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Original Article

Protective and antioxidant effects of *Opuntia ficus-indica* cladodes hydroethanolic extract on castor oil-induced diarrhea in rats

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Abstract

Gastrointestinal disorders, which significantly impact human health, are a major focus of research. Diarrhea is one such disorder, affecting individuals across all age groups. Conventional anti-diarrheal drugs are often characterized byside effects, so safer alternative treatments are needed. In this context, we investigated the effects of *Opuntia ficus-indica*, a plant known for its therapeutic properties, by evaluating the impact of its cladode hydroethanolic extraction castor oil-induced diarrhea in rat. The hydroethanolic cladodes extract (HECE) was prepared from fresh cladodes, and its bioactive compounds were identified using high-performance liquid chromatography (HPLC). The antidiarrheal potential of HECE was evaluated at increasing doses (100, 200, 400 and 600 mg/Kg) using the castor oil-induced diarrhea model, as well as intestinal transit and enteropooling assays in rats. Histological changes in tissues were assessed using a high-performance slide scanner (Zeiss Axioscan7), and biochemical analyses were conducted on gastric and intestinal tissue homogenates. HPLC analysis identified eight phenolic compounds known to have beneficial effects on gastrointestinal disorders. In vivo, HECE administration reduced the number of stools and wet stools, normalized the antioxidant enzymes activities (Superoxide dismutase and Catalase) and levels of mediators such as plasma calcium and free iron. Histological analysis further confirmed mucosal protection, showing preserved villi and reduced epithelial disruption in treated rats. These findings suggest that HECE from *Opuntia ficus-indica* exhibits potent antioxidant and anti-diarrheal properties in rats, supporting its potential as a natural therapeutic agent for diarrhea.

Keywords: *Opuntia ficus-indica*, Diarrhea, Intestinal fluid, Castor oil, Oxidative stress, Rat.

1. Introduction

Gastrointestinal disorders represent a complex group of conditions that affect the digestive tract, often associated with symptoms such as abdominal pain, diarrhea, intestinal obstruction, nausea, and vomiting[1]. These conditions are often linkedto the onset of oxidative stress, which is closely related to gut health [2].Indeed, diarrhea is a significant cause of mortality worldwide, particularly among children under 5 years of age [3]. It is characterized by the sudden evacuation of stools (≥3 times in 24 hours) that are excessively abundant and/or liquid. Various pathogens can cause diarrhea, including bacteria, such as *Salmonella spp*., *Shigella spp*., and *Clostridium difficile*, viruses like SARS-CoV-2 and parasites [4, 5].Depending on its duration, diarrhea is classified as acute if it lasts 14 days or less, or chronic /persistent when it extends beyond 14 days [6]. The pathophysiology of diarrhea involves several mechanisms, the main ones beingsecretory, osmotic, or inflammatory [7]. The first type results from an imbalance between water absorption and its secretion in the intestinal lumen. Osmotic diarrhea is caused by the presence of unabsorbed or poorly absorbable substances in the intestinal lumen. In contrast, inflammatory diarrhea occurs when the intestinal epithelium is disturbed by pathogens such as bacteria or viruses, leading to inflammation and impaired absorption [1]. For instance, castor oil-induced diarrhea is a used experimental model for evaluating anti-diarrhealactivity for natural agents [8]. It is a laxative agentextracted from the seeds of *Ricinus communis (Euphorbiaceae)* that induced intestinal hypermotility. Its active component, ricin oleic acid, triggers an inflammatory response in the intestines via acting on EP3 receptors of prostaglandin E_2 (PGE2) [9]. In fact, when administered, castor oil irritates the intestinal mucosa, inducing an inflammatory response by promoting the release of pro-inflammatory mediators [10]. Due to the limited effectiveness of standard treatments, there is increasing interest in searching for natural alternatives, particularly medicinal plants with antioxidant and anti-inflammatory properties [11]. In this context, we selected the cladode extract of *Opuntia ficus-indica* (prickly

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pear), as various parts of this plant are renowned for their health benefits and effectiveness in treating many inflammation-related pathologies [12, 13, 14]. Numerous studies have reported the therapeutic effects of *Opuntia* species in conditions such as inflammatory bowel disease (IBD) and cardiovascular disease, as well as hypercholesterolaemia and oxidative stress. Our recent study has shown that the aqueous cladode extract of *Opuntia ficus-indica* has potent anti-inflammatory and antioxidant properties [17]. Based on these results, the present study was carried out to investigate the protective effects of the hydroethanolic cladode extract (HECE) against castor oil-induced diarrhea in rats*in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

Phosphate-buffered saline (PBS), hydrogen peroxide (H_2O_2), 2,2′-azino-bis-3-ethylbenzothiazoline 6-sulfonic acid (ABTS), Ethylene diamine tetra acetic (EDTA), castor oil, Trolox, Gallic acid, Quercetin, Catechin, Ferrozine, Clonidine, Yohimbine hydrochloride, Charcoal meal and Gum arabic were from Sigma-Aldrich Co, St Louis, USA.

2.2. Plant material preparation and identification

The *Opuntia ficus-indica* plant material used was identified and authenticated (reference number: Ofi-C/LBE 22-001) by Professor Neziha Ghanem-Boughanmi at the Faculty of Sciences ofBizerte (University of Carthage). The cladodes were collected in the Menzel Bourguiba region of Bizerte (37° 9' 21" North, 9° 47' 37" East), cleaned, dried, and ground into powder. The dried, powdered material was extracted using a 70:30 ethanol-water solution by maceration at room temperature for 24 hours. The extract was then concentrated, lyophilised, and stored at 4 °C until use. For the experiments, the powder was dissolved in physiological saline solution (0.9% NaCl).

As the *Opuntia ficus-indica* plant is not listed as a protected species in Tunisia, permission for plant collection was not required.

2.3. Phytochemical composition, HPLC profiling and antioxidant activity of HECE

The total phenolic content (TPC) of the samples was determined using the Folin-Ciocalteu assay described by Dewanto et al. [18]. Total flavonoid content (TFC) was determined using the aluminium chloride method described by Zhishen et al. [19]. The antioxidant activity of HECE was evaluated using an ABTS assay according to Re et al. [20], as well as a Fe^{2+} chelating assay according to Chew et al. [21]. The phenolic compounds in HECE were identified using high-performance liquid chromatography (HPLC) with a C18 column (4.6 mm × 250 mm) and UV detection at 280 nm. An elution gradient of acetonitrile and phosphoric acid was used and separation was achieved under optimised conditions (flow rate: 0.6 mL/min; injection volume: 20 µL; column temperature: 40 °C). All analyses were performed in triplicate.

2.4. Animals

Adult male Wistar rats, weighing 200–220g, were purchased from the Pasteur Institute of Tunis (Tunisia). They were acclimated and housed six per cage. The animals were maintained under a standard controlled environment: Temperature 22 ± 2 °C, 12-hour light-dark cycle. Food (EL BADR, Utique-Bizerte, Tunisia) and water were provided ad libitum.All experimental procedures were conducted in respect with the guidelines the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The study protocol was approved by the ethics committee of the Tunisian Association of Laboratory Animal Sciences (ATSAL).

2.5. Evaluation of antidiarrheal activity of HECE

The antidiarrheal activity of HECE was evaluated according to Yu et al. [22].Groups of male Wistar rats (200-220g) were fasted for 18 h (but had access to water) and then divided into seven groups (n=6). The first group served as the negative control and received 0.9% NaCl solution by oral gavage. The second group served as the positive (diarrheal) control.The third group received the standard anti-diarrheal drug loperamide (10mg/Kg, intraperitoneally (*i.p*)). Groups 4 to 7 were administered HECE orally by gavage atdoses of 100, 200, 400 and 600 mg/Kg, respectively. After 60 minutes,all animals of each group-except those in the negative control group-were orally administrated 1mL of castor oil by gavage. Each rat was then placed in individually cage with non-wetting paper at the base. The number of defecations, the frequency of watery stools and the weight of both wet and dry fecal matter were recorded for up to 7h.

2.6. Enteropooling assay

This assay was performed using the method described by Robert et al. [23]. Male Wistar rats weighing 200–220 g were fasted for 18 hours and then randomly divided into seven groups of six rats each. The animals received the following pre-treatments: 0.9% NaCl (control group); castor oil (5 mL/Kg); increasing doses of HECE (100, 200, 400 and 600 mg/Kg) administered orally by gavage; and loperamide (10 mg/Kg, intraperitoneally) as the standard anti-diarrheal agent. Thirty minutes later, all groups except the control group, were given castor oil (5 mL/Kg, orally) to induce enteropooling. Two hours later, the animals were sacrificed. The entire small intestine, from the pylorus to the cæcum, was carefully excised after both ends were tied off with thread. The intestinal contents were collected to measure their volume. The intestine was then weighed both full and empty in order to calculate the percentage of fluid inhibition based on the weight difference.

2.7. Gastrointestinal motility measurement

The gastrointestinal transit (GIT) study was performed using the charcoal meal method, as previously described by Souli et al. [24]. Briefly, male Wistar rats were fasted and then divided into six groups. Group 1 (control) received 0.9% NaCl by oral gavage. Groups 2 and 3 were pretreatedby oral gavage with clonidine (1 mg/Kgbw) and yohimbine (2 mg/Kgbw), respectively. Groups 4, 5, and 6 received increasing doses of the HECE at 200, 400, and 600 mg/Kgbw, administered orally. Two hours after treatment, all groups were orally administered a charcoal meal consisting of 10% activated charcoal suspended in 5% gum arabic. Thirty minutes later, the animals were sacrificed and the small intestines were removed from the pylorus to the cæcum. The distance travelled by the charcoal marker was measured, and the percentage of GIT was

calculated using the following formula:

GIT (%) = Distance travelled by charcoal (cm) /Total intestinal length (cm) × 100.

2.8. Assessment of intestinal histological changes

Small intestine segments were fixed in 10% formalin. Following dehydration thoughta graded series of ethanol and xylene, the tissues were embedded in paraffinblocks. Sections of 5 µm thickness were prepared and stained with Hematoxylin and Eosin (H&E) for general histology, and Alcian blue–periodic acid–Schiff (AB–PAS) for mucin secretion. The stained sections were examined using a high-performance fluorescence slide scanner (ZEISS Axioscan 7). The villi/crypts ratio was calculated to assess mucosal integrity, while the surface area of mucins was measured using *ImageJ* software on multiple sections of the intestinal epithelium.

2.9. Biochemical assays in gastric and intestinal mucosa

Gastric and intestinal tissues were homogenized in a phosphate buffer (100 mM, pH 7.4) at 4°C using an ultra-turax (IKA-T10). The homogenates were then centrifuged at 4000 rpm for 20 minutes at 4°C. After centrifugation,the pellets were separated, and supernatants were collected, aliquoted, and stored at -20°C until analysis.The main antioxidants parameters responsible for counteracting oxidative stress, namely superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were quantified in gastric and intestinal tissues using the methods described by Misra and Fridovich [25], Aebi [26] and Yagi [27] respectively. Variations in absorbance were measured using a UV-visible spectrophotometer (Agilent Cary 3500).

2.10. Iron and calcium determination in plasma

Plasma free iron was measured using a colorimetric method based on ferrozine, as described by Leardi et al. [28]. In brief, iron was first dissociated from the transferrin–iron complex using a guanidine acetate solution, then reduced by ascorbic acid and subsequently reacted with ferrozine to form a colored complex, which is measured at 562 nm.

Calcium levels were determined using the Stern and Lewis[29] method. In this assay, calcium ions (Ca^2) precipitate as calcium oxalate, which subsequently forms a purple complex with o-cresol phthalein in an alkaline medium. The intensity of the color is measured spectrophotometrically at 570 nm to quantify the calcium concentration.

2.11. Statistical analysis

Statistical evaluationswascarried outthought one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to perform multiple comparisons among the experimental groups (GraphPad Prism 8, GraphPad Software Inc., San Diego, CA, USA). All tests were two-tailed, and a P value ≤ 0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

3. Results

3.1.Phytochemical analysis

Preliminary analysis revealed that HECE is rich in phenolics (608.84±14.94 mg of GAE/g) and flavonoids (227.34±0.03 mg of QE/g). Its antioxidant potential was confirmed by strong $ABTS^{\bullet+}$ radical scavenging activity (IC_{50} of 42.14± 0.87 µg/mL vs. 25.26 ± 0.23 µg/mL for Trolox), and Fe^{2+} chelation (IC_{50} of 52.81± 0.32 µg/mL vs. 36.51± 0.42 µg/mL for EDTA). Phytochemical analysis of HECE using HPLC technique revealed that HECE contains eight phenolic compounds identified as gallic acid (1), catechin, hydrated catechin (2, 3), caffeic acid (4), p-coumaric acid (5), rutin (6), quercetin (7) and kaempferol (8) (Fig.1). Multiple absorbance peaks were detected and classified based on their retention times, as detailed in Table 1.

3.2. Effects of HECE on castor oil-induced diarrhea

The results presented in Fig.2 demonstrate that cas-

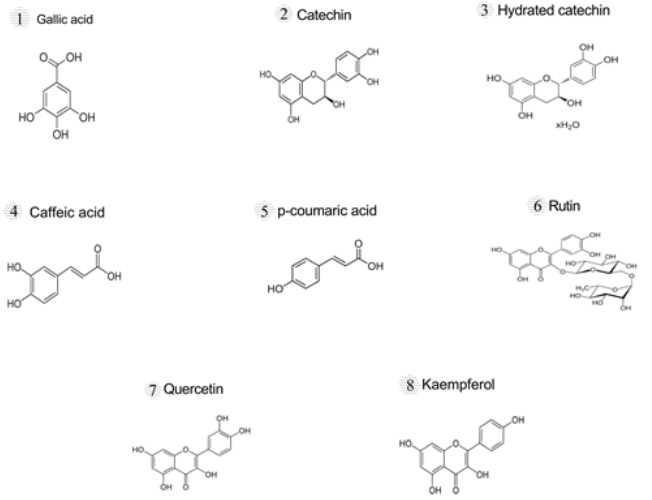


Fig. 1. HPLC chromatogram of major phenolic compounds in the hydroethanolic cladode extract (HECE) Identified compounds, along with their retention times and peak areas, are listed in Table1.

Table 1. Bioactive compounds identified in the hydroethanolic extract of *Opuntia ficus-indica* cladodes using HPLC at 280 nm.

Peak No.	Retention Time (min)	Compound Identified	Compound Type	Molecular Formula	Area [mAU*s]	Height [mAU]	Area %
1	10.832	Gallicacid	Phenolicacid	$C_7H_6O_5$	716.558	77.625	10.658
2	15.725	Catechin	Flavonoid	$C_{15}H_{14}O_6$	1379.872	34.841	20.524
3	15.725	Hydratedcatechin	Flavonoid	$C_{15}H_{14}O_6$	1379.872	34.841	20.524
4	22.735	Caffeicacid	Phenolicacid	$C_9H_8O_4$	27.484	1.841	0.408
5	27.858	p-coumaricacid	Phenolicacid	$C_9H_8O_3$	105.443	5.583	1.568
6	36.002	Rutin	Flavonoid	$C_{27}H_{30}O_{16}$	201.633	4.100	2.999
7	39.009	Quercetin	Flavonol	$C_{15}H_{10}O_7$	320.935	16.956	4.773
8	44.334	Kaempferol	Flavonol	$C_{15}H_{10}O_6$	26.950	1.953	0.400

tor oil administration significantly increased diarrhea, as evidenced by a higher average stool number (control: 0.38±0.05; castor oil 1.94±0.20; P<0.001) (Fig.2A) and elevated stool water content (control: 28.25±2.13; castor oil 73.1±11.95; P<0.001) (Fig.2B) compared to the control group. Oral administration of HECE effectively reduced diarrhea, as shown by a significant reduction in average stool number, with the most pronounced effects observed at doses of 200, 400, and 600 mg/Kg (200 mg/Kg: 0.94±0.05; 400 mg/Kg: 0.84±0.20; 600 mg/Kg: 0.5±0.05; P< 0.001 vs. castor oil group) (Fig.2A). Notably, this reduction was comparable to the effect of the reference drug, loperamide at doses 400, and 600 mg/Kg. These findings were further supported by the stool water content data, where the castor oil group had the highest percentage of water (73.1±11.95; P<0.001 vs. the control group). HECE treatment significantly reduced feces water content in a dose-dependent manner (200mg/Kg: 34.29±4.14; 400 mg/Kg: 28.68±2.37; 600 mg/Kg: 21.37±3.39; P < 0.001 vs. castor oil group), supporting the antidiarrheal effect (Fig.2B).

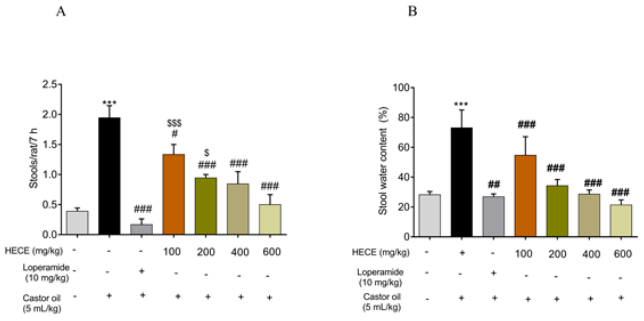


Fig. 2. The effect of HECE treatment on castor oil-induced diarrhea in rats. (A) Stool average and (B) stool water content. Rats were fasted for 18 hours and pretreated with saline solution, loperamide (10 mg/Kg b.w.) or increasing doses of HECE (100, 200, 400 or 600 mg/Kg). One hour later, all groups except the control group received 1 mL of castor oil (5 mL/Kg b.w.). The number of defecations and the water content of the stool were recorded over 7 hours. Values are presented as mean ± S.E.M. (n = 6), with ***P < 0.001 vs. the control group, $^{\#}$P < 0.01 and $^{\#\#\#}$P < 0.001 vs. the castor oil group and $^{\$\$\$}$P < 0.001vs. the referent group (Loperamide)

3.3. Effect of HECE on castor oil-induced enteropooling

Analysis of the intestinal content volume and weight (Table 2) showed that the castor oil group had significantly higher values compared to control group, with mean volumes of 1.987 ± 0.077 mL and intestinal content weights of 2.267 ± 0.086 g (P< 0.001). These results confirm that castor oil administration promotes intestinal fluid accumulation.The data showed that treatment with HECE at increasing doses significantly reduced castor oil-induced intestinal fluid content. This reduction in intestinal volume was more marked at higher doses of the extract (400 and 600 mg/Kg) (P< 0.01). For intestinal content weight, the reduction was significant at 200 mg/Kg (p< 0.05) and highly significant at 400 and 600 mg/Kg (P< 0.001). These effects were confirmed by the percentages of protection calculated for HECE at the different doses used, reaching over 40% protection.

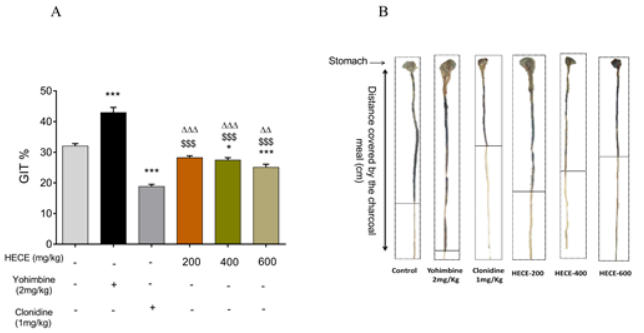


Fig. 3. The effect of HECE, clonidine and yohimbine on gastrointestinal transit. Rats in each group were pretreated with a saline solution, yohimbine (2 mg/Kg b.w.) clonidine (1 mg/Kg b.w) or increasing doses of HECE (200, 400 or 600 mg/Kg).After two hours, all rats received a charcoal meal (10% charcoal in 5% gum arabic, 5 ml/Kg) by oral route, and 30 minutes later they were sacrificed. Data are presented as the mean ± S.E.M. (n = 6), with ***P < 0.001 vs. the control group, $^{\$\$\$}$P < 0.001vs. the yohimbine group and $^{\Delta\Delta\Delta}$P < 0.001vs. the clonidine group.

3.4. Effects of HECE on Gastrointestinal transit (GIT)

To evaluate the effect of HECE on gastrointestinal transit, we measured the distance travelled by a charcoal meal within the gastrointestinal tract. As shown in Fig.3A , HECE treatment at various doses effectively inhibited gastrointestinal transit, as evidenced by a reduction in the distance travelled by the charcoal marker (Fig.3B). Specifically, as presented in Table 3, HECE significantly decreased the transitpercentage in a dose-dependent manner, achieving an inhibition rate ranging from 28.20% to 25.78%, compared with the effect of yohimbine (an α_2-adrenergic antagonist), used as a reference transit activator (47.2±1.54% increase ; P < 0.001 vs. the control group). This inhibitory effect of the extract was less pronounced

Table 2: Effect of HECE on castor oil-induced intestinal fluid accumulation in rats.

Groups	volume of intestinal content (mL)	Percentage protected (%)	Weight of intestinal content (g)	Percentage protected (%)
Control	1.018 ±0.053	-	0,969±0.182	-
Castor oil	1.987±0.077$^{\#\#\#}$	-	2.267±0.086$^{\#\#\#}$	-
loperamide	1.088±0.286	82.62	1.444±0.214	36.30
Castor oil+ HECE-100	1.425±0,2175	28.88	1.531±0.159	32.46
Castor oil+ HECE-200	1.225±0.085**	38.34	1.348±0.110*	40.53
Castor oil+ HECE-400	1.025±0.085***	48.41	1.154±0.110***	49.09
Castor oil+ HECE-600	0.945±0.037***	52.44	1.153±0.146***	49.139

*P< 0.05; ** P< 0.01; *** P< 0.001 vs Castor oil group and $^{\#\#\#}$P< 0.001vs Control group

Table 3. Effect of HECE, Yohimbine and Clonidine on gastrointestinal transit (GIT).

Groups	GIT (%)	Percentage change
Control	32,06 ±0.75	-
Yohimbine (2 mg/kg)	47,2 ± 1.54***	47.22 (+)
Clonidine (1 mg/kg)	18,81 ± 0,72***	41.32 (-)
HECE-200	28,20 ± 0.65*	12.03 (-)
HECE-400	27.41 ± 0,80**	14.50 (-)
HECE-600	25.78 ± 0,87***	19.58 (-)

Results are expressed as means ±SEM. *p < 0.05, **p < 0.01, *** p < 0.001 compared to control group. Abbreviations: (-), transit decrease; (+), transit increase.

than that of clonidine (α_2-adrenergic agonist), used as a reference inhibitor (18.81±0.72% decrease; P < 0.001 vs. the control group).

3.5. Microscopic evaluation of the effect of HECEon the intestine

Histological evaluationof the intestinal samples from the control group revealed intact intestinal layers, well-preserved villi with uniform epithelial lining, and normal crypt structures (black arrows) (Fig.4-Control). In contrast, the castor oil group showed marked structural alterations (black arrows), including shortened and atrophic villi, epithelial desquamation and disrupted mucosal architecture (Fig.4A-Castor oil). Interestingly, administration of HECE significantly improved these damages, particularly at the higher doses 400 and 600 mg/Kg (Fig.4A-HECE-treated groups), where the mucosa appeared nearly normal with restored villus height, reduced inflammation and re-epithelialization. To further quantify these histological observations, we calculated the ratio of villus height to crypt depth (VH/CD) (Fig.4C). The castor oil group, showed a significant decrease in the VH/CD ratio compared with the control group (p <0,001). Treatment with HECE restored VH/CD ratio, with statistically significant improvements observed at the higher doses 400 and 600mg /Kg (p < 0,001), suggesting improved mucosal recovery and intestinal function. Regarding mucin secretion, no significant differences were observed among the groups (Fig.4B and D).

3.6. Effect of HECE on castor oil–induced gastric and intestinal mucosal lipid peroxydation and on antioxidant enzyme activities

Oral administration of castor oil led to a marked decrease in antioxidant enzymes (CAT and SOD) and a significant increase in mucosal lipid peroxidation, as measured by malondialdehyde (MDA) levels, in both gastric (CAT: 26.39±2.35 vs. 68.3±7.75 in control group; SOD: 1.41±0.17 vs. 5.42±0.78 in control group; MDA:40.92±2.027 vs. 20.74±1.49 in control; P< 0.001 vs. control group) and intestinal mucosa (CAT: 13.19±3.93 vs. 40.76±4.34 in control group;SOD: 1.46±0.18 vs. 4.05±0.26 in control group; MDA:61.78±3.29 vs. 32.68±1.73 in control; P< 0.001 vs. control group), indicating gastrointestinal oxidative stress and tissue dysfunction (Figure 5). Treatment with HECE at increasing concentrations significantly restored the activities of both CAT and SOD enzymes in gastric and intestinal homogenates. For example, CAT activity in the stomach increased from 26.39±2.35 in the castor oil group to 56.41±7.42, 63.25±5.89 and 65.63± 4.94 at doses of 200, 400 and 600 mg/Kg, respectively (Fig.5A and D) (P<

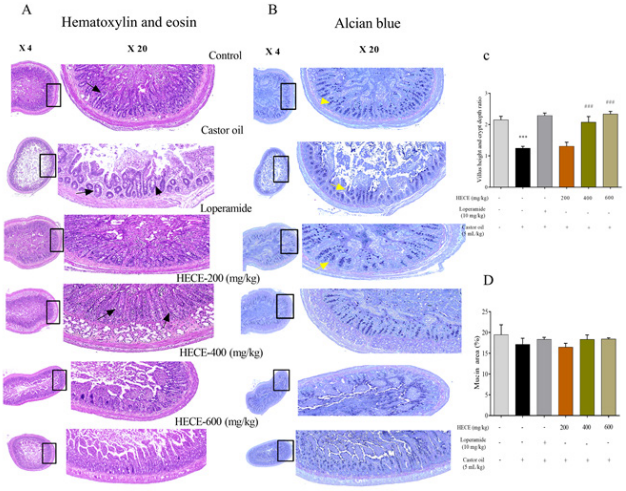


Fig. 4. The effect of HECE on castor oil-induced changes to intestinal morphology and mucin secretion (A) Intestinal sections stained with hematoxylin and eosin. (B) Intestinal sections stained with Alcian blue–periodic acid–Schiff (AB–PAS). (C) Represents the statistics of the villus height-to-crypt depth ratio. (D) Represents the percentage of mucin area.(Control) shows a section of intestine from rats that received an oral saline solution. (Castor oil) shows a section of intestine from rats that received an oral saline solution and a dose of castor oil. (Loperamide) shows intestines from rats treated orally with loperamide (10 mg/Kg b.w.) and a dose of castor oil. HECE-200, HECE-400 and HECE-600 show sections of intestine from rats treated with increasing doses of HECE (200, 400 and 600 mg/Kg, respectively) and a dose of castor oil. Black arrows represent villus architecture; yellow arrows represent goblet cells and mucin secretion. Intestinal sections were examined at two magnifications (4X and 20X).

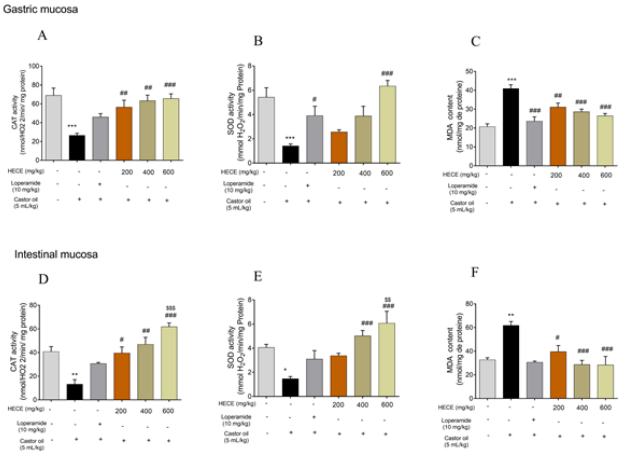


Fig. 5. Effects of pretreatment with HECE on the oxidative stress status of gastric and intestinal homogenates. Rats in each group received an oral administration of saline solution, castor oil (5 mL/Kg) or increasing doses of hydroethanolic cladode extract of *Opuntia ficus-indica*, (200, 400 or 600 mg/Kg) after which diarrhea was induced by an oral administration of castor oil (5 mL/Kg). Gastric and intestinal fragments were homogenised and the resulting supernatants were divided into portions. CAT activity (A, D), SOD activity (B, E) and lipid peroxidation, as well as MDA content (C, F), were determined. The results are presented as the mean ± S.E.M. (n = 6), with ***P < 0.001 vs. the control group, #P < 0.05, ##P <0.01, ###P < 0.001 vs. castor oil group and $^{\$\$}$P < 0.01, $^{\$\$\$}$P < 0.001vs. the referent group (Loperamide)

0.001). SOD activity in the intestine was restored from 1.46±0.18 in the castor oil group to 3.37±0.20, 5.01±0.45 and 6.06±0.99 at 200, 400 and 600 mg/Kg, respectively (Fig.5B and E) (P< 0.001 vs. castor oil group).For MDA levels, HECE effectively counteracted the castor oil-induced elevation. A significant reduction was observed at a dose of 200 mg/Kg (stomach: 31.07±2.15; intestine: 39.55±5.23; P< 0.01 and P< 0.05, respectively vs. castor oil group), and this reduction became highly significant at doses of 400 and 600 mg/Kg (stomach: 28.63±1.38 and 26.45±1.25; intestine: 28.7±3.56 and 28.35±7.21, respectively; P<0.001 in both stomach and intestine tissues) (Fig.5C and F).

3.7. Effect of HECE on Iron and calcium plasma levels

As shown in Fig.6, analysis of plasma $Ca^{2+}$and Fe^{2+}levels revealed that castor oil administration caused a significant increase in both minerals compared to the control group (Ca^{2+}: 94.06±1,76; Fe^{2+}: 28.67±0.53; P< 0.001), indicating systematic mineral imbalance (Fig.6A and 6B). Oral administration of HECE effectively attenuated these elevations, restoring Ca^{2+} and Fe^{2+}levels toward physiological ranges. Notably, HECE at doses of 400 and 600 mg/Kg significantly reduced plasma iron levels (Fe^{2+}: 19.75±0.92;19.2±1.76 P< 0.001) and Ca^{2+} levels (Ca^{2+}: 69.24±0.98;57.93±1.60 P< 0.01) compared to the castor oil group (Fig.6A and 6B).

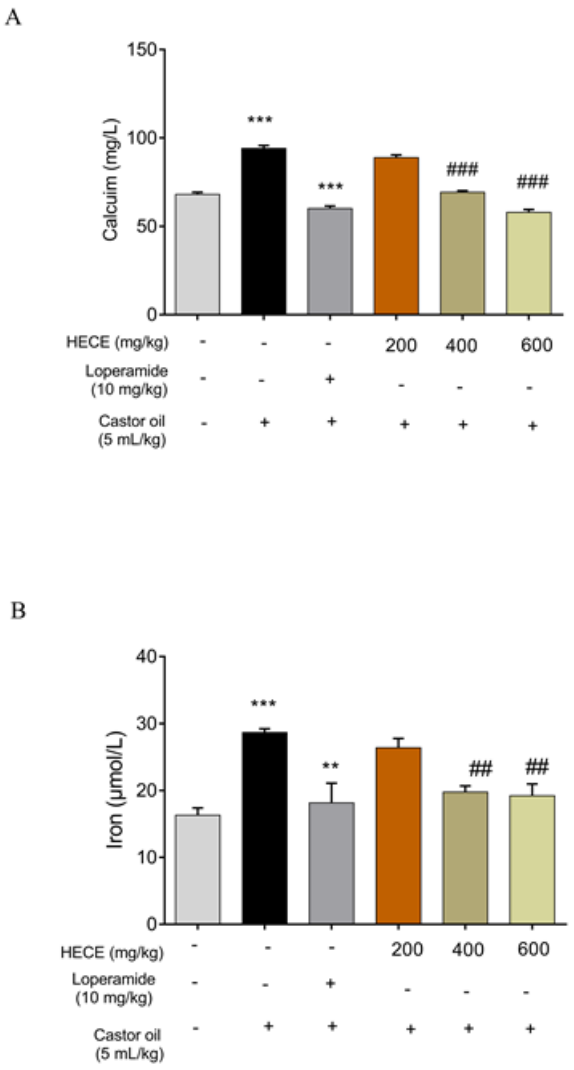


Fig. 6. Effect of HECE on iron and calcium levels in plasma. Rats in each group received an oral administration of saline solution, castor oil (5 mL/Kg) or increased doses of hydroethanolic cladode extract of *Opuntia ficus-indica* (200, 400 or 600 mg/Kg). Diarrhea was then induced by an oral administration of castor oil (5 mL/Kg). Plasma was collected from each group and calcium (A) and iron (B) levels were determined. The results are presented as the mean ± S.E.M. (n = 6), with ***P < 0.001 vs. the control group, and ##P < 0.01 and ###P < 0.001 vs. castor oil group.

4. Discussion

Diarrhea is a common gastrointestinal disease that is receiving increasing attention due to its significant contribution to morbidity and mortality worldwide. According to the World Health Organization (WHO), it is one of the leading causes of child mortality, primarily due to dehydration and associated complications. Natural products are recommended for managing diarrhea due to their safety and therapeutic potency [30]. This study focuses on the *Opuntia ficus-indica* plant, which is rich in bioactive compounds that have beneficial effects in the treatment of various diseases. Particular attention is given to the hydroethanolic extract of the cladodes (flattened stems), with the aim of evaluating its potential anti-diarrheal effect in an animal model. Castor oil-induced diarrhea is a well-established experimental model used to evaluate the efficacy of various antidiarrheal agents. Castor oil primarily acts through its active component, ricinoleic acid [9], which irritates the intestinal mucosa, inducing electrolyte hypersecretion and reducing water absorption. Our HPLC analysis of bioactive compounds revealed the presence of several phenolic compounds and flavonoids, which are renowned for their antioxidant properties [31, 32]. Some of these compounds had previously been identified in our study of the aqueous cladode extract [17], while the present analysis detected new compounds such as rutin, kaempferol and p-coumaric acid. This may be due to the inclusion of the alcoholic fraction, which likely enhanced the extraction of additional compounds.

The *in vivo* study showed that HECE significantly inhibited castor oil-induced diarrhea, as evidenced by reductions in key diarrheal parameters, including stool frequency, number of wet stools and percentage of diarrheal. Interestingly, the inhibitory effects observed were close to those of the reference drug, loperamide, particularly at higher doses (400 and 600 mg/Kg). Loperamide was used as thereference drug due to its well-documented efficacy in managing diarrhea. It acts by reducing intestinal motility and fluid secretion through the activation of μ-opioid receptors in the myenteric plexus of the large intestine [33]. This reduces peristalsis and increases fluid absorption, thereby limiting the frequency and volume of bowel movements [34].

The anti-diarrheal effects observed with HECE can be explained by the presence of phenolic compounds and flavonoids identified in the extract. Compounds such as quercetin, rutin, kaempferol and gallic acid are well recognized for their anti-diarrheal and anti-inflammatory properties. Gallic acid, for example, is known to reduce excessive intestinal secretions by tightening the intestinal mucosa, thereby reducing stool fluidity [31]. In addition, quercetin, rutin and kaempferol have been widely cited for their ability to reduce intestinal inflammation, a key factor in the pathophysiology of acute diarrhea [35, 36]. These mechanisms may explain the therapeutic efficacy observed in this study.In terms of its physiological action, HECE reduced

intestinal content by decreasing both volume and weight, reflecting a reduction in intestinal secretion. This effect can be linked to the presence of flavonoid and phenolic compounds such as quercetin, rutin and kaempferol, which are known to modulate intestinal secretion and permeability in animal models of gastrointestinal disorders [37]. Previous studies have shown that quercetin can reduce the activity of calcium channels involved in intestinal secretion, thereby limiting fluid loss [38]. In addition, gallic acid and rutin have astringent properties that help to tighten the intestinal mucosa, thereby tightening the intestinal wall and minimizing water loss [39, 40].This observed inhibition of enteropooling supports the overall anti-diarrheal efficacy of the extract. Beyond these intestinal effects, the identified phenolic compounds are also well known for their antioxidant and metals-chelating properties. Quercetin and rutin scavange ROS and up regulates antioxidant enzymes such as SOD and CAT [37], while gallic acid exhibits strong Fe^{2+} chelating capacity, preventing Fenton reactions and lipids peroxidation [40]. These combined effect likely explain the restoration of antioxidant balance and normalization of Ca^{2+} and Fe^{2+} levels observed in our study, thereby reinforcing the extract's overall protective role against oxidativeand secretory stress inducedcastor oil.

In terms of its effects on bowel motility, HECE demonstrated a reduction in gastrointestinal (GIT) motility relative to the reference drugs yohimbine (known to increase motility) and clonidine (known to decrease motility) [24]. These results suggest that HECE acts as an anti-diarrheal agent. In addition, microscopic examination provided strong evidence of an anti-diarrheal effect, showing significant restoration of intestinal mucosa architecture, evidenced by longer villi and preserved crypt architecture. This includes a marked improvement in the length and integrity of the intestinal villi. This is directly proportional to absorptive efficiency and overall intestinal function [41]. The observed increase in villi height suggests that HECE may help repair intestinal injury caused by diarrhea, promoting mucosal restoration and potentially improving absorptive capacity.

Moreover, several studies have reported that castor oil-induced diarrhoea in the small intestine may be associated with oxidative stress [42]. Measuring antioxidant enzymes (CAT and SOD) in intestinal and gastric homogenates after HECE administration shows that the extract significantly protects against oxidative stress associated with castor oil-induced diarrhoea. This finding corroborates our recent in vitro study, which examined the same plant extract and demonstrated its significant ability to scavenge free radicals, such as superoxide anion and hydrogen peroxide, thereby mitigating oxidative stress [17]. Recent literature has shown that diarrhoea is accompanied by increased plasma levels of $Fe^{2+}$and Ca^{2}, as well as increased hydrogen peroxide (H_2O_2) in the gastric and intestinal mucosa [43]. These radicalsare known to contribute to oxidative stress [44, 45]. H_2O_2 can react with Fe^{2+}via the Fenton reaction to generate $Fe^{3+}$and the highly reactive hydroxyl radical ($OH^{\cdot}$) leading to cellular damage. The observed reduction in Fe^{2+} levels after HECE treatment likely reflects the chelating ability of phenolic compounds such as gallic acid and quercetin. These compounds bind transition metals, thereby preventing the Fenton reaction and limiting oxidative damage [32, 33]. Regarding calcium, it has been reported that oxidative stress activates myeloperoxidase (MPO), an enzyme involved in the immune response. MPO produces hypochlorous acid (HOCL), a potent oxidant that reacts with protein thiol groups, leading to the inhibition of calcium ATPase pumps in the endoplasmic reticulum.This impairment leads to cytosolic Ca^{2+} accumulation, contributing to intracellular calcium imbalance[46, 47]. Interestingly, HECE appeared to attenuate castor oil-induced calcium elevation, and the role of polyphenols in regulating calcium levels is well documented, particularly for compounds such as quercetin and rutin, both of which are present in ourextract, are known to regulate intracellular calcium signaling and maintain ionic balance[48]. Altogether, these findings suggest that the normalization of $Fe^{2+}$and Ca^{2+} by HECE contributes to its antioxidant and antidiarrheal effect thought redox and ionic homeostasis restoration.

This research highlights that the hydroethanolic extract of *Opuntia ficus-indica* cladodes exhibits significant protective effects against castor oil-induced diarrhea in rats. These effects appear to be due to a combination of antioxidant activity and modulation of oxidative stress. The presence of bioactive phenolic compounds in the extract is likely to be responsible for its therapeutic properties. The results suggest that cladode extract shows promise as a therapeutic agent for treating diarrhea. Given the extract's antioxidant and gastrointestinal protective properties, future studies could explore its potential for clinical application and development as a natural treatment for digestive disorders such as diarrhea.

List of abbreviations

HECE (hydroethanolic cladode extract), **HPLC** (high-performance liquid chromatography), **PBS** (phosphate-buffered saline), **bw** (body weight), **QE** (quercetin equivalent), **GAE** (gallic acid equivalent), **CE** (catechin equivalent), **SOD (**superoxide dismutase), **MDA** (malondialdehyde), **GIT** (Gastrointestinal transit**), H&E** (Hematoxylin& Eosin), **AB–PAS** (Alcian blue–periodic acid–Schiff), **VH (**villus height), **CD** (crypt depth), **CAT** (Catalase), **IBD** (Inflammatory Bowel Disease), **H_2O_2** (hydrogen peroxide), **EDTA** (Ethylene diamine tetraacetic), **TPC** (Total Phenolic Content**), ABTS** (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), **TFC** (Total Flavonoid Content), **MPO** (Myeloperoxidase), **HOCL** (Hypochlorous Acid), **ANOVA** (one-way analysis of variance)

Conflicts interest

The authors declare no conflict of interest

Consent for publications

All authors have read and approved the final manuscript for publication.

Ethics statements

All animal protocols were conducted in compliance with national regulations and the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The study protocol was approved by the ethics committee of the Tunisian Association of Laboratory Animal Sciences (ATSAL).

Data availability

All Data generated during this study will be made available on request.

Authorship contribution statement

W.F.: Data curation, Conceptualization, Methodology, Investigation, Writing–original draft. N.Z.: Writing, Data curation, and formal analysis.P.M.-C.D.: Investigation Resources Validation, Visualization. A.K.: Data acquisition, formal analysis and validation. M.B-A.: Writing–review and editing Resources Validation, Visualization. J.E-B., and A.S.: Conceptualization; Funding acquisition, Project administration, Resources, Supervision, Writing–review and editing.

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