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Mechanisms of action underlying the gastric antiulcer activity of the *Rhizophora mangle* L.

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ABSTRACT

Ethnopharmacological relevance: Rhizophora mangle, the red mangrove, has long been known as a traditional antiulcer medicine. The present work evaluated the mechanisms of action involved in the anti-ulcer properties of the *Rhizophora mangle* bark extracts.
Materials and methods: Gastroprotection of *Rhizophora mangle* was evaluated in rodent experimental models (ethanol). To elucidate the mechanisms of action the antisecretory action and involvement of NO, SH, mucus and PGE_2 were evaluated. The acetic acid-induced gastric ulcer model, Western blotting assay (COX-1, COX-2 and EGF) and immunohistochemical localization of HSP-70, PCNA and COX-2 were also used to evaluate the *Rhizophora mangle* healing properties.
Results: Results showed that *Rhizophora mangle* bark crude extract (CE), as well as ethyl acetate (EtOAc) and butanolic fractions (BuOH) provided significant gastroprotection at all the tested doses. Thereby, the following protocols were performed using the lowest dose capable of producing the most effective gastroprotection, which was the BuOH 0.5 mg/kg ($P < 0.001$). Several mechanisms are involved in the antiulcer activity of *Rhizophora mangle*, such as, participation of NO, SH and mucus. The enhancement of PGE_2 levels and the *upregulation* of COX-2 and EGF seem to be directly linked to the antisecretory, cytoprotective and healing effects of BuOH. HSP-70 and PCNA are also involved in this cicatrisation process. No sign of toxicity was observed in this study, considering the analyzed parameters.
Conclusion: Our study reinforces its traditional medicinal use. Considering that the current therapies are based on the use of antisecretory or cytoprotective drugs, the *Rhizophora mangle* arises as a promising alternative antiulcer therapy.

1. Introduction

The stomach is constantly exposed to a wide range of substances that have the capacity to cause epithelial damage, such as hydrochloric acid and digestive enzymes (Wallace, 2005). Under normal conditions, mucosal integrity is maintained by defense mechanisms, which include an epithelial "barrier", mucus secretion, bicarbonate, prostaglandins, nitric oxide, growth factors, heat-shock proteins and continuous blood flow. Mucosal injury may occur when noxious factors "overwhelm" an intact mucosal defense or when the mucosal defense is somehow impaired (Laine et al., 2008).

Proton pump inhibitors (i.e., lansoprazole) are the most potent inhibitors of gastric acid secretion available; because of its efficacy, current guidelines recommend their use for the treatment of acid-related gastric diseases (Sheen and Tridafilopoulus, 2011). Treatments of gastric disorders with medicinal plants are quite common in traditional medicine worldwide (Schmeda-Hirschman and Yesilada, 2005). The beneficial effects of herbal and plant extracts (isolated or in combination) in the prevention of gastric injury have been evaluated in several experimental studies; three main functions including antisecretory, cytoprotective and antioxidant activities, isolated or in combination, are responsible for gastric mucosal protection (Al Mofleh, 2010).

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Rhizophora mangle, the red mangrove, has long been known as a traditional medicine, its bark has been used as astringent, antidysentery, anti-diarrheal, antihemorrhagic, antiseptic, hemostatic, with antifungic and antiulcerogenic properties (Roig, 1988; Pio-Corrêa, 1984). In recent years, various authors have described some activities such as antioxidant (Sánchez et al., 2006; de-Faria et al., 2009), antiulcer (Perera et al., 2001; Berenger et al., 2006; de-Faria et al., 2009) and wound healing (Fernández et al., 2002; de Armas et al., 2005). Based on these data, we focused the present work on the mechanisms involved in the anti-ulcer properties of the *Rhizophora mangle* bark extracts.

2. Materials and methods

2.1. Drugs and chemicals

The following drugs were used: lansoprazole (Medley, Campinas, Brazil), acetic acid (Sinth, SP, Brazil), absolute ethanol (©Merk KGaA, Darmstadt, Germany); cimetidine, carbenoxolone, indomethacin, L-NAME (N-G-nitro-L-arginine), NEM (N-ethylmaleimide), Alcian Blue and NaCl were from Sigma Chemical Co. (St. Louis, USA). The chemicals used in the buffers and other solutions were all of analytical grade. All drugs and reagents were prepared immediately before use.

2.2. Animals

Male Wistar rats Unib: WH (150–250 g) and male Swiss mice Unib: SW (25–35 g), both obtained from the breeding facility of the State University of Campinas (CEMIB/UNICAMP), were used. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark–12 h light, humidity (60 ± 1.0%) and temperature (21 ± 1%). Moreover, the animals were kept in cages with raised floors of wide mesh to prevent coprophagy. The UNICAMP Institutional Animal Care and Use Committee, following the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993), approved all of the employed protocols.

2.3. Plant material and preparation of the extracts

The barks of *Rhizophora mangle* L. (Rhizophoraceae) were collected in "Estuário de Santos", Santos, SP, Brazil. Professor M.Sc. Paulo Salles Penteado Sampaio authenticated the botanical identity of the plants and a voucher specimen (HUSC – P.S.P. Sampaio et al., 800) was deposited at the "Herbário da Universidade Santa Cecília-HUSC". The bark of *Rhizophora mangle* was dried for seven days at 40 °C (213 g) and powdered (3 μm). The acetone: water (7:3) extract was prepared by maceration and obtained a yield of 31.4% (66.9 g). The extract from *Rhizophora mangle* bark (CE) was submitted to liquid–liquid partition with solvents of increasing polarity, thereby semi-purified fractions. This methodology provides a proper clean up of the polar extracts (Yunes and Calixto, 2001). Approximately 20 g of the extract was partitioned between 150 mL of water and 50 mL of ethyl acetate in a separation funnel for 3 times. Then, the aqueous phase was also partitioned with 50 mL of *n*-butanol (for 3 times). All fractions were concentrated under vacuum to obtain the dried fractions: aqueous fraction (Aq; 6.34 g, 31.7%), ethyl acetate fraction (EtOAc; 4.82 g, 24.10%) and butanolic fraction (BuOH; 7.73 g, 38.65%).

2.4. Phytochemical

Chromatographic analyses of CE (10 μL), Aq (10 μL), EtOAc (10 μL) and BuOH (10 μL) were performed by TLC (Fluka Si-gel plateson glass, 20 cm × 20 cm × 0.25 mm) eluted with $CHCl_3$:MeOH:*n*-PrOH:H_2O (5:6:1:4, v/v/v/v) in a previous communication published by de-Faria et al. (2008). Flavonoids were detected by spraying the plates with natural products reagent and polyethylene glycol (NP/PEG). Phenolic compounds are detected after exposing the plates to ammonia vapors and immediate observation of fluorescent spots under UV light. Total phenols were detected with 5% ferric chloride solution in MeOH (Wagner et al., 1984).

2.5. Acute toxicity

The acute toxicity studies were performed on male and female Swiss mice ($n = 10$). A single dose of the extract was administered orally to groups of animals after a 12-h fast. Animals receiving saline 0.9% served as control. The signs and symptoms associated with the CE administration (5 g/kg, p.o.) were observed at 0, 30, 60, 120, 180 and 240 min after and then once a day for the next 14 days. At the end of the period, the number of survivors was recorded and the acute toxicological effect was estimated through the method described by Souza-Brito (1994).

2.6. Gastroprotection activity

2.6.1. Ethanol-induced gastric ulcer

The rats were divided into six treatment groups, fasted 24 h prior to receiving an oral dose of the vehicle (saline 10 mL/kg), lansoprazole (30 mg/kg), CE (50, 100, 250 and 500 mg/kg), Aq (6.25, 12.50, 25 and 50 mg/kg) and EtOAc and BuOH (0.5, 1.5, 3.0, 6.25, 12.5, 25 and 50 mg/kg). After 60 min, all groups were orally treated with 1 mL of absolute ethanol for the gastric ulcer induction. One hour later, the animals were sacrificed and their stomachs excised and gastric contents aspirated. Each stomach was incised along the greater curvature and examined for linear hemorrhagic lesions in the glandular region (Morimoto et al., 1991). Then, the stomachs were photographed and the extent of the lesions was measured (mm^2) by the program AVSoft BioView (Khan, 2004).

2.6.2. Role of endogenous nitric oxide (NO) and sulfhydril compounds (SH)

Male rats were divided into 6 groups and pretreated (i.p.) with saline, L-NAME (N-G-nitro-L-arginine, 70 mg/kg) an inhibitor of the NO synthesis or NEM (N-ethylmaleimide, 10 mg/kg) a blocker of SH compounds. Thirty minutes after the pretreatment, the animals were administered (p.o.) vehicle, carbenoxolone (100 mg/kg) or BuOH (0.5 mg/kg). After 60 min, all the groups received absolute ethanol (10 mL/kg) to induce gastric ulcers. One hour after receiving ethanol the rats were killed for the determination of the gastric lesions (Arrieta et al., 2003).

2.6.3. Gastric acid secretion

The determination of gastric secretion was performed using the method of Shay et al. (1945), with a few modifications. The rats were divided into groups ($n = 7$). After 24 h of fasting, the animals were anesthetized, the abdomen was incised and the pylorus ligated. Immediately after the pylorus ligature, BuOH was administered at a dose of 0.5 mg/kg, lansoprazole (30 mg/kg) was used as positive control, and 1 mL of vehicle (saline solution 0.9%) was administered as negative control. All the samples were administered intraduodenally. Four hours later, the animals were sacrificed by cervical dislocation; the abdomen was opened, and another ligature placed around the esophagus close to the diaphragm. The stomachs were removed and the gastric content collected and drained into a graduated centrifuge tube and centrifuged at 2000 × *g* for 15 min. The

supernatant volume and pH were recorded with a digital pH meter (PA 200, Marconi S.A., Brazil).

2.6.4. Determination of the mucus adhering to gastric wall

Rats were divided into groups ($n = 7$). After animals fasted for 24 h, BuOH (0.5 mg/kg), carbenoxolone (200 mg/kg) or the vehicle was administered orally. Sixty minutes later, under anesthesia, the abdomen was incised and the pylorus ligated. The animals were killed 4 h after the surgery. The stomach content was immersed in 10 mL of 0.02% Alcian blue 0.16 M sucrose/0.05 M sodium acetate solution, pH 5.8, and incubated for 24 h at 20 °C. The Alcian blue binding extract was centrifuged at 2000 × *g* for 10 min. The absorbency of supernatant was measured by spectrophotometry at 598 nm. The free mucus in the gastric content was calculated from the amount of Alcian blue binding the gastric mucus [mg/glandular tissue (g)]. This assay was performed according to the methodology described by Corne et al. (1974).

2.6.5. Determination of the PGE_2 levels

Animals fasted for 24 h and divided randomly into the groups sham, saline + indomethacin and BuOH + indomethacin. First, animals were treated with saline or BuOH (p.o.), 30 min after, indomethacin (dissolved in 5% sodium bicarbonate solution) 30 mg/kg was administered, s.c. Thirty minutes after indomethacin administration, the rats were killed, the stomachs removed, weighed and then placed in 1 mL of sodium phosphate buffer (10 mM, pH 7.4). The tissue was finely minced and then incubated at 37 °C for 20 min. The prostaglandin E_2 (PGE_2) level was quantified with an immune-enzymatic dosage kit from R&D Systems (USA). The methodology was according to Curtis et al. (1995).

2.7. Healing properties

2.7.1. Acetic acid-induced gastric ulcer

Ulcer induction was based on Okabe and Amagase (2005). Male Wistar rats ($n = 15$) after fasting for 24 h, were used in this experiment. Under anesthesia, laparotomy was performed on all animals through a midline-epigastric incision. After exposing the stomach, 0.05 mL (v/v) of a 30% acetic acid solution was injected into the subserosal layer in the glandular part of the anterior wall. The stomach was bathed with saline to avoid adherence to the external surface of the ulcerated region. The abdomen was then closed and the animals were fed normally.

The rats were divided into groups: saline (10 mL/kg, negative control), lansoprazole (30 mg/kg, positive control) and BuOH (0.5 mg/kg). The animals received the treatments by gavage once a day for 14 consecutive days, beginning 2 days after surgery. During this period, body weight was recorded daily to evaluate possible chronic toxicity induced by BuOH. Five animals of each group were killed at days 4, 8 and 14 after the induction of the gastric lesions to evaluate de evolution of the cicatrisation, and then, the stomachs were removed. The gastric lesions were evaluated by examining the inner gastric surface with a dissecting magnifying glass. The stomachs were then photographed and the extent of the lesions was measured (mm^2) by the program AVSoft BioView (Khan, 2004). The lesion was half-sectioned and one portion was fixed in ALFAC solution (alcohol, acetic acid and formaldehyde) for 24 h at 4 °C; the other portion of the stomachs was stored at −80 °C for biochemical analysis. The samples were then routinely processed for embedding in paraplast, cut into 10 μm-thick sections and put onto histological slides.

2.7.1.1. Toxicity evaluation. The toxicological parameters were set according to the method of Souza-Brito (1994). We evaluated the toxicity in the animals submitted to BuOH treatment under the aforementioned cicatrisation model. For a period of 14 days, BuOH effects were observed daily (body weight progression, hair and mucosal alteration). The following organs were weighed to detect any effect of the extract on their individual weights: heart, lungs, liver, kidneys and spleen.

2.7.2. Expression of COX-1, COX-2 and EGF

Frozen glandular stomachs samples were homogenized in 1 mL of ice cold buffer (PB 0.1 M, pH 7.4 and protease inhibitor 1%). Homogenates were centrifuged (12,000 × *g*, 15 min, 4 °C) and the supernatants were collected and stored at −80 °C. Protein concentration of the homogenate was determined following Bradford's colorimetric method (1976). Then, samples were treated with Laemmili buffer (PB buffer 0.5 M, pH 6.8; glycerol, sodium dodecyl sulfate (SDS) 10%, bromophenol 0.1%, β-mercaptoethanol) in a 1:1 proportion. Equal amounts of protein from samples (100 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: EGF (Santa Cruz Biotechnology, Inc., USA), COX-1 and COX-2 (Cayman Chemical, USA) at dilution of 1:500. Each membrane was washed three times for 10 min and incubated with anti-goat immunoglobulin G antibody (Zymed Laboratories, USA) for EGF and with anti-rabbit (Zymed Laboratories, USA) for COX-1 and COX-2, all diluted at 1:5000. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma–Aldrich, MO, USA). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (SuperSignal® West Femto Chemiluminescent Substrate, Pierce, IL, USA). Densiometric data were performed following normalization to the control (housekeeping gene) by AVSoft program.

2.8. Histological analysis

The slides were observed after haematoxylin and eosin (HE) staining (Behmer et al., 1976). The slides also underwent Periodic Acid Schiff (PAS) staining (Vacca, 1985), by which we observed mucus production. Histological analyses were made using a Leica microscope associated with Leica Q-Win Software 3.1 (Leica-England), from the image analysis laboratory of the Department of Morphology, UNESP-Botucatu.

2.8.1. Immunohistochemical localization of PCNA, HSP-70 and COX-2

Representative slides of gastric tissue were deparaffinized, rehydrated and immunostained by the peroxidase anti-peroxidase method. High temperature antigen unmasking technique was employed in 0.01 M citrate buffer pH 6.0 in a microwave oven, twice for 5 min each, except for HSP-70. Blocking of nonspecific reaction was performed with 3% non-fat milk, and sections were incubated with primary antibodies for PCNA mouse monoclonal antibody (Novo Castra NCL-PCNA) (1:100), HSP-70 (Santa Cruz Biotechnology SC-1060) (1:100), COX-2 (Cayman Chemical) (1:200). After rinsing in phosphate buffered saline (0.01 mol/l PBS, pH 7.4), the sections were incubated in secondary antiserum. They were then washed in PBS and incubated in ABC (avidine and biotine complex-Easy Path) and incubated in peroxidase reaction (3,3-diaminobenzidine tetrahydrochloride) (Sigma) containing 0.01% H_2O_2 in PBS buffer.

2.9. Statistical analysis

Results were expressed as mean ± standard error of means (S.E.M.). The statistical significance of each test group in relation

Table 1
Effect of CE, Aq, EtOAc and BuOH under the model of gastric ulcer induced by absolute ethanol. Ulceration lesion area (ULA) is presented as mean ± S.E.M. ANOVA followed by Dunnet's *t* test.

Treatments (p.o.)	Dose (mg/kg)	*n*	ULA (mm^2)	Inhibition (%)
Saline	–	7	225.30 ± 35.00	–
Lansoprazole	30	7	51.13 ± 13.77***	77.3
CE	500	7	0.00 ± 0.00***	100
	250	7	24.23 ± 12.51***	89.2
	100	7	3.62 ± 1.95***	98.4
	50	7	69.36 ± 16.63***	69.1
Saline	–	7	133.50 ± 19.04	–
Lansoprazole	30	7	35.31 ± 9.31**	73.5
Aq	50	7	104.50 ± 13.31	21.7
	25	7	88.40 ± 27.11	33.7
	12.5	7	23.94 ± 2.80***	82.0
	6.25	7	122.60 ± 26.12	8.1
Saline	–	7	95.93 ± 9.72	–
Lansoprazole	30	7	16.43 ± 3.22***	82.8
EtOAc	50	7	29.67 ± 8.07***	69.0
	25	7	33.27 ± 12.84***	65.3
	12.5	7	59.42 ± 3.60*	38.0
	6.25	7	40.07 ± 8.30***	58.2
	3.0	7	37.30 ± 8.64***	61.1
	1.5	7	39.39 ± 4.93***	58.9
	0.5	7	58.67 ± 9.12*	38.8
BuOH	50	7	17.83 ± 4.68***	81.4
	25	7	28.03 ± 8.90***	70.7
	12.5	7	25.22 ± 5.56***	73.7
	6.25	7	49.93 ± 10.50**	47.9
	3.0	7	57.39 ± 16.12*	40.1
	1.5	7	52.26 ± 16.09*	45.5
	0.5	7	18.30 ± 7.10***	80.9

* $P<0.05$ significantly different from saline group.
** $P<0.01$ significantly different from saline group.
*** $P<0.001$ significantly different from saline group.

to the control was calculated using ANOVA followed by Dunnet or Tukey's *t* test.

3. Results

3.1. Toxicity evaluation

CE was first investigated for acute toxicity in mice. A single oral dose of CE (5 g/kg) did not produce any visible signs or symptoms of toxicity in the treated animals. Since no acute toxicity was found using CE, we continued our studies evaluating the mechanisms underlying the anti-ulcer properties of *Rhizophora mangle*. After 14 days of administration of BuOH (0.5 mg/kg) in the acetic acid-induced gastric ulcer model, no animal died and no significant macroscopic changes in daily body (Fig. 2A) or organ weights were observed (data not shown).

3.2. Ethanol-induced gastric ulcer

In ethanol-induced gastric ulcer were tested all extracts obtained from the bark of *Rhizophora mangle*, CE (50; 100; 250 and 500 mg/kg), Aq (6.25; 12.5; 25 and 50 mg/kg) EtOAc (0.5; 1.5; 3.0; 6.25; 12.5; 25 and 50 mg/kg) and BuOH (0.5; 1.5; 3.0; 6.25; 12.5; 25 and 50 mg/kg). This experimental model provides the screening of doses and efficacy of the treatments against ulcer formation. Results showed that CE as well as EtOAc and BuOH provided significantly gastroprotection in all tested doses ($P<0.05$; 0.01 and 0.001), while Aq only produce protection at dose of 12.5 mg/kg

Table 2
Effect of oral BuOH treatment, under ethanol induced gastric ulcer, on animals pretreated with L-NAME or NEM. Data are presented as mean ± S.E.M. ANOVA followed by Tukey's *t* test.

Pretreatments (i.p.)	Treatments (p.o.)	Dose (mg/kg)	*n*	ULA (mm^2)	Inhibition (%)
Saline	Vehicle	–	7	176.60 ± 8.94	–
	Lansoprazole	30	7	41.55 ± 10.91***	76.4
	BuOH	0.5	7	56.01 ± 14.37***	68.3
L-NAME	Vehicle	–	7	258.80 ± 22.83*	–
	Lansoprazole	30	7	61.18 ± 15.74***	76.3
	BuOH	0.5	7	168.30 ± 23.31	35.0
Saline	Vehicle	–	7	195.80 ± 24.37	–
	Lansoprazole	30	7	49.60 ± 11.64***	74.6
	BuOH	0.5	7	71.15 ± 8.69***	63.6
NEM	Vehicle	–	7	413.60 ± 29.47***	–
	Lansoprazole	30	7	201.00 ± 19.01	51.4
	BuOH	0.5	7	251.50 ± 14.27	39.2

* $P<0.05$ significantly different from saline group.
*** $P<0.001$ significantly different from saline group.

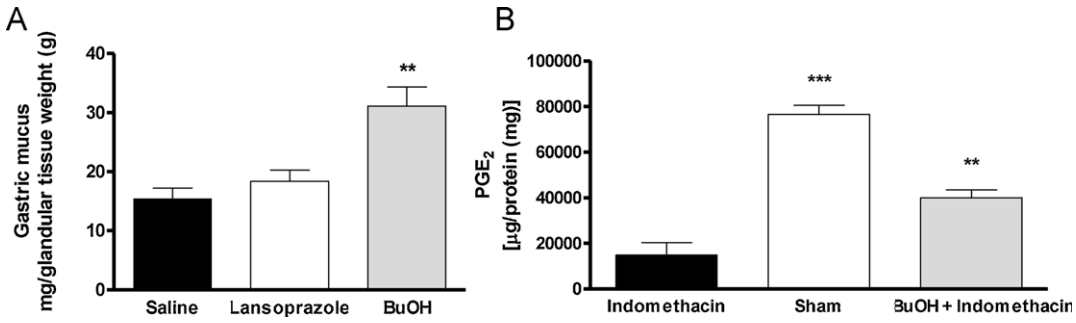

Fig. 1. Quantification of adherent mucus (A) and PGE_2 (B) in gastric mucosa of rats treated with BuOH from *Rhizophora mangle*. Results are presented as mean ± S.E.M. ANOVA followed by Dunnet's *t* test, $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from negative control groups saline (A) or indomethacin (B).

($P < 0.001$) (Table 1). Thereby we focused the following protocols using the minor dose capable to produce the more effective gastroprotection, which was the BuOH 0.5 mg/kg ($P < 0.001$), furthermore, glycosilated heterosides are the main constituents of BuOH, what contributes to an improved absorption, hence improving the biological activity.

3.3. Role of endogenous nitric oxide (NO) and sulfhydril compounds (SH)

As demonstrated by Table 2, the pretreatment with L-NAME or NEM led to the lost of the gastroprotection carried out by the BuOH treatment, suggesting that NO and SH are required by the treatment to provide its gastroprotection.

3.4. Gastric acid secretion

The intraduodenal (i.d.) administration of BuOH significantly reduced gastric juice volume ($P < 0.05$) and acidity ($P < 0.05$) while lansoprazole reduced the acidity ($P < 0.05$).

3.5. Determination of the mucus adhering to gastric wall and PGE_2 levels

The treatment with BuOH augmented in more than 100% the amount of mucus adhering to the gastric mucosa compared to control group (Fig. 1A), moreover, BuOH, concomitantly administered with indomethacin (a cyclooxygenase inhibitor) could enhance the levels of PGE_2 compared with control group which received indomethacin only (Fig. 1B).

3.6. Acetic acid-induced gastric ulcer

Chronic gastric ulcers were developed after 1 day of serosal application. The gastric ulcer showed progressive healing in control (saline), lansoprazole (30 mg/kg) and BuOH (0.5 mg/kg) treated groups of animals where ulcer area started reducing in size on day 4, a gradual decrease on day 8 and final healing on day 14 in each of the group. On day 4, lansoprazole ($P < 0.05$) and BuOH ($P < 0.001$) already showed significantly different from control group, BuOH ($P < 0.01$) also showed difference from lansoprazole group. After 8 days of treatment, the healing remained delayed in control group while the treatment with BuOH ($P < 0.001$) or lansoprazole ($P < 0.001$) improved the cicatrisation; again, the difference of lansoprazole versus BuOH was significantly ($P < 0.001$). Fourteen days after induction of lesions, both lansoprazole and BuOH were significantly different ($P < 0.001$) compared to control group (saline), while statistical difference between lansoprazole and BuOH was $P < 0.01$ (Fig. 2B).

3.7. Expression of COX-1, COX-2 and EGF

Western blotting analysis reveals that both COX-1 and COX-2 expression were found at the same values ($P > 0.05$) in all groups on 4th and 8th days of treatment. On 14th day, COX-1 remains unchanged. However, an *upregulation* of COX-2 and EGF expression in lansoprazole ($P < 0.05$) and BuOH ($P < 0.01$) groups were found when compared to control (saline).

3.8. Histological analysis

Histological analysis was performed by the HE and PAS staining. HE slices showed the architecture at the margin of the ulcer, even lansoprazole (30 mg/kg) as well as BuOH (0.5 mg/kg) treatments

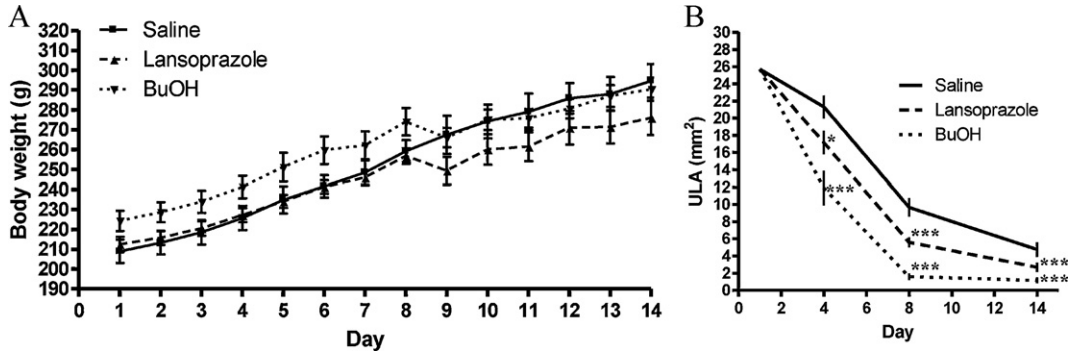

Fig. 2. Body weight in rats treated orally with vehicle (saline), lansoprazole and BuOH for 14 days after ulcer formation by acetic acid model (A). Evolution of the ulcer cicatrisation on days 4, 8 and 14 of treatment (B). Results are presented as mean ± S.E.M. ANOVA followed by Tuckey's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ significantly different from saline.

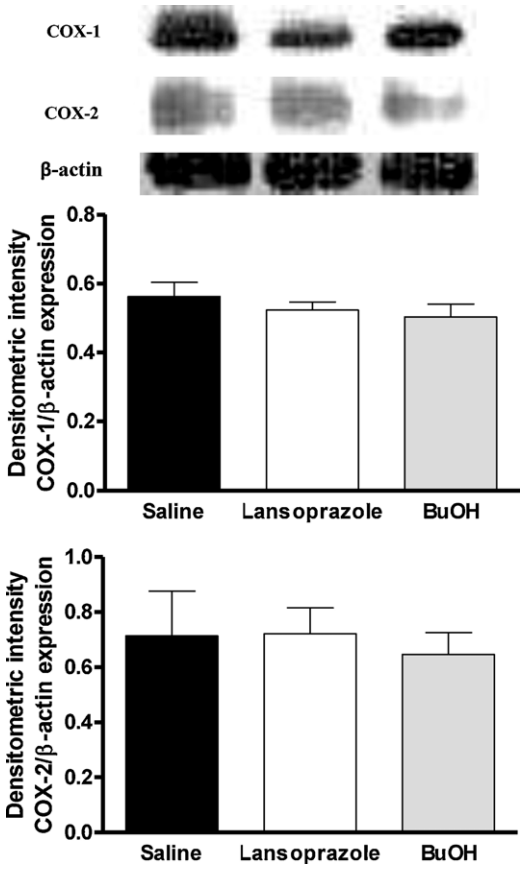

Fig. 3. Effects of oral administration during 4 days of BuOH on COX-1 and COX-2 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Densitometry was made following normalization with the control (housekeeping gene). Data are expressed as mean ± S.E.M. ANOVA followed by Dunnet's *t* test, no statistical differences were found.

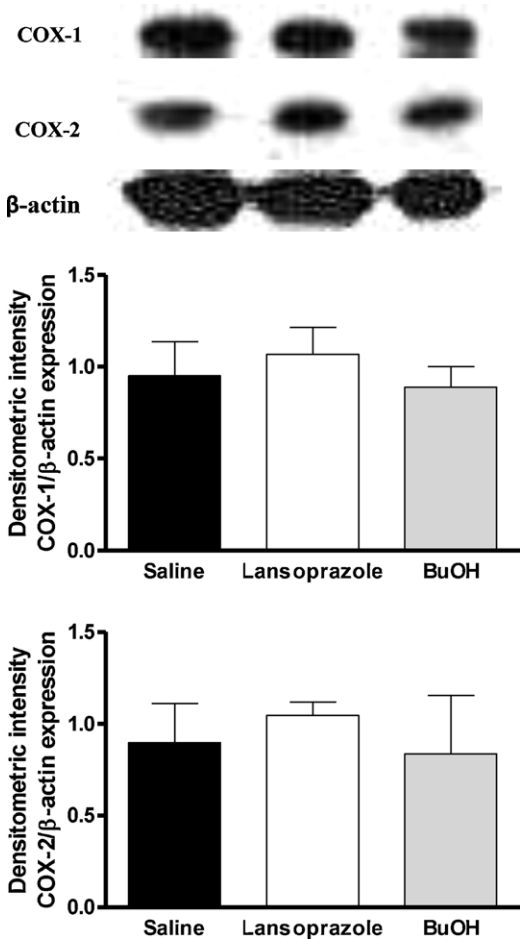

Fig. 4. Effects of oral administration during 8 days of BuOH on COX-1 and COX-2 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Densitometry was made following normalization with the control (housekeeping gene). Data are expressed as mean ± S.E.M. ANOVA followed by Dunnet's *t* test, no statistical differences were found.

showed gland preservation, and reduced granulation tissue. PAS also showed the preservation of inflamed tissue by the treatments, moreover, PAS staining showed presence of gastric mucus and the intact disposition of the glands in the groups treated with BuOH and lansoprazole.

3.9. Immunohistochemical localization of PCNA, HSP-70 and COX-2

The gastric tissues obtained in the chronic model of gastric ulcer were used for immunohistochemical localization of PCNA, HSP-70 and COX-2 antibodies. The analysis of histological slices showed a great number of proliferation cells in the stomach of animals treated with BuOH, the same profile was found to HPS-70, as well as COX-2. Thus, the results indicate that these proteins participated in the healing of the gastric ulcer treated with BuOH for 14 days.

4. Discussion

Various polyphenolic compounds, including tannins and other metabolites (e.g., epigallocatechin gallate, procyanidins), have been isolated from medicinal plants and their biological and pharmacological activities include anti-inflammatory, antisecretory and antiulcerogenic effects. Crude extracts from some medicinal tannin-rich plants are traditionally used worldwide to treat gastric ulcer (Okuda, 2005). Tannins are known to exist in mangrove species, which consist primarily of condensed tannins or proanthocyanidins (Rahim et al., 2008). According to Sánchez et al. (1996) *Rhizophora mangle* bark extract present 80% of polyphenols, represented in their majority by condensed tannin (80%) and 20% of hydrolysable tannin. Condensed tannins have attracted great attention due to rapid growing evidence associating these compounds with a wide range of potential health benefits (Zhang et al., 2010).

Ethanol-induced gastric ulcer models have been widely used for the evaluation of gastroprotective activity, since basic information can be provided by using it, as well as the determination of the therapeutic dose. Thereby the gastroprotective effects of the CE (50; 100; 250 and 500 mg/kg), Aq (6.25; 12.5; 25 and 50 mg/kg), EtOAc (0.5; 1.5; 3.0; 6.25; 12.5; 25 and 50 mg/kg) and BuOH (0.5; 1.5; 3.0; 6.25; 12.5; 25 and 50 mg/kg) were verified (Fig. 1). Ethanol is known to be one of many factors that increase the risk of gastric ulcer formation. Hemorrhagic ulceration of the stomach in humans and experimental animals is also caused by ingestion of elevated amounts of ethanol. The main features of ethanol-induced gastric ulcer are epithelial loss, mucosal edema and sub-epithelial hemorrhage. Ethanol rapidly penetrates the gastric mucosa, its injury is characterized by membrane damage, erosive hemorrhagic lesions with diffuse coagulative cell necrosis, cell exfoliation, multiple superficial erosions, marked vascular congestion and ulcer formation (Gazzieri et al., 2007; Li et al., 2008; Nassini et al., 2010).

Rhizophora mangle antiulcer activity assessment was firstly performed with CE in ethanol-induced gastric lesions, once its activity was confirmed, the study was carried out to evaluate its organic fractions. The results obtained in the ethanol model showed that CE as well as their Aq, EtOAc and BuOH fractions

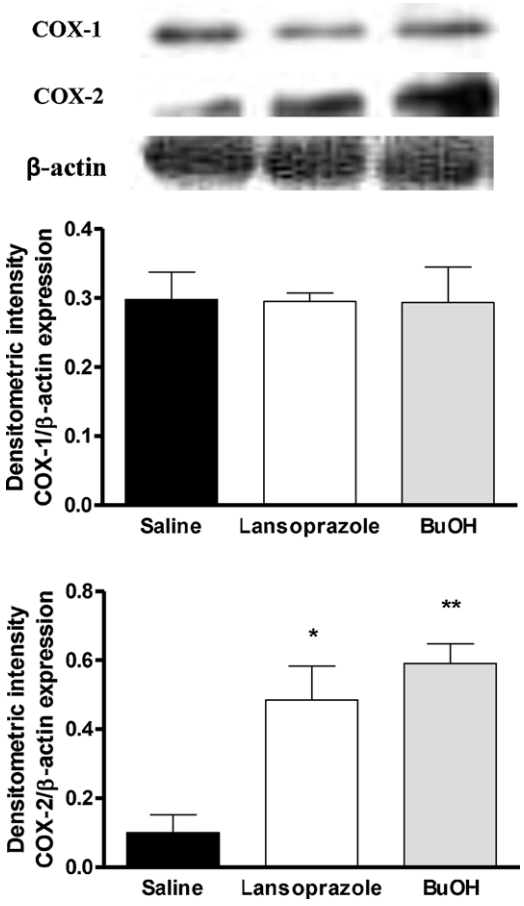

Fig. 5. Effects of oral administration during 14 days of BuOH on COX-1 and COX-2 expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Densitometry was made following normalization with the control (housekeeping gene). Data are expressed as mean ± S.E.M. ANOVA followed by Dunnet's *t* test, $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from saline (control group).

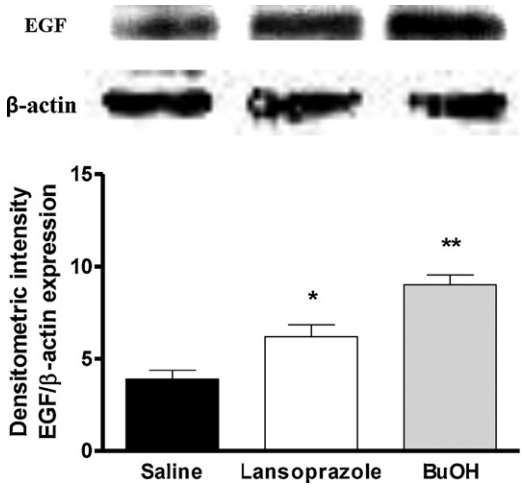

Fig. 6. Effects of oral administration during 14 days of BuOH on EGF expression in gastric mucosa of rats submitted to gastric ulcer induced by acetic acid. Densitometry was made following normalization with the control (housekeeping gene). Data are expressed as mean ± S.E.M. ANOVA followed by Dunnet's *t* test, $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from saline (control group).

possess gastroprotective activity. Once all treatments had their activity determined, we focused the succeeding experiments on using the most effective treatment with the lowest dose tested. As demonstrated in the results (Fig. 1), the BuOH treatment showed the most effective gastroprotection in the lowest dose used (0.5 mg/kg). Previous studies have already showed that tannins, specially condensed tannins, have pharmacological activity at low doses (Iwasaki et al., 2004) and as it has been demonstrated by de-Faria et al. (2009) the antiulcer property of *Rhizophora mangle* at low doses is linked to an antioxidant property whereas the treatment augmented the superoxide dismutase (SOD) activity against ischaemia/reperfusion-induced gastric ulcer.

In gastric ethanol injury, the endothelium is the first preferential target for ethanol damage. Along with an increase in systemic blood pressure, vasoconstriction of several vascular beds produced by the systemic administration of L-NAME, given to inhibit NO synthesis, would be certainly harmful, especially for gastric mucosa and its endothelium (Sikiric et al., 1997). As well as NO, the SH are important for the maintenance of gastric mucosa because of its protection against pro-oxidant agents, Luiz-Ferreira et al. (2010) reported that the administration of glutathione depletors (NEM) significantly potentiate the effects of ethanol on gastric mucosa injury. As presented in Table 2, the administration of L-NAME or NEM increased the ulcer area in animals submitted to ethanol-induced gastric ulcer, the BuOH treatment completely loses its gastroprotection when L-NAME is co administered. Thus, our result suggests that BuOH gastroprotective effect depends on the participation of mucosal sulfhydril compound levels and endogenous nitric oxide.

As aforementioned above, the antiulcer activity of BuOH suggests the participation of antioxidant properties which are, well known, linked to the maintenance of SH. On the other hand, the relationship between BuOH gastroprotection and endogenous NO could be explained based on the hypotension action of some tannins and other flavan-3-ol molecules, since this activity is a consequence of endogenous stimulation of NO production by nitric oxide synthase as related by Crestani et al. (2009).

Gastric acid has been known for many decades to be a key factor in normal upper gastrointestinal functions, including protein digestion, calcium and iron absorption, as well as providing some protection against bacterial infections. However, inappropriate levels of gastric acid underlie several wide spread pathological conditions, including gastric ulcer (Olbe et al., 2003). Proton pump inhibitors (PPI) have an excellent safety profile and have become one of the most commonly prescribed class of drugs in primary and specialty care. Their mechanism of action involves inhibition of the H+,K+-ATPase enzyme that is present in gastric mucosal parietal cells. PPIs are the most potent inhibitors of gastric acid secretion available, with efficacy superior to histamine-2 receptor antagonists (Sheen and Tridafilopoulus, 2011). Our results, Table 3, showed the inhibitory effect of the administration of BuOH on gastric juice and acid secretion. According to Murakami et al. (1992) the antiulcer activity of tannins may be related to its ability to bind and precipitate proteins suggesting a directly inhibition of the H+,K+-ATPase. Another possibility could be related to cytoprotective property, once we found an interesting augmentation in mucus and PGE_2 levels by the BuOH administration (Fig. 1A and B, respectively).

Table 3
Anti-secretory effect of intra-duodenal treatment with BuOH on rats submitted to pylorus ligature model. Data are presented as mean ± S.E.M. ANOVA followed by Dunnet's *t* test.

Treatments (i.d.)	Dose (mg/kg)	*n*	Gastric juice volume (mL/4 h)	pH (units)
Saline	–	7	1.78 ± 0.07	2.38 ± 0.05
Lansoprazole	30	7	1.51 ± 0.14	3.04 ± 0.23*
BuOH	0.5	7	1.41 ± 0.06*	3.09 ± 0.09*

* $P < 0.05$ significantly different from saline group.

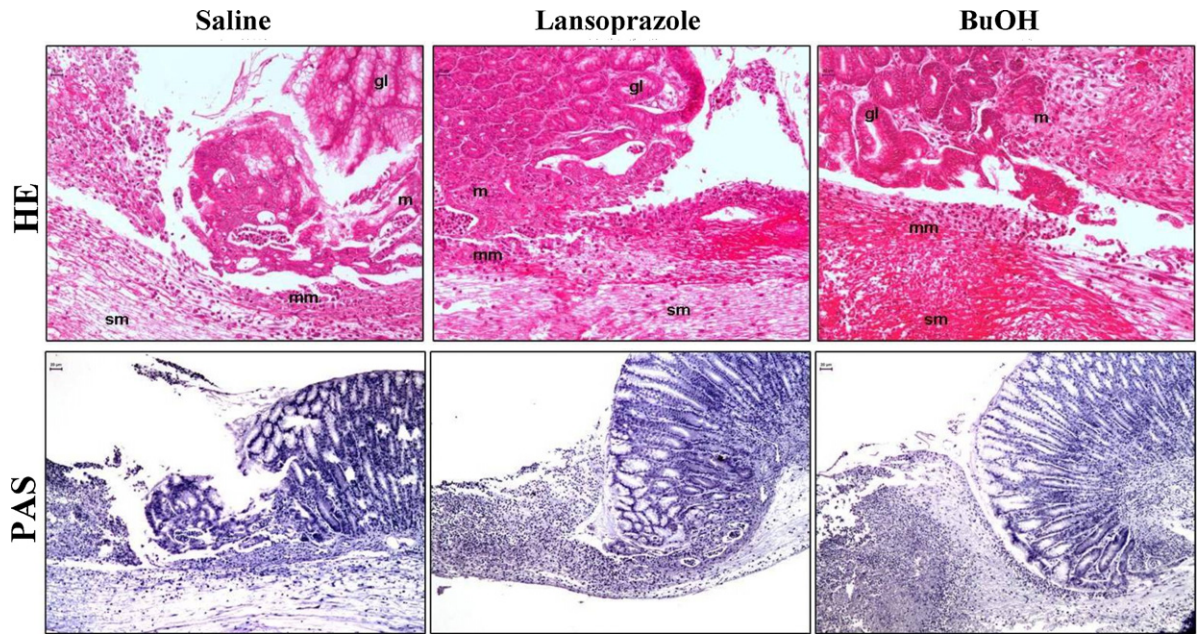

Fig. 7. Histological slices of the stomachs from rats submitted to acetic acid induced gastric ulcer assay. HE staining shows comparison of the tissue architecture between treatments, the legends found in HE image presented the gastric mucosa (m), sub-mucosa (sm), muscular (mm) and glands (gl). PAS staining presented the mucus in the glands.

The term *cytoprotection* was described by Robert et al. (1979) since they noticed that prostaglandin was capable of suppressing gastric acid secretion, although they found out that doses far below those necessary for the antisecretory effect produced a protective effect against oral administration of necrotizing agents. Although suppressors of acid secretion (e.g., H_2 receptor antagonists, PPI) have been a mainstay for promotion of ulcer healing for three decades, there has been an increasing interest in recent years in the mechanisms through which ulcers heal, and the possibility that both the speed and quality of healing may be pharmacologically modulated (Wallace, 2005).

Prostaglandins accelerate ulcer healing in experimental models. The mechanisms responsible for this effect are not fully understood, but the above-mentioned ability of prostaglandins to reduce gastric acid secretion would contribute to acceleration of ulcer healing (Wallace, 2008). In this context, the results obtained for mucus and PGE_2 levels are in agreement with the finding for antisecretory activity of the BuOH, according to our findings. Alanko et al. (1999) reported that phenolic compounds, such as tannins and flavonols, have dual effect on prostaglandin biosynthesis; low concentrations stimulate and high concentrations inhibit. These substances stimulate prostaglandin synthesis by reducing substrates for the oxidized intermediates of prostaglandin H synthase (PGHS), thereby accelerating the peroxidase cycle and by protecting PGHC from self-catalyzed inactivation by removing the free radical of PG.

It has been reported that PGE_2 not only prevents the formation of irritant-induced gastric ulcer but also enhances gastric ulcer healing. For the effective treatment of gastric ulcers, not only the prevention of further ulcer formation, but also the enhancement of ulcer healing is important. The ability of prostaglandins to stimulate mucus and bicarbonate secretion may also significantly contribute to the promotion of ulcer healing. The endogenous prostaglandins that contribute to ulcer healing are mainly derived from COX-2, the beneficial effects of PGE_2 on gastric ulcer healing in rodents appear to be mediated via EP4 receptor (Wallace, 2008).

In order to evaluate the healing properties of the BuOH treatment, acetic acid-induced gastric ulcer method was carried out. This ulcer model highly resembles human ulcers in terms of pathological features and healing process (Okabe and Amagase, 2005). Animals were sacrificed 4, 8 and 14 days after the treatment with saline, lansoprazole and BuOH as demonstrated by Fig. 2B. The acceleration of healing in BuOH was more effective than lansoprazole group in all days of evaluation. This could be explained by the dual effect of BuOH; antisecretory and cytoprotective properties. In this context, Okabe and Amagase (2005) showed that anti-secretory drugs, prostaglandin analogs, mucosal defense agents and various growth factors, all significantly enhance healing of acetic acid ulcers.

Hence, Western blotting analysis of COX-1 and COX-2 were performed on days 4, 8 and 14 of treatment, while EGF expression and histological evaluation were done on the 14th day. As can be seen in Figs. 3 and 4, we found no differences in COX-1 and COX-2 expression between the experimental groups on 4th and 8th days. On the other hand, on the 14th day, an increase in COX-2 was found for lansoprazole and BuOH treatment. Tsuji et al. (2002) found that lansoprazole increased the expression of COX-2 but not COX-1. Our data are in agreement with their findings; the BuOH treatment showed similar results on both COX-1 and COX-2 expression, however its efficacy was more significant than presented by the lansoprazole group.

While COX-1 is the predominant isoform expressed in the healthy gastric mucosa, COX-2 expression can be upregulated very rapidly. Substantial increased COX-2 expression can be seen following a mucosal exposure to a hapten, induction of ischemia or when COX-1 activity is suppressed by aspirin. This upregulation of COX-2 appears to be a defensive and anti-inflammatory response aimed at enhancing mucosal defense (Wallace, 2008) (Fig. 5).

Healing requires angiogenesis in the granulation tissue at the base of the ulcer, together with replication of epithelial cells at the ulcer margins and subsequent re-establishment of glandular architecture. Epithelial and endothelial cell proliferation is largely driven by growth factors (Wallace, 2005). The major growth factor receptor expressed in gastric progenitor cells is the epidermal growth factor receptor (EGF-R), EGF itself is absent in normal gastric mucosa, however it is present in the gastric lumen, derived from salivary and esophageal glands, and can stimulate progenitor cell proliferation in case of injury (Laine et al., 2008). Takayama

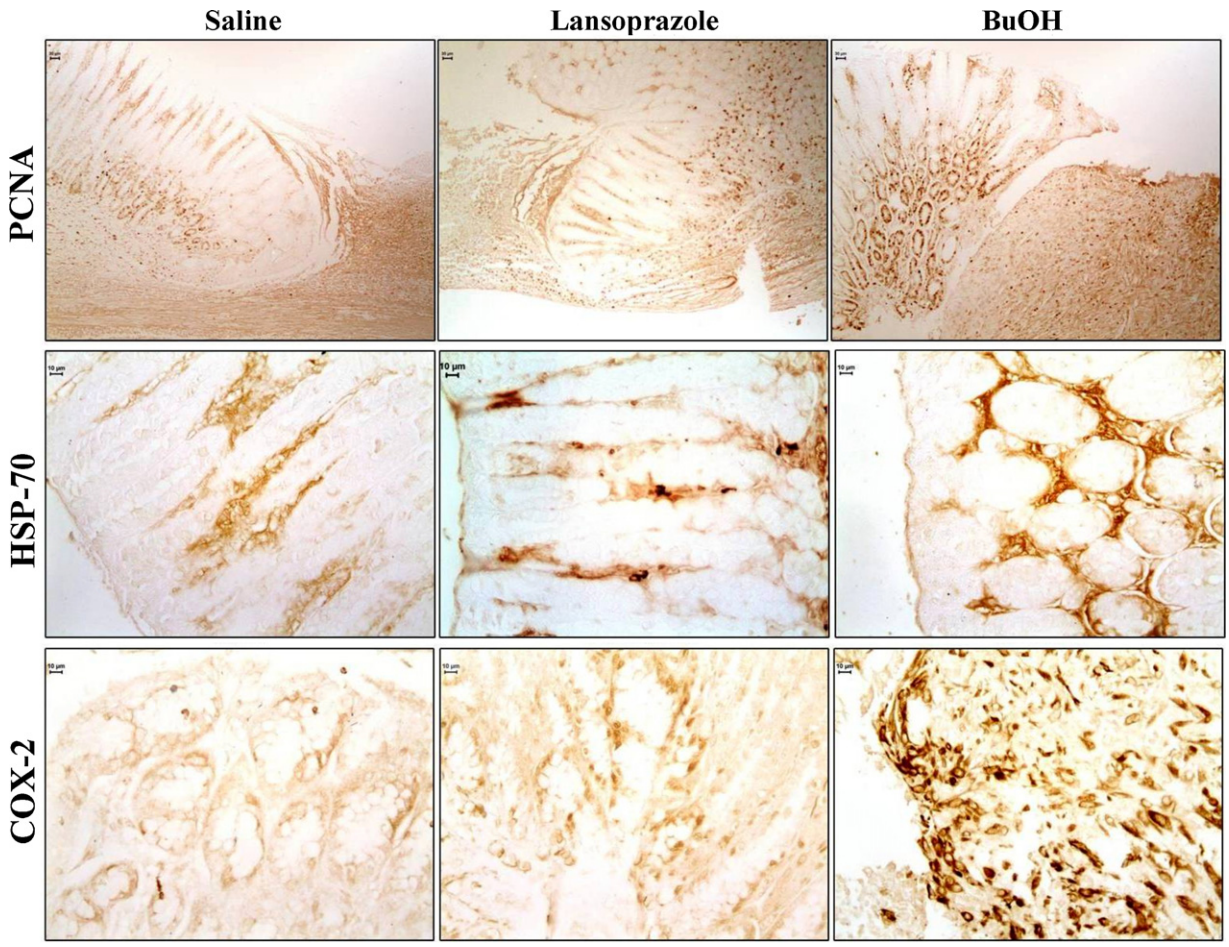

Fig. 8. Histological analysis of gastric mucosa of rats treated with saline (vehicle), lansoprazole (30 mg/kg) and BuOH (0.5 mg/kg) for 14 days. Images show immunohistochemistry localization of PCNA, HSP-70 and COX-2.

et al. (2011) reported the participation of EGF in the process of healing of chronic gastric ulcers. We also found an increase in the EGF expression (Fig. 6) of animals treated with lansoprazole or BuOH for 14 days. In addition, histological analysis are in agreement with the above findings, the HE and PAS staining (Fig. 7) showed the maintenance of glands integrity by the BuOH treatment, which also presented, by immunohistochemical localization, more presence of proliferation cell natural antigen (PCNA), heat-shock protein-70 (HSP-70) and COX-2 (Fig. 8).

As already mentioned, gastric ulcer healing is a process involving cell proliferation and migration at the gastric ulcer margin and angiogenesis in granulation tissue. When cells are exposed to gastric irritants, expression of heat shock proteins is induced, making the cells resistant to the irritants. Recently, Ishihara et al. (2011) examined the role of HSP-70 in gastric ulcer healing, providing the evidence that HSP-70 accelerates the process of healing by increasing the level of PGE_2 and expression of growth factor, thereby stimulating cell proliferation at the gastric margin and angiogenesis in granulation tissue. Immunohistochemical localization showed a great quantity of PCNA in the gastric mucosa of animals treated with lansoprazole or BuOH. COX-2 immunolocalization just confirmed the Western blotting data.

5. Conclusion

Our study on the pharmacological mechanisms involved in the antiulcer activity of *Rhizophora mangle* reinforces its traditional medicinal use. The treatment did not show any toxic effects, but instead, demonstrated to have cytoprotective, antisecretory and healing properties, possibly, due to the stimulation of PGE_2 by the upregulation of COX-2. Considering the current therapies are based on the use of antisecretory or cytoprotective drugs, the *Rhizophora mangle* arises as a promising alternative antiulcer therapy.

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