mice treated with TNBS. Administration of EH (1 g/kg i.p.) six and thirty hours after TNBS reduced the mRNA expression of iNOS, ArgI, COX-2, TNF-α, IL-1β and IL-6 in colonic tissue. Bars represent mean ± s.e.m., *P < 0.05, **P < 0.01 and ***P < 0.001, vs all experimental groups in the graph.

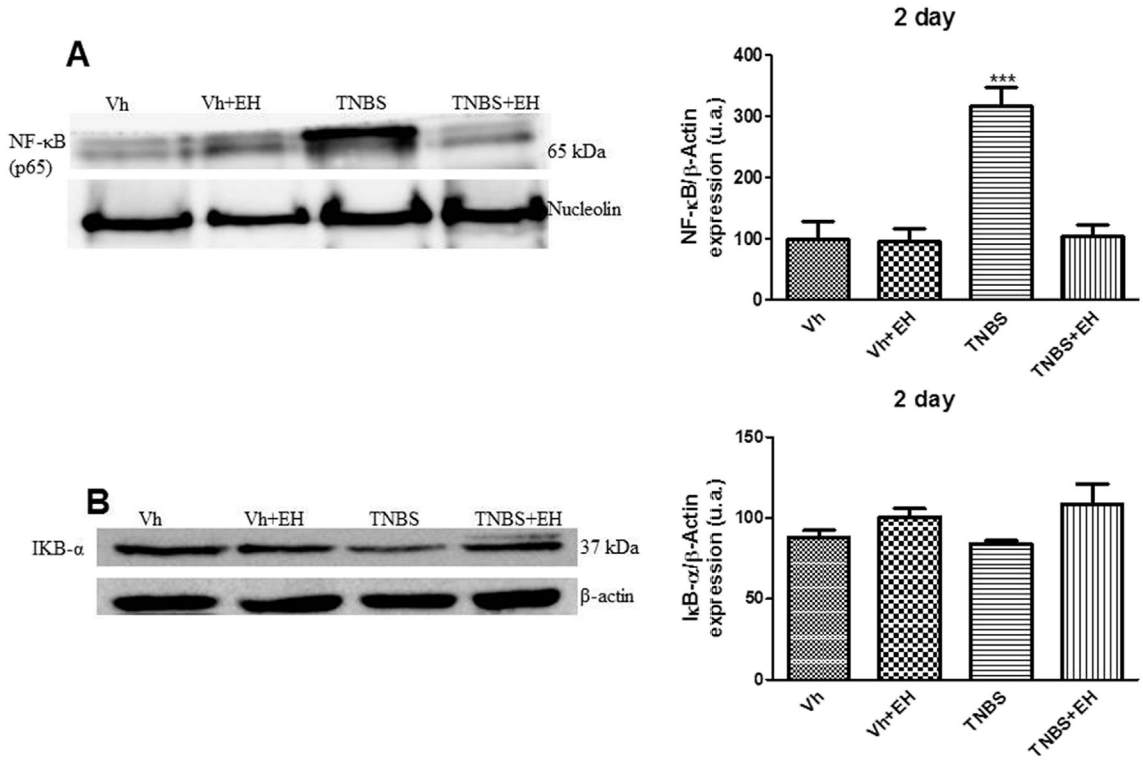

Fig. 5. Effects of the ethanolic extract of *H. polyrhizus* on NF-κBp65 protein levels and IκB-α degradation in colonic mucosa of mice treated with TNBS. Administration of EH (1 g/kg i.p.) six and thirty hours after TNBS reduced nuclear NF-κBp65 protein levels and IκB-α degradation. Representative western blots showing nuclear NF-kB p65 and cytoplasmic IκB-α protein levels in the colon of mice receiving different treatments. Graphs show quantification of protein expression of NF-κBp65 and IκB-α standardized to nucleolin or β-actin, respectively. Bars represent mean ± s.e.m., of at least three experiments. ***P < 0.001, vs all experimental groups in the graph.

significantly modify Il-10 expression in TNBS-treated mice but induced a significant increase in vehicle-treated animals (Fig. 4).

3.4. The ethanolic extract of H. polyrhizus decreases nuclear NF-κB protein levels and IκB-α degradation in colonic tissue of TNBS-treated mice

In order to understand the cellular mechanisms activated by the EH we proceeded to analyze IκB-α and NF-κB protein levels by Western blot. TNBS administration slightly reduced IκB-α levels compared with vehicle-treated mice. IκB-α degradation was detected in parallel with increased levels of NF-kB protein in the nucleus. Administration of EH significantly prevented IκB-α degradation induced by TNBS and reduced nuclear NF-kB (Fig. 5).

3.5. Composition of the ethanolic extract of H. polyrhizus by LC–MS/MS analysis

Finally we characterized by LC–MS/MS the composition of EH of *H. polyrhizus*. We observed eight different compounds, three of which were detected by positive screening (Table 2) and five of them by negative detection (Table 3). These organic compounds correspond to structures of flavonoids and phenolic compounds attached to carbohydrates.

4. Discussion

Earlier studies on the red dragon fruit cactacea (*Hylocereus* sp.) have analyzed the chemical composition of several extracts and

Table 2
The compounds identified in positive mode in *H. polyrhizus* fruit ethanolic extract.

Retention time (min)	Compound	Molecular formula	$[M+H]^+$
0.77	Ellagic acid arabinoside	$C_{19}H_{14}O_{12}$	435.05
3.70	Chrysoeriol 7-O-(6″-malonyl-apiosyl-glucoside)	$C_{30}H_{32}O_{18}$	681.16
490	Luteolin7-O-beta-D-diglucuronide	$C_{27}H_{26}O_{16}$	639.11

Table 3
The compounds identified in negative mode in *H. polyrhizus* fruit ethanolic extract.

Retention time (min)	Compound	Molecular formula	$[M-H]^-$
5.08	Tridecarboxylated hylocerenin	$C_{27}H_{34}N_2O_{11}$	561.20
6.5	O-Coumaric acid	$C_9H_8O_3$	163.04
7.45	p-HPEA-AC	$C_{10}H_{12}O_3$	179.07
7.45	4-Vinylsyringol	$C_{10}H_{12}O_3$	179.07
7.7	Oleoside 11-methyl ester	$C_{17}H_{24}O_{11}$	403.12

showed the antioxidant effects [7,8], as well as their therapeutic benefit in metabolic syndrome diseases [18]. In the present study we demonstrated for the first time that the ethanolic extract of *H. polyrhizus* prevents murine colitis through an anti-inflammatory action.

The experimental model of colitis induced by a single administration of TNBS to Balb/c mice was associated with loss of body weight, distortion of mucosal architecture and cellular infiltration which are characteristics similar to those observed in human Crohn's disease [19]. These changes peaked two days after injury and afterwards mice started to recover. The intraperitoneal administration of EH, at doses lower than those reported to lack toxicity [8] significantly avoided the loss of body weight induced by TNBS and reduced the macroscopic and histological colonic damage. Mice treated with TNBS presented hemorrhagic colon and the histological analysis revealed erosions, ulceration and cellular permeability in the mucosa. Of interest, animals that had received the EH lack of macroscopic signs of inflammation and damage and the histological analysis revealed a preserved architecture with some abnormal crypts and poor cellular infiltration.

In the TNBS model of colitis, myeloperoxidase activity (MPO) contributes to dysfunction and inflammation of the mucosa. Our results reveal an increase in MPO activity in the colon of mice treated with TNBS which is thought to be caused by the neutrophilic infiltration [20]. These infiltrated cells are also associated with the production and liberation of pro-inflammatory cytokines which initiate and maintain mucosal inflammation [21]. Our results reveal in colonic tissue of TNBS-treated mice an increase in mRNA expression of *iNOS*, *COX2*, *Arginase I* and several cytokines such as *TNF-α*, *IL-1β* and *IL-6*. Of interest, the administration of EH significantly prevented both the increase in MPO activity and the induction of pro-inflammatory cytokines observed in TNBS-treated mice which would be in line with the poor cellular infiltration observed in these animals. Taken together results in the present study strongly suggest an antiinflammatory activity of the ethanolic extract of *H. polyrhizus*.

The transcription factor, NF-kB has been associated with the inflammatory activity of TNBS [22] and its activity is controlled by the steady state levels of IKappa-Bα. Our results reveal in the colon of TNBS-treated mice, a slight degradation of cytoplasmic IKappa-Bα and a significant up-regulation of NF-κB in the nucleus and both effects are significantly altered by the administration of EH to TNBS-treated mice. Considering that members of the NF-kB family have been shown to control the transcriptional activity of proinflammatory gene promoters' such as those of *TNF-α*, *IL-1β*, *COX-2* and *iNOS*, surface cell receptors and adhesion molecules [23], results point to an antiinflammatory effect of the EH mediated by its ability to inhibit the transcriptional activity of NF-κB. Activation of NF-kB has been reported in IBD patients and regulation of its expression has been proposed as a good therapeutic target [24]. Data in the present study strongly suggest that the fruit of *H. polyrhizus*, due to its antiinflammatory properties could be used as a complement in the treatment of IBD patients. Further clinical studies are required to address this point.

Finally, we performed a LC–MS/MS analysis to determine the potential antiinflammatory compounds present in the ethanolic extract of the fruit of *H. polyrhizus*. Data reveal the presence of several compounds such as, flavonoids, polyphenols, and fatty acid esters. Among them we found ellagic acid, luteolin and p-HPEA-AC which have been reported to exert anti-inflammatory effects [14,25–27]. Furthermore, it has been reported that the antiinflammatory activity of the ellagic acid is mediated by the inhibition of NF-κB transcriptional activation [28] which suggest that the antiinflammatory activity of EH observed in the present study is mediated by this compound. However it is important to point out that we found both ellagic acid and luteolin bound to glucosides. Although no literature analyzing the relevance of this binding with ellagic acid has been found, it has been described a reduction in the antiinflammatory activity of luteolin when it is bound to glucosides [26]. Further studies are required to characterize the final responsible for the antiinflammatory effects of the EH of *H. polyrhizus*. Of interest, it has been described that the ellagic acid bound to a glucoside exerts prebiotic properties [29], which could be also involved in the protective effects shown by the EH against colitis.

In addition to polyphenols and fatty acids, the methanolic extract of *H. polyrhizus* has been shown to contain metabolites that are precursors of the synthesis of bethalains (betanines and betaxanthins) which exhibit antiinflammatory properties [10,11]. Our results show that the ethanolic extract of *H. polyrhizus* contains tridecarboxylated hylocerenin which is a monodecarboxilated betaxathin, but some concerns exit about the antiinflammatory activity of the decarboxylated betaxanthin [30]. Currently we are carrying on studies in order to analyze the effect of the methanolic extract of *H. polyrhizus* in a murine model of colitis.

5. Conclusions

The present study demonstrates that the ethanolic extract of *H. polyrhizus* exerts an anti-inflammatory activity and prevents colitis induced by TNBS in mice, what suggests that the EH maybe a therapeutic alternative for inflammatory diseases such as IBD.

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Statement of authorship

DM, CH, SC, MDB conceived and designed the experiments.

DM, JC, DO, PS performed the experiments and analyzed the data.

DM, MDB wrote the manuscript.

Conflict of interest

The authors declared no conflict of interest.

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