JPP Journal of Pharmacy and Pharmacology

JPP 2008, 60: 931–936

Received December 13, 2007
Accepted March 31, 2008
DOI 10.1211/jpp.60.7.0016
ISSN 0022-3573

Bioassay-guided isolation of an anti-ulcer diterpenoid from *Croton reflexifolius*: role of nitric oxide, prostaglandins and sulfhydryls

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Abstract

Croton reflexifolius H. B. K (Euphorbiaceae) is a very common medicinal plant in the Huastecan region of Mexico that, according to local folk medicine, is considered useful in the treatment of gastritis and gastric ulcer. We have aimed to test the validity of this practice by using the experimental model of an ethanol-induced gastric ulcer in male Wistar rats. The results showed that *C. reflexifolius* had gastroprotector activity, that the hexane extract had the highest protective activity (64.38 ± 7.72%), and that polyalthic acid isolated from this extract was the main active gastroprotector agent. Rats treated orally with polyalthic acid showed a gastroprotective effect similar to that elicited by carbenoxolone. As with carbenoxolone, the effect elicited by polyalthic acid was attenuated by pretreatment with either N^G-nitro-L-arginine methyl ester (70 mg kg^{-1}, i.p.), a nitric oxide (NO) synthase inhibitor, or N-ethylmaleimide (10 mg kg^{-1}, s.c.), a blocker of sulfhydryl groups. This suggested that the gastroprotective mechanism of this diterpenoid involved the participation of both NO and endogenous sulfhydryl groups. Contrary to carbenoxolone, the gastroprotective effect of polyalthic acid was not affected by the inhibition of prostaglandin synthesis with indometacin (10 mg kg^{-1}, s. c.). In conclusion, *Croton reflexifolius* contains compounds with gastroprotector activity. Polyalthic acid, which was isolated from this plant, was the main compound with gastroprotector activity, having effectiveness similar to that found with the use of carbenoxolone. Whereas NO and sulfhydryl groups were involved in the mechanisms of gastroprotective action of polyalthic acid, prostaglandins were not.

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Funding and acknowledgements: This work was supported by a grant from the Escuela Superior de Medicina del Instituto Politécnico Nacional (México) (Proyecto 20070451). We thank Bruce Allan Larsen for his advice.

Introduction

It is generally accepted that gastric ulcers are caused by a lack of balance between gastric aggressive factors and mucosal defensive mechanisms. The gastric mucosa is continuously exposed to potentially injurious agents such as gastric acid, pepsin, bile acid, food ingredients, bacterial products, and drugs. These agents increase gastric acid and pepsin secretion, decrease the gastric blood flow, suppress the endogenous generation of prostaglandins, inhibit mucosal growth and cell proliferation, and modify the gastric motility (Toma et al 2002). On the other hand, defensive mechanisms of the gastric mucosa consist mainly of functional, humoral and neural factors. Mucus–alkaline secretions, microcirculation and motility act as functional factors, prostaglandins (PGs) and nitric oxide (NO) as humoral factors, and capsaicin-sensitive sensory neurons (CPSN) as neuronal factors (Calatayud et al 2001; Tsukimi & Okabe 2001). Although many drugs have been effectively employed in the treatment of gastroduodenal ulcer and peptic diseases, all of these compounds have shown major shortcomings, such as the therapeutic failures observed in certain cases, adverse effects or high cost (Toma et al 2002). In the search for new therapeutic options, traditional medicinal plants are one source of natural products, such as triterpenes, diterpenes and flavonoids, among others with gastroprotective activity (Borreli & Izzo 2000). The genus *Croton*, which comprises 700 species (Catalán et al 2003), is well known for its diterpenoid content, and many different types of diterpenes, such as phorbol esters, clerodane, labdane, kaurane, trachylobane etc. have been isolated from it (Block et al 2004). Some of

these substances have anti-hypertensive (Baccelli et al 2005), antifeedant (Nihei et al 2006) or anti-ulcerogenic (Rodriguez et al 2004) activity.

Croton reflexifolius H.B.K. (Euphorbiaceae) is a Huastecan medicinal plant called 'huilocuahuitl', and its leaves are commonly used as an infusion to treat cough, diabetes and gastric ulcers (Estrada 1985). Although this plant is commonly used by the healers and shamans of the Huasteca (Hidalgo State, Mexico) to cure gastric ulcers, there is no scientific report either validating or invalidating this therapeutic practice. Therefore, we decided to test the gastroprotector activity of *C. reflexifolius* and, upon validating such protective action, proceeded to identify the active compound or compounds. A bioassay-guided fractionation was performed and the compounds obtained were tested using the absolute ethanol-induced gastric ulcer experimental model in Wistar rats. The role of endogenous NO, sulfhydryl groups and prostaglandins in the gastroprotective effect was evaluated to provide information about the mechanism of action of these compounds. The results were compared with the effect of carbenoxolone.

Materials and Methods

General procedures

The ^{1}H and ^{13}C NMR spectra were recorded in a $CDCl_3$ solution on a Bruker AVANCE-DMX 500 spectrometer, working at 500 and 125 MHz, respectively.

Plant material

Leaves of *C. reflexifolius* H.B.K were collected at Tehuetlan, in the state of Hidalgo, Mexico, during May of 2007. A specimen of the original collection can be found in the Herbarium of the Division de Ciencias Forestales, at the Universidad Autónoma Chapingo, with the voucher number CHAP60955.

Extraction and preliminary fraction

The leaves of *C. reflexifolius* were dried at room temperature (22 ± 2°C) in the shade. After grinding 5.2 kg leaves, compounds were successively extracted from them by maceration at room temperature (22°C ± 2) for three days, first with hexane (32 L × 3), then dichloromethane (32 L × 3) and finally methanol (32 L × 3). Evaporation of the solvents in vacuum gave 142, 128 and 401 g of syrupy residues, respectively. The hexane extract obtained from the leaves of *C. reflexifolius* showed the most active gastroprotective effect (Table 1). Thus 40 g of this extract was subjected to percolation over a silica gel column (0.063–0.200 mm, 250 g) by using a step gradient of hexane (1.7 L, fraction 1 (F1)), hexane/EtOAc (9:1, 1.7 L, F2), hexane/EtOAc (7:3, 1.7 L, F3), hexane/EtOAc (1:1, 1.7 L, F4), EtOAc (1.7 L, F5) and MeOH (1.7 L, F6). Fraction 3 (F3), which was the most active, was chromatographed on a silica gel column (300 g). Elution with hexane, hexane/EtOAc mixtures, EtOAc and MeOH afforded 50 fractions of 20 mL each. Fractions 17–40 (hexane/EtOAc, 95:5) yielded a white solid (1.0 g, mp 97–100°C) identified as polyalthic acid (Figure 1); its LC-MS spectrum showed a purity of 91% (Carreras et al 1998), as well as yielding β-sitosterol and β-lupeol.

Table 1 Gastroprotective effect of the extracts of *Croton reflexifolius* on ethanol-induced ulceration in rats

Treatment	Dose ($mg\,kg^{-1}$)	n	Gastroprotection (%)
Hexane extract	1	8	22.57 ± 10.71*
	3	8	32.83 ± 7.02*
	10	8	49.50 ± 7.80*
	30	8	57.22 ± 7.01*
	100	8	64.38 ± 7.72*
Dichloromethane extract	10	8	44.24 ± 9.90*
	30	8	40.49 ± 6.83*
	100	8	53.83 ± 5.15*
Methanol extract	10	7	4.82 ± 9.67
	30	7	23.64 ± 8.81*
	100	7	35.74 ± 7.75*

*$P < 0.05$ compared with control group. The ulcer index in the control group for this evaluation was 63.48 ± 8.64.

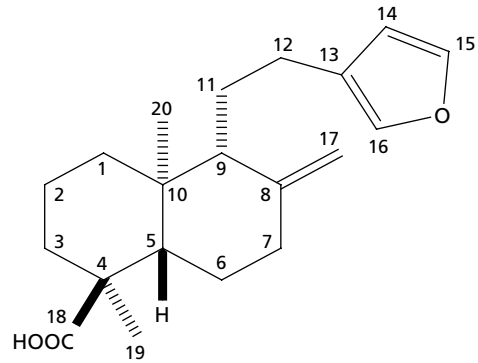

Figure 1 The structure of polyalthic acid.

Phytochemical data

Polyalthic acid, ^{1}H NMR ($CDCl_3$): $\delta = 7.35$ (1H, t, $J = 1.7$ Hz, H-15), 7.19 (1H, s, H-16), 6.26 (1H, s, H-14), 4.88 (1H, s, H-17), 4.59 (1H, s, H-17'), 1.15 (3H, s, H-19), 0.72 (3H, s, H-20), ^{13}C NMR ($CDCl_3$): $\delta = 185.2$ (C-18), 147.7 (C-8), 142.7 (C-15), 138.6 (C-16), 125.4 (C-13), 110.9 (C-14), 107.0 (C-17), 56.0 (C-9), 49.4 (C-5), 47.5 (C-4), 38.8 (C-10), 37.9 (C-1), 37.7 (C-7), 37.0 (C-3), 26.8 (C-6), 23.9 (C-12), 23.4 (C-11), 18.4 (C-2), 16.2 (C-19), 14.7 (C-20). These data corresponded with those in the literature (Carreras et al 1998).

Animals

All the experiments were performed with male Wistar rats (180–220 g; from the animal house of the Universidad Autónoma Chapingo). Procedures involving animals and their care were conducted in conformity with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and in compliance with international rules on care and use of laboratory animals. Unless otherwise

specified, the rats were placed in single cages with wire-net floors and deprived of food 24 h before experimentation, but were allowed free access to tap water throughout. All experiments were carried out using 7–10 animals per group.

Drugs and dosage

Carbenoxolone (Sigma-Aldrich Co.) was used as the gastroprotective reference drug. The drugs were prepared freshly each time, suspended in 0.5% Tween 80 and administered by the intragastric route. Control rats received the vehicle (0.5% Tween 80) in the same volume (0.5 mL/100 g) and by the same route. N^G-nitro-L-arginine methyl ester (L-NAME), N-ethylmaleimide (NEM) and indometacin were purchased from Sigma Chemical Co. (USA).

Acute gastric ulcer induced by absolute ethanol

Gastric ulcer was induced by administering absolute ethanol orally (1 mL) as described by Robert (1979). The extracts or drugs were administered to different groups 30 min before ethanol administration. Two hours after ethanol administration, the animals were killed in a CO_2 chamber. The stomach and duodenum were dissected out, inflated with 10 mL formalin, and then placed in 2% formalin for 5 min to fix both the inner and outer layers. The duodenum was opened along its anti-mesenteric side and the stomach along the greater curvature. The damaged area (mm^2) was measured under a dissection microscope (×10) with an ocular micrometer. The sum of the area of all the lesions in the corpus of each animal was calculated and served as the ulcer index. Gastroprotection (%) was calculated according to: % gastroprotection = (UIC–UIT) × 100/UIC, where UIC is the ulcer index in control and UIT is the test animal's index (Matsuda et al 1999; Arrieta et al 2003).

Ethanol-induced gastric mucosal lesions in L-NAME pretreated rats

To investigate the involvement of endogenous NO in the gastroprotective effects of the compounds, L-NAME (70 $mg\,kg^{-1}$ dissolved in saline solution) was intraperitonally injected in the three experimental groups 30 min before the administration of either the vehicle, polyalthic acid (30 $mg\,kg^{-1}$) or carbenoxolone (30 $mg\,kg^{-1}$) by the oral route (Matsuda et al 1999). Absolute ethanol was given to each rat in these groups 30 min later, and the animals were killed 2 h after the administration of ethanol to measure the ulcer index. Two control groups (L-NAME-treated and non-L-NAME-treated) were included in this evaluation.

Ethanol-induced gastric mucosal lesions in indometacin-pretreated rats

To investigate the involvement of endogenous prostaglandins in the gastroprotective effect of the compounds, a control group received a subcutaneous injection of $NaHCO_3$ 5 mM in saline solution and another group an injection of indometacin (10 $mg\,kg^{-1}$, dissolved in $NaHCO_3$ 5 mM) by the same route (Wan & Gottfried 1985). After 75 min, the animals in each of these two groups received one of three oral treatments (saline solution, 30 $mg\,kg^{-1}$ polyalthic acid or 30 $mg\,kg^{-1}$ carbenoxolone). Absolute ethanol was given to each rat 30 min after polyalthic acid or carbenoxolone administration and the rats were killed 2 h later in a CO_2 chamber. The stomachs were subsequently removed to measure the ulcer index, as mentioned previously.

Ethanol-induced gastric mucosal lesions in NEM pretreated rats

To investigate the involvement of endogenous sulfhydryls in the protective effects of polyalthic acid and carbenoxolone, NEM (10 $mg\,kg^{-1}$, dissolved in saline solution) was subcutaneously injected in the three experimental groups of animals 30 min before the oral administration of either the vehicle, polyalthic acid (30 $mg\,kg^{-1}$) or carbenoxolone (30 $mg\,kg^{-1}$) (Matsuda et al 1999). Absolute ethanol was given to each rat 30 min later and rats were killed 2 h after the administration of ethanol to measure the intensity of the gastric ulcer. Two control groups (NEM-treated and non-NEM-treated) were included in this experiment.

Statistics

Data were presented as the mean ± s.e.m. from seven to ten rats per group. Statistically significant differences between the treatments were tested by the Kruskal–Wallis test followed by Dunn's multiple comparison test. Probability (P) values less than 0.05 were considered significant.

Results

Gastroprotective effect

The gastroprotective activity of different extracts of *C. reflexifolius* on ethanol-induced ulcerations in rats are given in Table 1. The hexane extract elicited a dose-dependent gastroprotective effect (maximum 64.38 ± 7.72% gastroprotective effect with 100 $mg\,kg^{-1}$) and was more active than the dichloromethane or methanol extracts. Of the six fractions (F1–F6) obtained from the silica gel percolation of the hexane extract, F3 was found to be the most active (maximal 83.28 ± 4.52% gastroprotective effect; see Table 2). A pure

Table 2 Gastroprotective effect of the fractions of hexane extract (F1–F6) on ethanol-induced ulceration in rats

Treatment	Dose ($mg\,kg^{-1}$)	n	Gastroprotection (%)
F1	100	10	52.12 ± 7.54*
F2	100	10	65.59 ± 8.70*
F3	100	10	83.28 ± 4.52*
F4	100	10	52.73 ± 9.69*
F5	100	10	56.30 ± 8.61*
F6	100	10	52.96 ± 8.96*

*$P < 0.05$ compared with control group. The ulcer index in the control group for this evaluation was 76.10 ± 8.61.

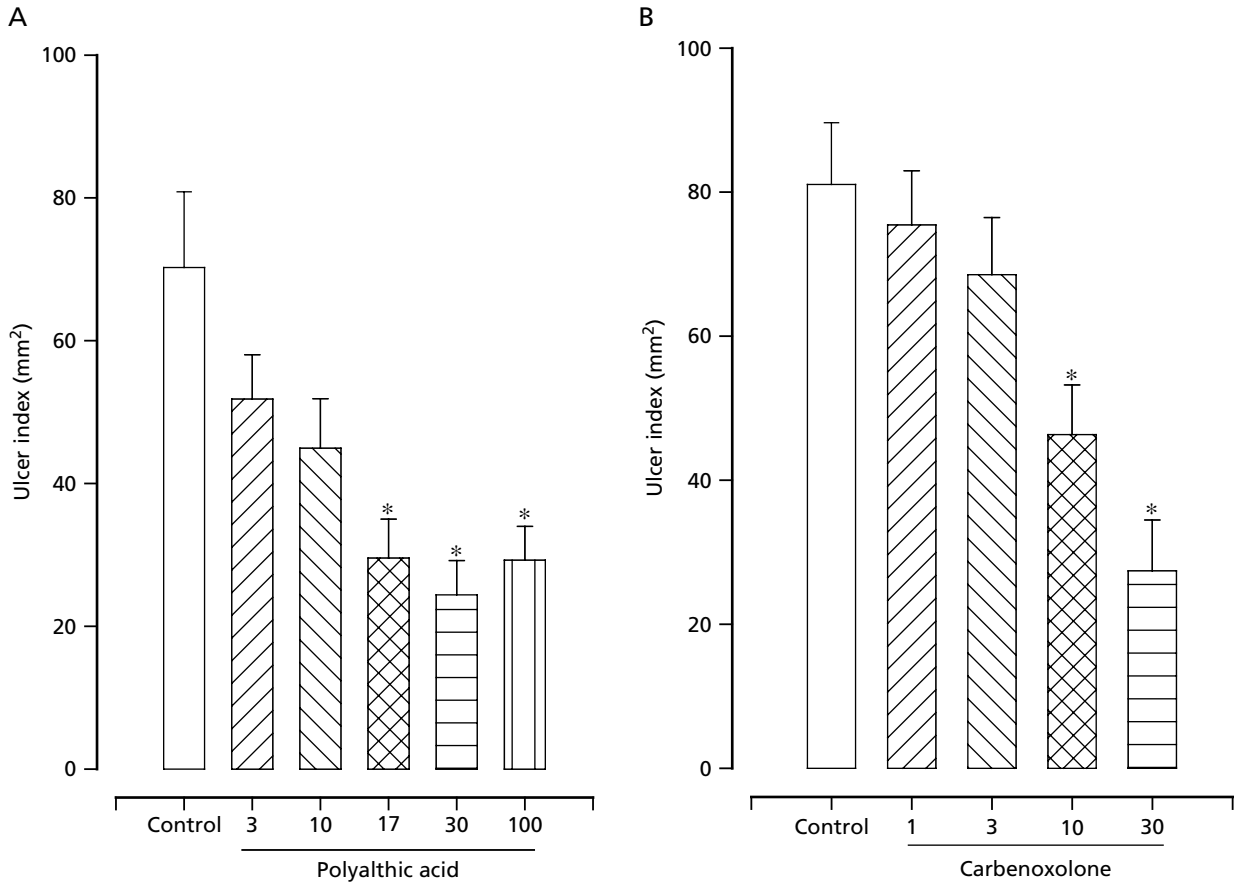

Figure 2 Effect of different doses of (A) polyalthic acid (3–10 mg kg^{-1}) and (B) carbenoxolone (1–30 mg kg^{-1}) on gastric lesions induced in rats by absolute ethanol. Bars represent the mean ± s.e.m., n = 7–10. *$P < 0.05$ compared with the respective control; Dunn's multiple comparison test after Kruskal–Wallis test.

compound identified as polyalthic acid, which showed a dose-dependent gastroprotective effect, was obtained from F3. The maximal gastroprotective effect, obtained with 30 mg kg^{-1} polyalthic acid, was 65.26 ± 7.71% (Figure 2A). It is worth mentioning that in addition to polyalthic acid, β-sitosterol (50 mg) and β-lupeol (60.52 mg) were isolated from the F3 fraction. They were not considered in this work because of the scarce quantity obtained and the fact that their gastroprotective effect is already very well known (Lewis & Hanson 1991; Arrieta et al 2003).

As previously mentioned, for comparative reasons the effect of carbenoxolone was studied. The results showed that this compound elicited a dose-dependent inhibition of gastric ulcers (Figure 2B) that represented no statistically significant difference in relation to that inhibition produced by polyalthic acid. The maximal gastroprotective effect induced by carbenoxolone was 66.7871 ± 7.8152% at 30 mg kg^{-1}.

Effect of indometacin, L-NAME and NEM on the gastroprotective effect

The ulcer index of the rats treated with 70 mg kg^{-1} L-NAME (72.11 ± 9.11 mm^2, Figure 3A), 10 mg kg^{-1} indometacin (64.90 ± 6.72 mm^2, Figure 3B), or 10 mg kg^{-1} NEM (73.62 ± 9.94 mm^2, Figure 3C) was not significantly different ($P < 0.05$) compared with controls treated with saline solution only (79.11 ± 9.88, 71.71 ± 9.94 and 67.50 ± 9.53 mm^2, respectively). Previously, it was reported that these doses of inhibitors were high enough to block prostaglandin synthesis, NO synthase and endogenous sulfhydryls, respectively, without producing ulcers or aggravating those previously present (Arrieta et al 2003).

Pretreatment with L-NAME (70 mg kg^{-1}, s.c.) attenuated the gastroprotective effect of both polyalthic acid (30 mg kg^{-1}) and carbenoxolone (30 mg kg^{-1}) (Figure 3A). The ulcer index obtained in the rats treated with polyalthic acid (76.7727 ± 10.6027 mm^2) or carbenoxolone (53.7188 ± 7.8411 mm^2) was not significantly different ($P < 0.05$) from the L-NAME-pretreated controls (72.6563 ± 9.1146 mm^2).

Polyalthic acid (30 mg kg^{-1}) produced inhibition of an ethanol-induced gastric ulcer in indometacin-pretreated animals (10 mg kg^{-1}). The maximum ulcer index obtained for polyalthic acid in this case was 37.17 ± 7.92 mm^2, which was significantly different ($P < 0.05$) from that of the indometacin-pretreated control (64.90 ± 6.72 mm^2). On the other hand, the value of the ulcer index obtained with 30 mg kg^{-1} carbenoxolone (74.65 ± 9.47 mm^2) was not significantly different ($P < 0.05$) from that of the control (Figure 3B).

Oral administration of polyalthic acid to NEM-pretreated (10 mg kg^{-1}) rats did not inhibit the ethanol-induced gastric

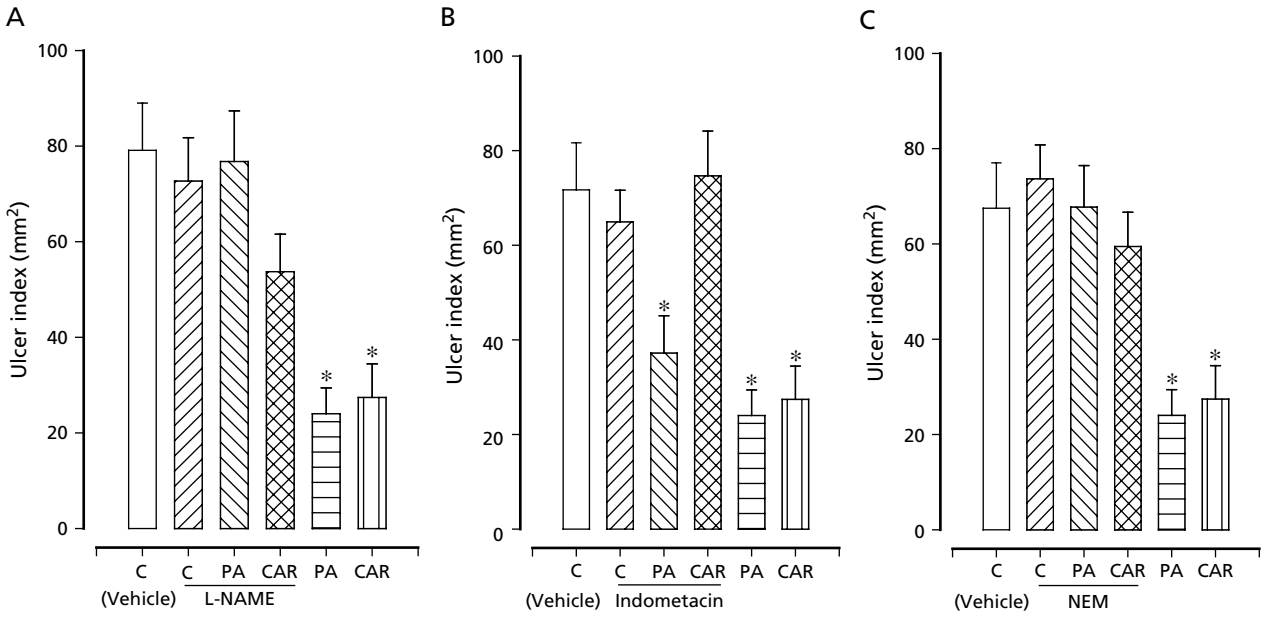

Figure 3 Effect of polyalthic acid (PA) and carbenoxolone (CAR) at 30 mg kg^{-1} on gastric lesions induced by ethanol in rats pretreated with (A) L-NAME (70 mg kg^{-1}), (B) indometacin (10 mg kg^{-1}) or (C) NEM (10 mg kg^{-1}). Bars represent the mean ± s.e.m., n = 7–10. *$P < 0.05$ compared with the respective control; Dunn's multiple comparison test after Kruskal–Wallis test.

lesions. In this case, the ulcer index obtained following the administration of polyalthic acid was 67.70 ± 8.75 mm^2, a value not significantly different ($P < 0.05$) from that of the NEM-pretreated control (Figure 3C). Carbenoxolone also showed no statistically significant difference in the inhibition of ethanol induced gastric lesions after pretreatment with NEM when compared with the control (Figure 3C).

Discussion

We have provided preliminary scientific support to the popular practice of administering *C. reflexifolius* in the treatment of gastric ulcers (Estrada 1985). It was found that extracts obtained from the leaves of this plant had gastroprotective activity in the experimental rat model of ethanol-induced gastric ulcers. The hexane extract was the most potent, with a maximal 64.38 ± 7.72% gastroprotective effect. The bioassay-guided fractionation showed that polyalthic acid isolated from F3 was one of the main active ingredients, with a maximal gastroprotective effect of 65.26 ± 7.71%. Interestingly, the maximal effect of polyalthic acid, a pure compound, was lower than that produced by F3 (83.28 ± 4.52%). This was probably due to the fact that relatively small amounts of two compounds, β-sitosterol and β-lupeol, both with well-known gastroprotector activity (Lewis & Hanson 1991; Arrieta et al 2003), were also isolated from F3.

Polyalthic acid is a diterpenoid and the genus *Croton* is well known for its diterpenoid content (Block et al 2004). Other plants containing diterpenoids have also demonstrated their anti-ulcer activity in several experimental ulcer models (Melo et al 2003; Zhang et al 2006). Therefore, the anti-ulcer effect of polyalthic acid presented in this study was another property of this substance, apart from the previously published biological effects as a repellent against the monodonta sea snail (Ohta & Nawamaki 1978) and an antimutagenic agent (Miyazawa et al 1995).

In an attempt to provide information about the mechanism of the gastroprotective action of polyalthic acid, the participation of NO was evaluated by pretreating the rats with L-NAME (a nitric oxide synthase inhibitor). It is well known that NO is involved in the modulation of gastric mucosal integrity and is important in the regulation of acid and alkaline secretion, mucus secretion and gastric mucosal blood flow (Calatayud et al 2001). The results of this study showed that in the presence of L-NAME the gastroprotective effect of polyalthic acid was inhibited (76.77 ± 10.60 mm^2), as was the protective effect of carbenoxolone (Figure 3A). Thus NO was involved in the gastroprotection elicited by polyalthic acid and carbenoxolone, the latter being an anti-ulcer drug derived from a natural source used as a comparator drug in the study of the mechanism of action of the former.

Further evidence was provided about the mechanism of action of polyalthic acid by investigating the role of endogenous sulfhydryl compounds. It has been demonstrated that the development of ethanol-induced gastric damage is accompanied by a decrease in mucosal sulfhydryl compounds. These compounds are neutralized when they bind to the free radicals that are produced following tissue injury by noxious agents. In addition, sulfhydryl compounds cause mucus subunits to be joined by disulfide bridges with the reduction of free radicals, the latter of which are eliminated since the mucus is rendered water-soluble (Ávila et al 1996; Maity et al 1998). Thus we pretreated animals with NEM, a blocker of sulfhydryl compounds (Figure 3C), to investigate the possible involvement of these compounds in the gastroprotective

effect of polyalthic acid. The results indicated that pretreatment with NEM inhibited the gastroprotective effect of polyalthic acid ($67.70 \pm 8.75\,mm^2$) and carbenoxolone ($59.45 \pm 7.18\,mm^2$). This indicated that the endogenous sulfhydryls may have been involved in the gastroprotection of the diterpenoid and the reference compound.

Prostaglandins, especially PGE_2 and PGI_2, which are responsible for mucus production and the maintenance of cellular integrity in the gastric mucosa (Rujjanawate et al 2005), are also involved in the protection of the gastric mucus. The results obtained in this study showed that prostaglandins did not participate in the mechanism of gastroprotection of polyalthic acid, as the effect of this compound was not changed by pretreatment with indometacin, a cyclooxygenase inhibitor (Wan & Gottfried 1985). In contrast, the gastroprotective action elicited by carbenoxolone was inhibited by pretreatment with indometacin (Arrieta et al 2003).

Conclusion

Scientific evidence for the effectiveness of *C. reflexifolius* in the treatment of gastric ulcer have been provided with this study. Polyalthic acid was identified as the main active gastroprotective agent in this traditional medicinal plant. The mechanism of the gastroprotective action of polyalthic acid was related to endogenous NO and sulfhydryl groups, but not to prostaglandins.

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