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Antiulcerogenic activity of *Combretum leprosum*

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This study investigates the effects of an ethanolic extract from the stem bark of *Combretum leprosum* Mart. & Eiche (*Combretaceae*) on experimental ulcers induced by ethanol and indomethacin and on gastric secretion and mucus content in pylorus-ligated rats. The effects were compared with those of ranitidine and carbenoxolone. *Combretum leprosum* orally administered elicited a complete inhibition of the appearance of gastric lesions induced by ethanol and a partial reduction when indomethacin was used as an ulcerogenic agent. Moreover, the protection against gastric ulceration induced by ethanol was decreased with indomethacin pretreatment. The intraduodenal administration of *Combretum leprosum* in four-hour pylorus-ligated rats increased the volume and pH of gastric juice while decreasing the acid output and produced a significant increase in gastric wall mucus content. The major compounds detected in a preliminary phytochemical screening were triterpenes, flavonoids, taninns and saponins. This study provides evidence that the ethanolic extract of *Combretum leprosum* possesses gastroprotective and anti-ulcerogenic effects, which are related to the inhibition of the gastric acid secretion and an increase of mucosal defensive factors such as mucus and prostaglandin.

1. Introduction

Gastric ulcer is a multifaceted, pluricausal illness. The pathophysiology of the disease remains incompletely understood. This illness is resultant of an interaction and balance between aggressive factors like hydrochloric acid, pepsin, free radicals, ischemia, leukotrienes, ethanol, nonsteroidal anti-inflammatory drugs (NSAIDs) and stress, and defensive factors like nitric oxide, prostaglandins, mucus, bicarbonate, sulfhydryls, mucosal blood flow and enzymatic activity of superoxide dismutase and catalase (Glavin and Szabo 1992).

Most of the available drugs used on the treatment of gastric and duodenal ulcers are thought to act on the aggressive factors. Antacids neutralize acid secretion while histamine-2 (H_2) receptor blockers (ranitidine, famotidine), anticholinergics (pirenzepin, telezipine) and proton pump blockers (omeprazole, lansoprazole) interfere with acid secretion (Rao et al. 2004).

Currently, medicinal plants are among the most attractive sources of new drugs available as therapeutic agents (Calixto 2005). In Brazil, a large number of herbal extracts are used in folk medicine to treat various types of digestive disorders (Hiruma-Lima et al. 2002). A scientific verification of plant use is important for the assessment of their quality, safety and therapeutic efficacy.

Combretum leprosum Mart. & Eiche, commonly called "mofumbo", is a *Combretaceae,* widely distributed in the Northeastern region of Brazil and popularly used in the state of Piaui as an expectorant and in the treatment of hemorrhages and flu (Freire et al. 1992). Lira et al. (2002) have demonstrated analgesic properties and a low oral toxicity (LD_{50} 4722 mg/kg) for this specie. Facundo et al. (1993) reported the isolation and the identification of two triterpenes and two flavonoids from the leaves and roots of this plant, and Pietrovski et al. (2006) described an antinociceptive effect of a triterpene isolated from the flowers of *C. leprosum* in several chemical and thermal behavioral models of pain. Other species of the same botanical genus, such as *Combretum dolichopetalum* (Asuzu and Njoku, 1992), and of the same botanical family, such as *Terminalia pallida* (Gupta et al. 2005) and *Guiera senegalensis* (Aniagu et al. 2005), have been reported to show a gastric antiulcer property. Since no data concerning the presence of this property in *Combretum leprosum* are available in the literature, the present study was carried out to investigate the possible anti-ulcerogenic and gastroprotective effect of this plant's ethanolic extract (CLEE) on acute experimental models of gastric ulceration induced by ethanol and indomethacin and on gastric secretion and mucus content in pylorus-ligated rats.

2. Investigations and results

2.1. Effects of CLEE on acute gastric ulcer induced by ethanol

In the experimental model of ethanol-induced gastric ulceration in rats, the *Combretum leprosum* ethanolic extract (CLEE) was found to possess remarkable ulcer-protective properties at orally administered doses of 60, 125, and 250 mg/kg. The inhibition of ulceration was dose-related

Table 1: Effects of different doses of *Combretum leprosum* ethanolic extract (CLEE) on gastric ulcers induced by ethanol (1 mL/animal, p.o.) in the absence or presence of pretreatment with indomethacin (30 mg/kg, s.c.) in rats

Treatment (p.o.)	Dose (mg/kg)	N	Ulcer index*	Inhibition (%)
Ethanol (1 mL/animal, p.o.)				
Control	–	18	14.05 ± 1.09	0
CLEE	30	6	12.43 ± 2.06[b]	12
	60	8	9.19 ± 1.32[ab]	35
	125	8	5.06 ± 0.73[ab]	64
	250	6	0.33 ± 0.17[ab]	98
Carbenoxolone	250	8	0.41 ± 0.33[a]	97
Ethanol (1 mL/animal, p.o.) after indomethacin (30 mg/kg, s.c.)				
Control	–	6	16.82 ± 2.40	0
CLEE	250	7	4.30 ± 1.21[a]	74
Carbenoxolone	250	6	5.57 ± 0.51[a]	70

* Data are presented as the mean ± S.E.M. [a] Student's two-tailed t-test: $p < 0.05$ compared to control group. [b] ANOVA: $p < 0,05$ compared to CLEE doses

and the maximal effect was reached with CLEE 250 mg/kg (98%). The standard drug carbenoxolone (250 mg/kg) showed 97% protection (Table 1).

2.2. Effects of CLEE on acute gastric ulceration induced by ethanol with pretreatment of indomethacin

Pretreatment with indomethacin (30 mg/kg, s.c.) weakened the protection against gastric ulceration induced by ethanol from 98% to 74% for CLEE (250 mg/kg) and from 97% to 70% for carbenoxolone (250 mg/kg) (Table 1).

2.3. Effects of CLEE on gastric ulcer induced by indomethacin

CLEE orally administered at doses of 250 and 500 mg/kg significantly inhibited the appearance of gastric lesions induced by indomethacin in rats. The percentage of inhibition varied from 71% to 76%. The standard drug ranitidine (60 mg/kg), a H_2-receptor blocker, reached the level of 95% protection (Table 2).

2.4. Effects of CLEE on acid gastric secretion

Intraduodenal treatment with CLEE (125, 250 and 500 mg/kg) in four-hour pylorus-ligated rats provoked a significant increase in pH and a reduction in the volume and total acidity of gastric juice produced. The response elicited by ranitidine (50 mg/kg) was similar to that obtained with CLEE (500 mg/kg). However ranitidine's effects on the total acidity and pH were significantly greater than those of CLEE (Fig. 1).

Table 2: Effects of different doses of *Combretum leprosum* ethanolic extract (CLEE) on gastric ulcers induced by indomethacin (30 mg/kg, s.c.) in rats

Treatment (p.o.)	Dose (mg/kg)	N	Ulcer index	Inhibition (%)
Control	–	11	4.1 ± 0.70	0%
CLEE	125	7	4.1 ± 0.69	0%
	250	7	1.2 ± 0.49[a]	71%
	500	8	1.0 ± 0.28[a]	76%
Ranitidine	60	6	0.2 ± 0.05[a]	95%

* Data are presented as the mean ± S.E.M. [a] Student's two-tailed t-test: $p < 0.05$ compared to control group

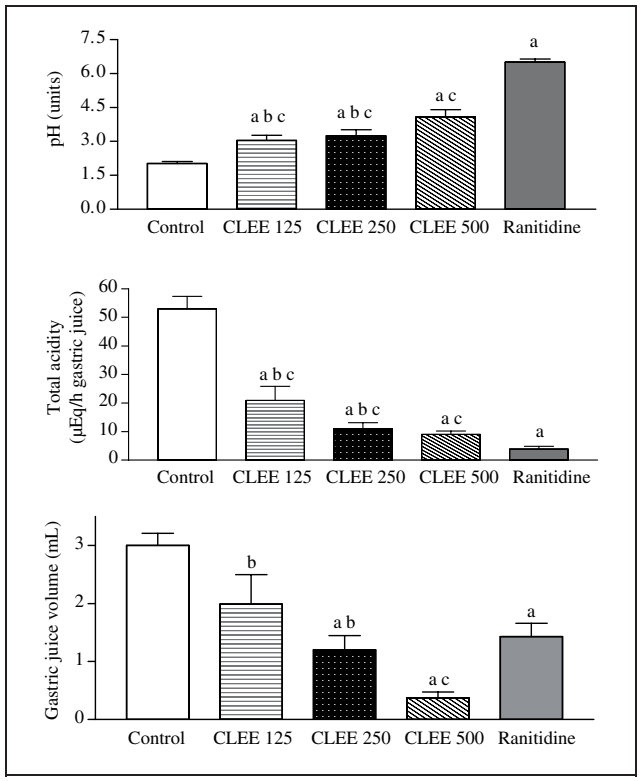

Fig. 1: Effect of *Combretum leprosum* ethanolic extract (CLEE) and ranitidine (50 mg/kg) on pH and total acidity of gastric secretion and gastric juice volume from pylorus ligated rats. Each column represents the mean ± S.E.M. of 12 animals. [a] $p < 0.05$ Student's two-tailed t-test compared to control; [b] $p < 0.05$ ANOVA compared to CLEE 500 mg/kg; [c] $p < 0.05$ Student's two-tailed t-test compared to ranitidine

An interesting finding was that the extract presented its effect when administered by intraduodenal route, indicating a systemic activity after the duodenal absorption of the active constituents of the plant ethanolic extract.

2.5. Effects of CLEE on gastric mucus

The effects of CLEE on changes in the amounts of gastric wall mucus in four-hour pylorus-ligated rats are depicted in Fig. 2. The intraduodenal administration of CLEE at doses of 250 and 500 mg/kg elicited a significant increase of 39% and 50% in the free mucus of the animals' gastric mucosa, respectively.

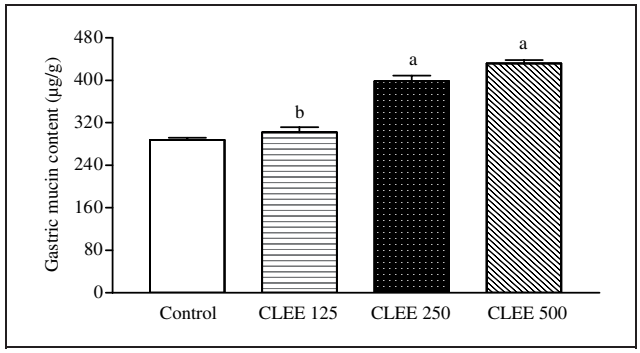

Fig. 2: Effect of *Combretum leprosum* ethanolic extract (CLEE) on gastric wall mucus content from pylorus ligated rats. Each column represents the mean ± S.E.M. of 12 animals. [a] $p < 0.05$ Student's two-tailed t-test compared to control; [b] $p < 0.05$ ANOVA compared to CLEE 500 mg/kg

2.6. Phytochemical screening

The preliminary qualitative phytochemical screening of CLEE revealed the presence of triterpenes, flavonoids, tannins and saponins.

3. Discussion

The results of this study showed that *Combretum leprosum* ethanolic extract (CLEE) is an effective anti-ulcerogenic and gastroprotective agent. The gastric mucosa of rats was protected by the oral administration of CLEE against ethanol- and indomethacin-induced acute mucosal damage. After intraduodenal administration of CLEE in pylorus-ligated rats, gastric mucus content increased significantly, acid output decreased, gastric juice pH was elevated and gastric fluid volume increased significantly. Comparatively, the extract was a better inhibitor on the gastric lesions induced by ethanol than in the indomethacin model.

Ethanol and indomethacin are among the most commonly utilized experimental models for the evaluation of gastric antiulcer drugs on rats. Gastric mucosal lesions induced by ethanol are caused by direct toxic effects through the reduction of mucus production, gastric mucosal blood flow and bicarbonate secretion. Ethanol also lowers endogenous glutathione and prostaglandin levels and increases the release of histamine, influx of calcium ions, generation of free radicals and production of leucotrienes (Glavin and Szabo 1992). The prostaglandine E_2 may exert its cytoprotective effect against ethanol-induced hyperemic lesions by diminishing the responsiveness of the gastric vasculature to histamine or leucotriene C_4 (Oates and Hakkinen 1988). It is well documented that ethanol-induced gastric mucosal lesions are not inhibited by anti-secretory agents such as cimetidine (Robert et al. 1979), but are primarily inhibited by agents that enhance mucosal defensive factors (Cho and Ogle 1992). CLEE's success in protecting against ethanol-induced ulcers suggests that it enhances mucosal defensive factors.

Gastric ulcer induced by NSAIDs like indomethacin are caused by the interference with prostaglandins synthesis and the increase of acid secretion and back diffusion of H^+ ions (Schoen and Vender 1989). Prostaglandins have a vital protective role in the stomach. They stimulate the secretion of bicarbonate and mucus, maintain mucosal blood flow and regulate mucosal cell turnover and repair (Hayllar and Bjarnason 1995). The suppression of prostaglandin synthesis by NSAIDs results in increased susceptibility to mucosal injury and gastroduodenal ulceration (Atay et al. 2000). The partial reduction (about 76%) of the indomethacin-induced gastric mucosal lesions by CLEE and the lowered protection against gastric ulceration induced by ethanol with pretreatment of indomethacin indicates that the gastroprotective effect of this plant extract partly depends on the involvement of prostaglandins.

Pylorus ligature (Shay et al. 1945) is an important experimental procedure used to investigate the changes in physicochemical parameters of gastric content in rats. It is widely used in studies of anti-ulcer drugs that could decrease the acid output and increase the amount of gastric mucus secretion. Mucus contributes to mucosal defense by providing a physical barrier to bacteria, thereby reducing bacterial adherence to the epithelium and invasion of the mucosa. Mucus also acts as a lubricant to reduce physical abrasion of the mucosa and participates in the protection of the mucosa from damage induced by acid and other luminal toxins and aggressive agents (Wallace and Miller 2000). Our data clearly demonstrated dose-related reductions of gastric acid secretion and increases in the free mucus content of the animals' gastric mucosa by CLEE, indicating that the protective action of this extract involves the reduction of an aggressive factor and the increase of a defensive one.

Nitric oxide (NO) is an important factor involved in gastric defense mechanisms through the regulation of acid and alkaline secretion, epithelial fluid, mucus secretion, and mucosal blood flow (Wallace and Miller 2000; Wallace 2001).

Some studies have suggested that the generation of oxygen-derived free radicals and lipid peroxidation are important mechanisms involved in the pathogenesis of gastric ulcer. Antioxidants are known to inhibit lipid peroxidation and scavenge free radicals (Di Carlo et al. 1999). Naik et al. (2004) showed that *Terminalia chebula*, another species of *Combretaceae*, is a potent antioxidant and a probable radioprotector. Phytochemical literature for anti-ulcer molecules shows that this activity comprises several types of compounds including terpenoids (Arrieta et al. 2003; Oliveira et al. 2004), flavonoids (Rao et al. 1997; Sannomiya et al. 2005), tannins (Hiruma-Lima et al. 2006) and alkaloids (Toma et al. 2004). The mechanisms of the mucosal protective action of CLEE may be partly related to the antioxidant properties of flavonoids, tannins and terpenoids present in this species.

A preliminary study performed by our group (Galvão et al. 2006) has shown that CLEE significantly reduced the *in vitro* lipid peroxidation level of rat brain homogenate as evaluated by the quantification of malondialdehyde using the thiobarbituric acid test (CLEE $Q_{1/2} = 0.37 \pm 0.01$ μg/mL versus butylated hydroxytoluene $Q_{1/2} = 0.37 \pm 0.1$ μg/mL), indicating antioxidant properties of CLEE.

In conclusion, this study provides evidence that the ethanolic extract of *Combretum leprosum* possesses a gastroprotective effect, which is related to the inhibition of gastric acid secretion and to a stimulation of gastric mucus production. Our results additionally show that the gastroprotection mediated by this plant extract could be related both to a local and systemic effect after the intestinal absorption of CLEE compound(s). Terpenoids, flavonoids and tannins present in CLEE revealed by preliminary qualitative phytochemical screening may be responsible for the potent antiulcer effect on the rat gastric mucosa but a clear understanding of the mechanisms of this plant extract requires complementary studies with isolated and chemically identified compounds and other experimental protocols.

4. Experimental

4.1. Plant material

The stem bark of *Combretum leprosum* was collected in July, 2005 at the Agrarian Science Center, Federal University of Piaui, Teresina, state of Piaui, Brazil. Voucher specimen (N° 10557) has been deposited in the Graziela Barroso Herbarium, at the same institution. The plant material was shade-dried at 40 ± 1 °C and the stem bark powder was extracted with 70% ethanol, evaporated in a vacuum at 50 °C and lyophilized to obtain a dry extract which was stored under refrigeration until further use. The extract was freshly prepared as a sonicated suspension in distilled water for the experiments.

4.2. Animals stock

Male Wistar rats aged 2–3 months weighing 250–300 g were used for the study. Animals were provided with a rodent-pellet diet (Purina Chow) and water *ad libitum*. They were maintained under standard conditions of light cycle (12 h light/12 h dark), 44–56% humidity and a temperature of 24 ±

2 °C. The rats were randomly assigned to different control and treatment groups. The experimental protocols were conducted in accordance with the guidelines of the Ethical Committee at the Federal University of Piaui and the Brazilian Council of Animal Experimental Investigation.

4.3. Experimental protocols

4.3.1. Ethanol-induced gastric ulcer

Rats maintained under standard conditions, as described above, were fasted for 24 h and orally received distilled water (5 mL/kg), CLEE (30, 60, 125 and 250 mg/kg) or carbenoxolone (250 mg/kg). One hour later, absolute ethanol (1 mL/animal) was orally administered. Animals were sacrificed 30 min after ethanol administration by ether inhalation. After each rat was sacrificed, the stomach was removed, opened along the lesser curvature and washed with normal saline. The glandular portion of the stomach was examined in a blinded manner. The lesion area (mm^2) was measured under a stereo-microscope (PZO-Labimex) and the ulcer index was calculated and expressed as a percentage of the total area (mm^2) of gastric corpus (Robert et al. 1979). The ulcer index mean values obtained for each group was compared and the percentage inhibition of ulceration was determined for each group.

4.3.2. Pretreatment with indomethacin on ethanol-induced gastric ulcer

Rats maintained under standard conditions, as described above, were fasted for 24 h with free access to water then divided in groups according to the respective treatment. All animal groups received a subcutaneous injection of indomethacin (30 mg/kg), a cyclooxygenase inhibitor. After 20 min, each group received the respective treatment orally (distilled water, CLEE 250 mg/kg or carbenoxolone 250 mg/kg). After 1 h, 1 mL of absolute ethanol was orally administered. The stomachs were removed 30 min later. The gastric mucosal lesions were evaluated and the ulcer index was calculated as described above.

4.3.3. Indomethacin-induced gastric ulcer

Rats maintained under standard conditions, as described above, were deprived of food for 24 h. The animals were divided into five groups ($n = 6$ to 11 rats). The rats in group **1** served as control which received distilled water (5 mL/kg, p.o.). Animals in groups **2**, **3** and **4** were administered with CLEE at the doses of 125, 250 and 500 mg/kg, p.o., respectively. Group **5** received the reference drug ranitidine (60 mg/kg, p.o.). The animals were treated with water, CLEE or ranitidine 30 min before and 3 h after indomethacin administration (30 mg/kg, s.c.) and were sacrificed 3 h after the last treatment. Each stomach was then opened along the lesser curvature, rinsed with normal saline and examined under a stereo-microscope (PZO-Labimex) for gastric ulcers in a blinded manner. The extent of gastric mucosal lesions was expressed as an ulcer index scored by the modified criteria of Robert et al. (1979) and calculated according to the quantity and the diameter of the erosion: Ulcer Index $= [3A + 2B + 1C]/6$, (A: number of small erosion up to 1 mm; B: number of erosion up to 3 mm; C: number of linear erosion greater than 3 mm). Mean scores for each group were calculated and compared. From this data, the percentage inhibition of ulceration was determined for each group.

4.3.4. Pylorus ligated rats

The animals were acclimatized under standard conditions, as described above, for at least 7 days in individual, metabolic, wire-bottomed cages to avoid coprophagy. The food was withdrawn 24 h before the experiment but there was free access to drink a 5% glucose solution to reduce fasting stress. The control and experimental groups consisted of twelve rats each. All experiments were done in the morning.

Pylorus ligation was performed as described by Shay et al. (1945) and was done through a midline abdominal incision under ether anesthesia. CLEE was administered intraduodenally in 125, 250 and 500 mg/kg of body weight doses to the animals in a 5 mL/kg volume as a suspension in distilled water. Control animals received distilled water (5 mL/kg) and the standard group received ranitidine (50 mg/kg). The abdomen was sutured and the animals were allowed to recover from anesthesia. Rats were sacrificed 4 h after treatment by ether overdose, the abdomen was opened and another ligature was placed around the esophagus close to the diaphragm. The stomachs were removed and gastric juice solution was collected. Distilled water (3 mL) was added and the total solution was centrifuged at 3500 rpm for 30 min. The content (mL), the pH and the total acidity in gastric secretion were determined in the supernatant volume. The total acidity output was determined by titration to pH 7.0 with 0.1 N NaOH in a pH-Meter (WTW 330i) and expressed as μEq/h gastric juice.

Additionally, glandular segments from the stomachs were excised for determination of gastric wall mucus content, as described below.

4.3.5. Determination of gastric wall mucus content

Gastric wall mucus was determined by the Alcian blue method (Corne et al. 1974). Stomachs excised from pylorus-ligated rats were opened along the lesser curvature. Glandular segments from the stomachs were removed and weighed. Each segment was transferred immediately to 7 mL of 0.25% w/v Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8) and incubated for 2 h at room temperature. The free dye was removed by two successive rinses at 15 and 45 min in 0.25 M aqueous sucrose solution. The gastric wall mucus bounded dye was extracted by immersion in 5 mL of 0.5 M $MgCl_2$ for 2 h with 1 min agitation every 30 min. A 4 mL sample of the blue extract was then shaken vigorously with an equal volume of diethyl ether and the resulting emulsion was centrifuged at 3000 rpm for 10 min. The optical density of Alcian blue in the aqueous layer was read against distilled water at 598 nm by a visible spectrophotometer (Celm E225D). The quantity of mucin was expressed as μg of Alcian blue extracted per weight (g) of wet stomach glandular tissue.

4.4. Statistics

Results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) was used to compare the values for each experimental group. Student's two-tailed t-test for unpaired data was used to compare each experimental group with the control or standard group. The p-value of less than 0.05 ($p < 0.05$) was considered a statistically significant difference.

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