PHARMACEUTICAL BIOLOGY
2022, VOL. 60, NO. 1, 1207–1213
https://doi.org/10.1080/13880209.2022.2087690

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RESEARCH ARTICLE

Gastroprotective activity of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, a compound isolated from *Heliotropium indicum*: role of nitric oxide, prostaglandins, and sulfhydryls in its mechanism of action

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ABSTRACT

Context: The gastroprotective effect of *Heliotropium indicum* L. (Boraginaceae), a plant traditionally used in Mexico to treat gastric ulcers, has been previously reported. However, no active compound was identified.

Objective: The current contribution aimed to isolate, through a bioassay-guided study, at least one compound from *H. indicum* with considerable gastroprotective activity, examine its effect on ethanol-induced gastric lesions in mice, and explore possible mechanisms of action.

Materials and methods: Three extracts (hexane, dichloromethane, and methanol) were obtained from *H. indicum* leaves. Their 30 and 100 mg/kg doses were assessed on ethanol-induced gastric lesions in male CD1 mice. Since the dichloromethane extract was the most active, successive chromatographies were carried out leading to the identification of the most active compound. This compound (at 3–100 mg/kg) was compared to carbenoxolone (at 10–100 mg/kg) in biological evaluations in mice. Pre-treatments with indomethacin (10 mg/kg, s.c.), L-NAME (70 mg/kg, i.p.), and NEM (10 mg/kg, s.c.) were performed independently to determine the participation of prostaglandins, nitric oxide, and/or sulfhydryl groups, respectively, in the mechanism of action of the compound.

Results: (*E*)-Ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, a compound isolated from *H. indicum*, afforded dose-dependent gastroprotective activity. The maximum effect was observed at 100 mg/kg (90.13 ± 3.08%), with an ED_{50} of 5.92 ± 2.48 mg/kg. Gastroprotection was not modified by pre-treatment with indomethacin, L-NAME, or NEM.

Conclusions: (*E*)-Ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, isolated from *H. indicum*, was found to produce a substantial gastroprotective effect. Prostaglandins, nitric oxide, and non-protein sulfhydryl groups are not involved in its mechanism of action.

ARTICLE HISTORY
Received 14 February 2022
Revised 26 April 2022
Accepted 5 June 2022

KEYWORDS
Gastric ulcers; bioassay-guided study; gastroprotection; medicinal plants; traditional medicine

Introduction

Peptic ulcers result from a circumstantial loss of the epithelial mucosa (Gong et al. 2021). This disorder occurs when gastric juice is capable of damaging the tissue and reaching the underlying submucosa. It is among the most frequent gastrointestinal diseases, affecting 10–15% of the population worldwide (Abreu Miranda et al. 2015). In some cases, peptic ulcers cause perforations leading to peritonitis (Weledji 2020).

Various factors are known to participate in the aetiology of gastric ulcers, including alcohol, tobacco, stress, *Helicobacter pylori* infection, and the consumption of some medications (e.g., non-steroidal anti-inflammatory drugs) (Li et al. 2018). Under conditions of homeostasis, the integrity of the epithelial barrier of the gastric mucosa is maintained by a balance between protective factors (the mucosa-bicarbonate layer, the endogenous antioxidant system, prostaglandins, nitric oxide (NO), and blood flow) and aggressive compounds (hydrochloric acid, pepsin, and bile acids). Peptic ulcers are caused by an imbalance between the same factors (Khan et al. 2018) and are characterised by distinct stages: necrosis, neutrophil infiltration, decreased blood flow, increased oxidative stress, and inflammation (Sharifi-Rad et al. 2018). The most common symptom is upper abdominal pain, associated with dyspepsia, satiety, inflammation, and/or nausea (Costa et al. 2018).

Although the therapeutic strategy for peptic ulcers currently focuses on relieving pain, healing the injury, and reducing the rate of recurrence (Sharifi-Rad et al. 2018), the available drugs mainly diminish gastric acidity and strengthen the gastric mucosal barrier (Khan et al. 2018). The medications complying with these functions are either mucosal protective agents or antisecretory drugs. Proton pump inhibitors (ATPase-H^+/K^+) are antisecretory drugs and the most effective type of medication available for treating gastroesophageal reflux and peptic ulcers (Peng et al. 2018). According to recent studies, however, prolonged use of these inhibitors (e.g., omeprazole) provokes irreversible adverse

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effects, especially a decreased absorption of vitamin B_{12} that may lead to dementia, neurological damage, anaemia, hypergastrinemia (Abreu Miranda et al. 2015), acute myocardial infarction (Shah et al. 2015), and/or gastric or pancreatic cancer (Peng et al. 2018).

As can be appreciated, it is necessary to seek alternative treatments for peptic ulcers. Medicinal plants are one of the principal sources of new compounds with therapeutic activity (Torres-Rodríguez et al. 2016). *Heliotropium indicum* L. (Boraginaceae) is a traditional medicinal plant that contains tannins, saponins, steroids, oils, and glycosides, and has been employed to clean and heal wounds, alleviate fever, relieve eye infections, and treat menstrual problems, nervous disorders, kidney disease, and ulcers (Adelaja et al. 2008; Nethaji et al. 2013). Since the key compounds responsible for the gastroprotection activity of the plant have not yet been identified, the aim of the current contribution was to isolate, through a bioassay-guided study, at least one such compound, evaluate it with ethanol-induced gastric lesions in mice, and explore the possible participation of prostaglandins, NO, and sulfhydryl groups in its mechanism of action.

Materials and methods

Animals

Male CD1 mice (25–30 g) were acquired from the animal house of the Universidad Autónoma Metropolitana, Xochimilco Campus, in Mexico City. The care and handling of animals was conducted in accordance with the Mexican official guidelines for laboratory animals (NOM-062-ZOO-1999) and international norms. The study was approved by the Internal Committee for the Care and Use of Lab Animals (CICUAL, according to the initials in Spanish) of the Escuela Superior de Medicina, Instituto Politécnico Nacional, with registration number ESM.CICUAL-01/14-03-2018. Unless otherwise specified, the mice were placed in individual cages with wire-net floors and deprived of food 24 h before experimentation. They were allowed free access to water during all procedures. All experiments were carried out with 7 animals per group.

Drugs

All drugs were prepared immediately before use. Carbenoxolone, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), and *N*-ethylmaleimide (NEM) were dissolved in water, while indomethacin was dissolved in 5 mM $NaHCO_3$. These compounds were purchased from Sigma Chemical Co. (St. Louis, MO, USA). (*E*)-Ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, extracts, and fractions were suspended in 0.5% Tween 80.

Plant material

During July of 2020, the leaves of *H. indicum* were collected in Copainalá in the State of Chiapas, Mexico. The plant was identified and registered by Manuel de Jesús Gutiérrez Morales from the Flora Department of the Chip Herbarium, which is part of the Botanical Garden of the Secretary of Environmental Protection, Housing and Natural History of the State of Chiapas, Mexico. A specimen of the original collection can be found with the voucher number 27855.

Table 1. Ulcer index (UI) of *Heliotropium indicum* extracts on ethanol-induced ulceration in mice.

Treatment	Dose (mg/kg)	n	UI (mm^2)
Control	–	7	11.32 ± 1.59
Carbenoxolone	100	7	3.41 ± 1.21*
Hexane extract	30	7	4.57 ± 0.59*
	100	7	0.54 ± 0.16*
Dichloromethane extract	30	7	2.16 ± 0.75*#
	100	7	0.50 ± 0.22*
Methanol extract	30	7	10.10 ± 3.43
	100	7	9.86 ± 0.75

Data are expressed as the mean ± SEM ($n = 7$). *$p \leq 0.5$ vs. the respective control; the Kruskal-Wallis test was followed by Dunn's multiple comparison. #$p \leq 0.5$ based on the Mann-Whitney U test for comparing dichloromethane vs. hexane extracts at 30 mg/kg.

Table 2. Ulcer index (UI) of the fractions of the dichloromethane extract on ethanol-induced ulceration in mice.

Treatment	Dose (mg/kg)	*n*	UI (mm^2)
Control	–	7	10.01 ± 0.60
Carbenoxolone	100	7	3.71 ± 1.30*
F1	100	7	8.88 ± 3.76
F2	100	7	4.34 ± 1.80*
F3	100	7	4.02 ± 1.73*
F4	100	7	1.54 ± 0.35*
F5	100	7	0.0 ± 0.0*

*$p \leq 0.5$ vs. the respective control; the Kruskal-Wallis test was followed by Dunn's multiple comparison.

Isolation of (E)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate

The leaves of *H. indicum* were dried at room temperature in the shade. After grinding the leaves (9 kg), they were extracted successively by maceration at room temperature (22 ± 2 °C), first with hexane (33 L × 3), then dichloromethane (33 L × 3), and finally methanol (33 L × 3). Evaporation of the solvents under reduced pressure afforded 230, 260, and 200 g of syrupy residue, respectively. The gastroprotective activity of the distinct *H. indicum* extracts was examined by administering them at 30 and 100 mg/kg doses to gastric lesions in mice induced by 0.2 mL ethanol. Since the dichloromethane extract generated the best effect at the dose of 30 mg/kg (Table 1), it was subjected to silica gel column chromatography involving large changes in polarity. In the evaluation of the resulting fractions, gastroprotection was detected for F2, F3, F4, and F5, with the latter displaying the highest activity (Table 2). Hence, column chromatography was carried out with F5, obtaining a white solid from fractions 74–92 eluted with a 9:1 hexane/EtOAc mixture.

Characterisation of (E)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (at 3, 30, and 100 mg/kg, p.o.)

The ^1H and ^{13}C NMR spectra were acquired in deuterated chloroform ($CDCl_3$) in a Bruker Ascend NMR spectrometer (Bruker Daltonics, Billerica, MA, USA) at 750 MHz. Electrospray ionisation (ESI) analysis was performed on a Bruker micrOTOF-Q II apparatus (Bruker Daltonics, Billerica, MA, USA). Samples were dissolved in methanol and injected directly into the spectrometer. The peaks related to the compound were found in positive and negative ion mode (ESI^+ or ESI^-). The capillary potential was −4.5 kV, the drying gas temperature 200 °C, and the drying gas flow 4 L/min. Total ion chromatograms were recorded from m/z 500 to 3,000. MS data were processed with PolyTools 1.0 (Bruker Daltonics, Billerica, MA, USA).

Gastric lesions induced by absolute ethanol

The extracts, fractions, active compound, carbenoxolone (the reference drug), and vehicle were administered orally (0.1 mL/10 g) to the corresponding groups of mice. Thirty min later, absolute ethanol (0.2 mL) was given orally to all animals to provoke gastric lesions. After another 2 h, the animals were sacrificed in a CO_2 chamber. The stomachs were dissected and filled with formalin, then placed in 2% formalin for 5 min before being opened along the greater curvature. Subsequently, the gastric lesions were quantified under a stereoscopic microscope (×10) with an ocular micrometer. The ulcer index was calculated as the sum of all lesions (area in mm^2) of each stomach. The gastroprotection was determined with the formula: (UIC-UIT) × 100/UIC, where UIC is the average ulcer index of the control group and UIT is the average ulcer index of the experimental group (Sánchez-Mendoza et al. 2020).

Participation of prostaglandins

To examine the possible role of prostaglandins in the mechanism of action of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, three groups of mice were subcutaneously injected with indomethacin (10 mg/kg) dissolved in saline solution with 5 mM $NaHCO_3$ (0.1 mL/10 g). Only $NaHCO_3$ (5 mM) dissolved in saline was applied to the control (by the same route and in the same volume). Seventy-five min later, the control animals were orally administered Tween 80 (0.5%), while the other groups each received one of three treatments: Tween 80 (0.5%), (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, or carbenoxolone. Upon completion of 30 min, all animals received 0.2 mL of ethanol. After 2 h, the animals were sacrificed to calculate the ulcer index (Sánchez-Mendoza et al. 2020).

Participation of nitric oxide

To evaluate the possible contribution of NO in the mechanism of action of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, three groups were injected with L-NAME (70 mg/kg, 0.1 mL/10 g, i.p.) dissolved in saline solution and the control was given the saline solution only. Thirty min later, the L-NAME pre-treated groups received one of three treatments: Tween 80 (0.5%), (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, or carbenoxolone, while the control received Tween 80 (0.5%). Upon completion of another 30 min, all groups of animals were treated with 0.2 mL of ethanol. After 2 h, the animals were sacrificed and the ulcer index was determined (Sánchez-Mendoza et al. 2020).

Participation of sulfhydryl groups

To explore the potential role of sulfhydryl groups in the mechanism of action of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, three groups were injected with NEM (10 mg/kg, 0.1 mL/10 g, s.c.), and the saline solution only was delivered to the control animals. Thirty min later, the NEM pre-treated groups received one of three treatments: Tween 80 (0.5%), (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, or carbenoxolone, while the control received Tween 80 (0.5%). Upon completion of another 30 min, ethanol was applied. After 2 h, the animals were sacrificed to establish the ulcer index (Sánchez-Mendoza et al. 2020).

Statistical analysis

Data are expressed as the mean ± SEM (n = 7). The differences between treatment groups were examined for statistical significance by the Kruskal-Wallis test followed by Dunn's multiple comparison, with significance considered at $p \leq 0.05$. The Mann-Whitney *U* test was employed to compare two groups.

Results

Bioassay-guided study of Heliotropium indicum

The effect of the hexane, dichloromethane, and methanol extracts of *H. indicum* was assessed on ethanol-induced gastric lesions in mice (Table 1). No significant difference in the ulcer index was detected between the vehicle control ($11.32 \pm 1.59\ mm^2$) and the group exposed to the methanol extract (at 30 or 100 mg/kg). Consequently, this extract is not active. In the mice treated with the hexane and dichloromethane extracts, the area of the gastric lesions was less than that observed in the control animals (Table 1). The dichloromethane extract was significantly more active than the hexane extract (2.16 ± 0.75 vs. $4.57 \pm 0.59\ mm^2$, respectively; $p \leq 0.05$) at 30 mg/kg. After fractionation of the dichloromethane extract by column chromatography, the biological activity of all fractions was evaluated. The F5 fraction proved to have the highest level of gastroprotection (100%, Table 1).

Compared to the ulcer index obtained from the 100 mg/kg dose of carbenoxolone (the reference drug), lower values were found for the hexane extract, dichloromethane extract, and F5 at the same dose (Tables 1 and 2), indicating a greater gastroprotection provided by the latter three treatments.

Fractionation of F5 by column chromatography afforded 1.11 g of white crystals (melting point = 87–88 °C), which corresponded to the compound responsible for the biological activity of this fraction. The crystals were analysed by means of nuclear magnetic resonance (NMR) and electrospray ionisation (ESI) mass spectrometry (MS).

According to the NMR spectra, the crystals are an unsaturated dihydroxy long-chain ester: (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (Figure 1). The phytochemical data are as follows: ^{1}H NMR (750 MHz, $CDCl_3$) δ 7.06 − 7.01 (m, 1H), 6.10 − 6.08 (m, 1H), 4.24 (dd, J = 6.0, 2.7 Hz, 1H), 4.14 (dd, J = 7.5, 2.7 Hz, 1H), 3.98 (tt, J = 17.8, 6.6 Hz, 1H), 1.72 − 1.65 (m, 1H), 1.62 − 1.41 (m, 4H), 1.38 − 1.22 (m, 29H), 0.89 (ddd, J = 20.1, 13.9, 6.8 Hz, 5H). ^{13}C NMR (189 MHz, $CDCl_3$) δ 166.09 (C = O), 146.49 (C-3), 122.97 (C-2), 85.19 (C-4), 71.28 (C-5), 61.46 (C-1′), 33.08, 33.00, 30.80, 30.78, 30.74, 30.69, 30.49, 26.31, 23.74, 23.05, 14.46. ESI-HRMS *m/z* 341.2699 ($C_{20}H_{36}O_4$).

The structure was confirmed with ESI MS. ESI (+) showed a molecular ion at *m/z* 341.2699 $[M^+H]^+$ (calc. *m/z* 341.2686), which is consistent with the molecular formula $C_{20}H_{36}O_4$ (*m/z*). The sodium adduct was observed at *m/z* 363.25 ($[M^+Na]^+$; calc. *m/z* 363.2505) (Figure 2). However, the ESI spectra displayed a family of compounds derived from this structure with -C_2H_4- added at the alcoholic moiety. Two more compounds were

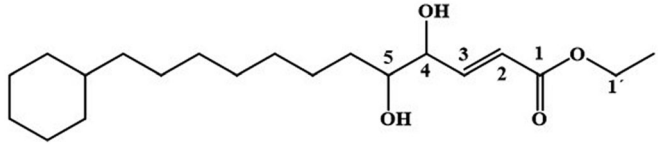

Figure 1. The structure of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate.

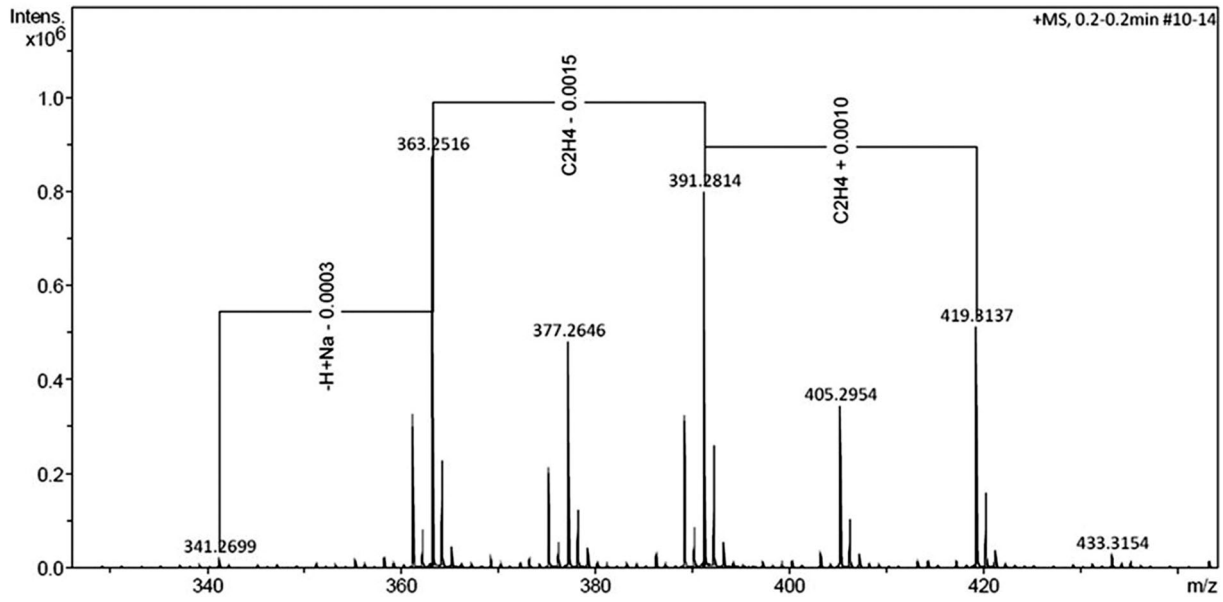

Figure 2. Electrospray spectrum acquired in the positive mode for the most active fraction.

identified from the molecular ions at *m/z* 377.2646 and 419.3137 (Figure 2).

The administration of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (at 3-100 mg/kg, p.o.) produced dose-dependent gastroprotective activity in mice (Figure 3(A)), with an ED_{50} of 5.92 ± 2.48 mg/kg. The greatest gastroprotection was exhibited by the 100 mg/kg dose (90.13 ± 3.08%), followed by 30 mg/kg (75.32 ± 6.13%), and finally 3 mg/kg (42.00 ± 3.73%). The oral application of carbenoxolone (the reference compound) also inhibited gastric lesions in a dose-dependent manner (Figure 3(B)), reaching the maximum gastroprotective activity at 100 mg/kg (94.28 ± 0.38%), with an ED_{50} of 19.65 ± 4.38 mg/kg. A lesser effect was generated by 30 mg/kg of carbenoxolone than the same dose of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (51.78 ± 7.99% vs. 75.32 ± 6.13%).

Effect of L-NAME, indomethacin, and NEM on the gastroprotection provided by (E)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate

The ulcer index values for the groups pre-treated with the three inhibitors were significantly higher than the value for the control treated with the vehicle only. Thus, some of the protective factors were inhibited by each of the pre-treatments. Accordingly, 28.29 ± 3.62 mm^2 was found for the pre-treatment with indomethacin (Figure 4(A)), 32.87 ± 2.56 mm^2 for L-NAME (Figure 4(B)), and 35.28 ± 3.02 mm^2 for NEM (Figure 4(C)), compared to 22.78 ± 5.22 mm^2 for the control.

Pre-treatment with 10 mg/kg of indomethacin, a non-specific inhibitor of COX, did not modify the gastroprotective activity of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, which afforded an ulcer index of 2.79 ± 0.68 mm^2 vs. 22.78 ± 5.22 mm^2 for the vehicle-treated control (Figure 4(A)). As can be appreciated, prostaglandins do not participate in the mechanism of action of the compound. Similarly, pre-treatment with L-NAME (a non-specific inhibitor of NO synthase) and NEM (a blocker of sulfhydryl groups) did not modify the protective effect of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (with an ulcer index of 8.62 ± 2.56 and 12.06 ± 2.53 mm^2, respectively; Figure 4(B, C)), indicating that NO and sulfhydryl groups do not contribute to the gastroprotective mechanism of action. Regarding carbenoxolone, the results are in agreement with the previously published data (García-Martínez et al. 2016).

Discussion

The drugs currently available to treat gastric ulcers not only providing gastroprotection, also have harmful side effects (da Silva et al. 2018). There is a clear need to search for new drugs capable of eliciting better protection with a minimum of adverse events.

Medicinal plants represent an important source of new drugs. *H. indicum* has a demonstrated gastroprotective effect, although the active compounds have not been identified (Adelaja et al. 2008; Nethaji et al. 2013). The present study isolated one of the metabolites with substantial gastroprotective activity and analysed the possible involvement of prostaglandins, NO, and sulfhydryl groups in its mechanism of action.

Oral administration of the hexane and dichloromethane extracts (at all doses herein examined) protected the gastric mucosa from ethanol-induced damage, revealing that *H. indicum* has more than one active compound. Greater efficacy was shown by the dichloromethane vs. hexane extract at a 30 mg/kg dose (Table 1). Hence, fractionation of the dichloromethane extract was carried out, finding gastroprotective activity for fractions F2, F3, F4, and F5 (Table 2). F4 and F5 were the most active, but only F5 gave rise to 100% gastroprotection. Once again, evidence was found of the existence of several compounds in *H. indicum* capable of generating gastroprotection. Since fractions F4 and F5 were much more active than carbenoxolone evaluated at the same dose, the compounds responsible for the effect of these fractions are probably more potent than the reference drug.

(*E*)-Ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate was isolated from F5 (Figure 1) based on fractionation by column chromatography and was the main compound in the fraction that afforded gastroprotection. This compound has not been previously reported for *H. indicum* and appears to be totally new. Considering that it did not provide 100% gastroprotection, its activity must be slightly enhanced by another compound in F5. Compared to carbenoxolone, (*E*)-ethyl-12-cyclohexyl-4,5-

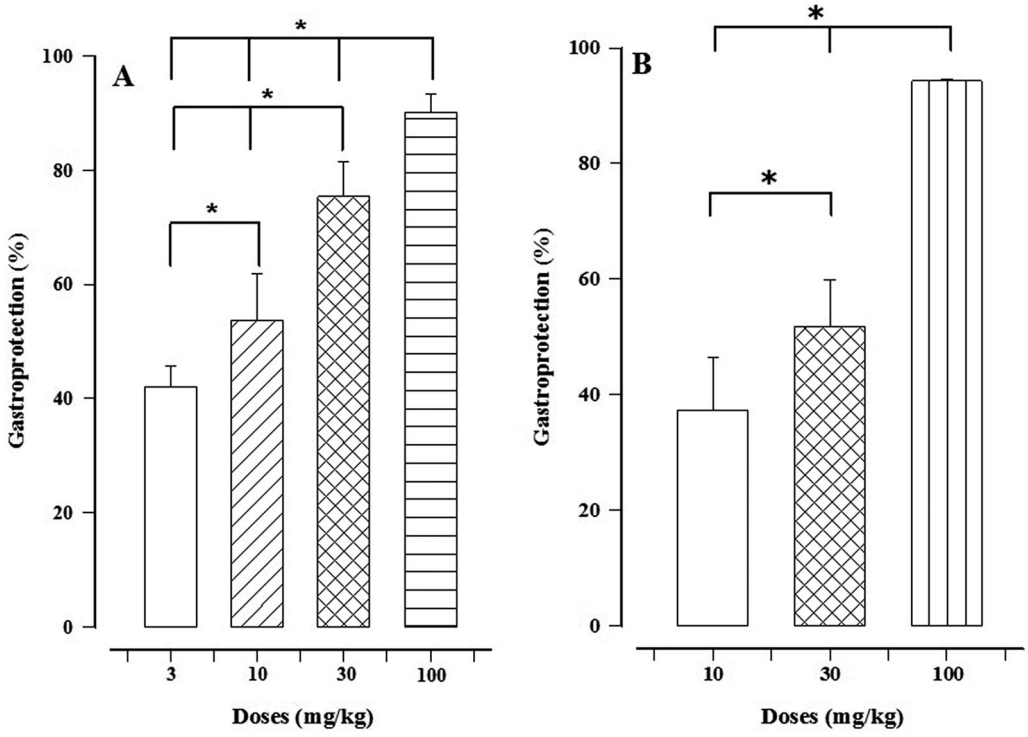

Figure 3. Gastroprotective effect of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (A) and carbenoxolone (B). Bars denote the mean ± SEM ($n = 7$). *$p \leq 0.05$, based on the Kruskal-Wallis test followed by Dunn's multiple comparison.

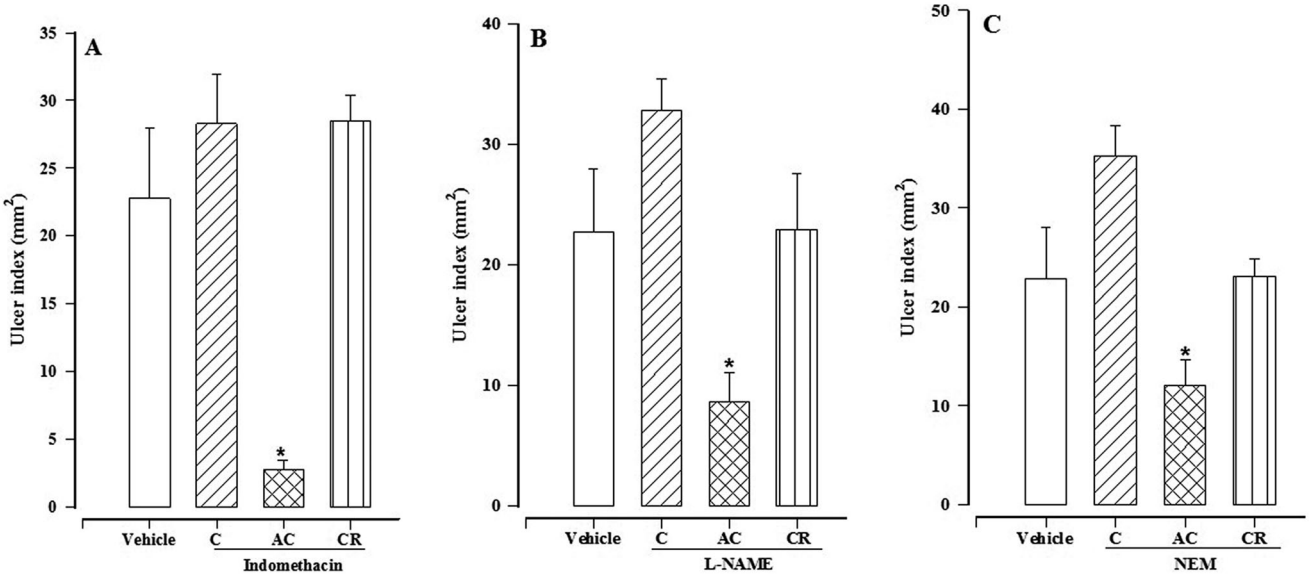

Figure 4. Effect of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate (AC) and carbenoxolone (CR) on ethanol-induced gastric lesions in mice pre-treated with indomethacin (10 mg/kg) (A), L-NAME (70 mg/kg) (B), or NEM (10 mg/kg) (C). C = the control group for the distinct inhibitors. Bars denote the mean ± SEM ($n = 7$). *$p \leq 0.05$ vs. the respective control, based on the Kruskal-Wallis test followed by Dunn's multiple comparison.

dihydroxydodec-2-enoate produced a greater effect at the doses of 10 and 30 mg/kg. However, the efficacy was the same for the two compounds because the percentage of gastroprotection was similar at the 100 mg/kg dose (Figure 3).

In ethanol-induced gastric lesions, several factors contribute to tissue damage. Ethanol promotes the secretion of gastric acid and pepsin, alters the mucus-bicarbonate barrier and mucosal microcirculation, and triggers the inflammatory response and the infiltration of inflammatory cells into the gastric mucosa (La Casa et al. 2000; Chang et al. 2017). Experiments were conducted to establish whether or not the mechanism of action of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate is related to prostaglandins, NO, and/or non-protein sulfhydryl groups, as these compounds are capable of counteracting some of the ethanol-induced damaging mechanisms.

Since prostaglandins, especially PGE_2, play a crucial role in protecting the gastric mucosa by stimulating mucus and bicarbonate secretion, preserving blood flow, and decreasing acid secretion, they facilitate the resistance of the gastric mucosa to damage (Takeuchi and Amagase 2018). Indomethacin, a non-

selective COX inhibitor, was used to explore the possible participation of prostaglandins in the gastric protective activity of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate. The gastroprotective effect was not reversed by indomethacin (Figure 4(A)), indicating that prostaglandins are not implicated in the mechanism of action of this compound. In contrast, indomethacin reversed the gastroprotective effect of carbenoxolone (Figure 4(A)), in agreement with previous reports (Sánchez-Mendoza et al. 2020). Consequently, prostaglandins are involved in the mechanism of action of the reference compound.

NO has an essential function in the protection of the gastric mucosa. It is mainly synthesized by NO synthase (NOS) isoforms, consisting of three subtypes in the gastrointestinal tract: neuronal, endothelial, and inducible NOS. Neuronal and endothelial NOS help to maintain the integrity of the gastrointestinal mucosa through the regulation of gastric mucosal blood flow and the secretion of mucus and bicarbonate, thus strengthening the gastric mucosal barrier. Contrarily, the NO synthesized by inducible NOS takes part in the inflammatory response and therefore helps to promote tissue damage (Liang et al. 2021). Pre-treatment with L-NAME, a non-specific inhibitor of NOS (Engevik et al. 2020), did not modify the gastroprotective activity of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, indicating that NO synthesized from endothelial or neuronal NOS do not contribute to the mechanism of action of gastroprotection. On the other hand, pre-treatment with L-NAME did indeed inhibit the effect of carbenoxolone (Figure 4(B)), demonstrating the involvement of NO in its mechanism of action. This result does not differ from published data (Sánchez-Mendoza et al. 2020).

Non-protein sulfhydryl groups play an important role in the protection of the gastric mucosa. They participate in the secretion of gastric mucus and are also contained in the mucus to protect it from reactive oxygen species (ROS), such as those generated by the administration of ethanol (Sidahmed et al. 2013). Sulfhydryl groups are diminished by substances capable of damaging the gastric mucosa (e.g., ethanol). Pre-treatment with NEM, a blocker of sulfhydryl groups, did not modify the effect of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, revealing that sulfhydryl groups do not form a part of the mechanism of action of gastroprotection. Contrarily, NEM pre-treatment did indeed alter the effect of carbenoxolone, which is consistent with descriptions in the literature (Sánchez-Mendoza et al. 2020).

According to a previous study, compounds with α,β-unsaturated carbonyl groups are able to bind to cysteine residues of proteins through a Michael-type reaction (Umemura et al. 2008), as occurs with the active metabolites of omeprazole (Esplugues 2005). Hence, the α,β-unsaturated carbonyl group of (*E*)-ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate can probably bind to the cysteine residues of ATPase H^+/K^+, thus inhibiting this enzyme and acting as an antisecretory agent. In agreement with the aforementioned hypothesis is a report on the inhibition of acid secretion by the ethanolic extract of *H. indicum* leaves (Nethaji et al. 2013). Further research is needed in this sense.

Conclusions

(*E*)-Ethyl-12-cyclohexyl-4,5-dihydroxydodec-2-enoate, isolated from the dichloromethane extract of *H. indicum*, was shown to be one of the main metabolites responsible for the gastroprotective effect of the plant. Its mechanism of action is not related to prostaglandins, endogenous NO, or sulfhydryl groups.

Acknowledgements

We thank Biologist Manuel de Jesús Gutiérrez Morales (in charge of the office of the Herbario CHIP of the Subsecretaria de Desarrollo Forestal del Jardín Botánico Dr. Faustino Miranda) for the collection of the plant.

Author contributions

María Elena Sánchez-Mendoza and Jesús Arrieta conceived and designed the experiments; Yaraset López-Lorenzo and Adriana Guadalupe Perez-Ruiz performed the experiments; Yaraset López-Lorenzo and Daniel Arrieta-Baez analysed the data and contributed to the preparation of the manuscript; María Elena Sánchez-Mendoza and Jesús Arrieta were responsible for writing, reviewing, and editing the manuscript. All authors were involved in discussing the manuscript, and all read and approved the final version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This research was supported from the Escuela Superior de Medicina of the Instituto Politécnico Nacional by grants [SIP 20200425 and SIP 20210991).

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