Protective and antioxidant effects of *Rhizophora mangle* L. against NSAID-induced gastric ulcers

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Abstract

The bark of *Rhizophora mangle*, the red mangrove, has been used traditionally in folk medicine of Caribbean countries due to its antiseptic, astringent, haemostatic and antifungal properties. Aqueous extracts are rich in tannins and have been proven experimentally to possess antibacterial, wound healing and antiulcerogenic effects. This work was designed to determine the gastroprotective effect of *Rhizophora mangle* in a model of diclofenac-induced ulcers in rats and to study the mechanisms involved, using the proton pump inhibitor omeprazole as a comparison. The lyophilized extract was given by oral gavage (125 and 62.5 mg/kg) three times at 12 h intervals before administering diclofenac 100 mg/kg. Pretreatment with the extract resulted in a significant decrease of the ulcerated area (P < 0.01). *Rhizophora mangle* induced a recovery of PGE2 levels, which had been depleted by diclofenac. No anti-inflammatory effect was observed ex vivo or in vitro. The highest dose of the extract provoked a marked increase in glutathione peroxidase and superoxide dismutase activity, which was comparable to omeprazole. Furthermore, lipid peroxidation levels were inhibited in a dose-dependent manner. These results suggest that the gastroprotective effect of *Rhizophora mangle* in this experimental model appears through an antioxidant and prostaglandin-dependent way.

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1. Introduction

*Rhizophora mangle* L. (Rhizophoraceae), the red mangrove, is widely distributed along the tropical and subtropical coasts of America from Bermuda to Florida, Occidental Africa and the islands of Fiji, Tonga and New Caledonia (Chapman, 1970) and is a characteristic tree of the lower and swampy zones of the Cuban archipelago.

One of the principal uses of this plant is the extraction of tannins, which appear in the dry bark in a proportion ranging from 15 to 36% (Chapman, 1976).

In traditional medicine, *Rhizophora mangle* has been used as astringent, antiseptic and haemostatic with antifungal and antifungal properties (Roig, 1974). Furthermore, the red mangrove has been widely used in folk medicine for the treatment of diarrhea, dyspepsia, epistaxis, eye ailments, inflammations, sore throat and wounds (Morton, 1981). The aqueous extract has shown antibacterial activity (Melchor et al., 2001) along with efficacy in the healing of open surgical wounds (Fernandez et al., 2002). Moreover, *Rhizophora mangle* exerted a remarkable protective effect on gastric ulcers induced by ethanol-hydrochloric acid (Sánchez Perera et al., 2001).

Gastric and duodenal ulcers are common pathologies that may be induced by a variety of factors, such as stress, smoking, nutritional deficiencies and noxious agents, including non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are worldwide used for the treatment of pain, rheumatic and cardiovascular diseases, and more recently for the prevention of colon cancer and Alzheimer’s disease (Teo et al., 2001). The mechanism by which NSAIDs cause injury to the gastric mucosa is mainly due to the inhibition of cyclooxygenase enzyme (COX) and suppression of prostaglandin (PG)-mediated effects on mucosal pro-
tection (Wallace et al., 2000). Besides, it has been proposed that neutrophil and oxygen radical-dependent microvascular injuries may be important processes that lead to mucosal damage in response to NSAID administration (Wallace and Granger, 1992). These agents cause the activation of neutrophils and their adherence to the vascular endothelium, hence blocking capillaries and reducing local gastric blood flow.

Most of the available gastroprotective drugs act on the offensive factors neutralizing acid secretion like antacids, H₂ receptor blockers like ranitidine, anticholinergics like pirenzepin, proton pump inhibitors like omeprazole, lansoprazole, etc. which interfere with acid secretion (Rao et al., 2004).

The aim of this study was to assess the gastroprotective effect of Rhizophora mangle in comparison with omeprazole in a model of diclofenac-induced gastric ulcer in rats and to elucidate the underlying mechanisms. For this purpose, we studied the role of the red mangrove in oxidative stress by measuring changes in glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity. In addition, the inhibition of lipid peroxidation was assessed in vitro in rat liver membranes. We also evaluated PGE₂ levels and myeloperoxidase (MPO) activity as a marker of neutrophil infiltration.

2. Materials and methods

2.1. Plant material

The barks of Rhizophora mangle L. were collected in Cabarién, Las Villas, Cuba. The Department of Botany, National Center of Animal and Plant Health (CENSA), Cuba, authenticated the botanical identity of the plants and a voucher specimen (no. 6538, HAJB) was deposited to the Systematic and Ecology Institute, La Havana, Cuba.

2.2. Preparation of the extract

Fresh bark in a proportion of 1:10 distilled water was extracted at 90°C during 90 min. The plant material was separated by filtration and the aqueous extract was concentrated and freeze-dried to preserve it.

2.3. Animals

Male and female Wistar rats (180–200 g) kept in standard laboratory conditions, were fasted overnight in single wire-net floor cages with free access to tap water and were randomly assigned to groups of 10–12 animals. All experiments followed a protocol approved by the local animal Ethics Committee and the Local Government and were in accordance with the recommendations of the European Union.

2.4. Dose selection and mode of administration

Two different doses of lyophilized extract, 62.5 and 125 mg/kg body weight, were used in the present study, since Rhizophora mangle in the range of 125–500 mg/kg previously has been proven to yield gastric protection in a model of ethanol–hydrochloric acid (Sánchez Perera et al., 2001). Diclofenac (Sigma Chemical Co.) was used as ulcerogenic agent at a dose of 100 mg/kg and the dose of Omeprazole (Cepa) was 20 mg/kg.

2.5. Ex vivo experimental protocols

Rhizophora mangle (freeze-dried in aqueous suspension) was administered three times to the different animal groups at 12 h intervals by oral gavage in a volume of 1 mL/100 g body weight. One hour after the last administration animals received diclofenac 100 mg/kg by the same route. Rats were sacrificed by cervical dislocation 6 h after NSAID administration. Vehicle-treated (sham) and diclofenac-treated rats were included as controls in all experiments. Experimental times were decided following the results of former experiments of our group that reflect the damage induced by the assayed NSAID peaks 6 h after oral administration (Sánchez et al., 2002).

2.5.1. Ulcer assessment

The stomachs were harvested, opened along the greater curvature and the mucosa was exposed to macroscopic evaluation. The ulcerated area was assessed using planimetry by a person unaware of the type of treatment received by the animals, and the ulcer index (UI, mm²) was calculated as the arithmetic mean for each treatment. Following the analysis, the mucosa layer was blotted dry, and scraped off the underlying muscularis externa and serosa. Homogeneous mixture of mucosa, damaged and macroscopically healthy tissue, was snap-frozen in liquid nitrogen, and stored at −70°C before biochemical studies.

2.5.2. Histological evaluation

Ulcerated portions from two to three stomachs of each experimental group were cut out with a scalpel and were fixed for 4 h in 4% buffered paraformaldehyde, then dehydrated gradually in ethanol and embedded in paraffin using xylene as intermediate solvent. Serial sections (6 µm) were obtained by cutting the block in a plane perpendicular to the mucosal surface with a microtome. Coded gastric sections were stained with haematoxylin and eosin before light microscope evaluation.

2.5.3. Myeloperoxidase activity

MPO activity was assessed as a marker of neutrophil infiltration according to the method of Grisham et al. (1990). The tissue was homogenized in phosphate-buffered saline (PBS), pH 7.4, and centrifuged, and the pellet was again homogenized in PBS, pH 6.0, containing hexadecyl-trimethylammonium bromide (HTAB) and ethylenediamine tetraacetic acid. This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication. A sample of homogenate (0.5 µL) was added to a 0.5 mL reaction volume containing PBS, pH 5.4, HTAB and 3,3′,5,5′-tetramethylbenzidine. The mixture was incubated at 37°C. The reaction started by the addition of H₂O₂, and was terminated by the sequential addition of catalase and sodium acetate, pH 3.0. The changes in absorbance at 655 nm were measured with a microplate reader (Labsystem Multiskan Ex).
2.5.4. Prostaglandin E2 levels

One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min at 37 °C and the results were expressed as U/mg protein. Protein content was assessed following the Bradford assay (1976).

2.5.6. Superoxide dismutase activity

The enzymatic activity of SOD is based on the inhibition of the reduction of cytochrome c according to the method of McCord and Fridovich (1969). Samples of gastric mucosa were homogenized (1:150) in a mixture of 50 mM PBS and 100 μM EDTA (pH 7.6). The homogenate was supplemented with 0.1% Triton. The assay method used 10 μM ferricytochrome c, 50 μM xanthine, as source of O$_2^-$, and sufficient milk xanthine oxidase (5 nM) to give a rate of increase in absorbance of 0.025/min at pH 7.8 and 25 °C. The reaction kinetic was measured in a spectrophotometer at 550 nm at a rate of 0–80 s. Results were expressed as U/mg protein.

2.6. In vitro experimental protocols

2.6.1. Leukocyte isolation

Peritoneal mast cells were isolated as previously reported (Wang and Teng, 1990). Mixed rat peritoneal cells were collected by peritoneal lavage with heparinized Tyrode’s solution and were purified by centrifugation through a 30% BSA density gradient.

Purified mast cells were washed and re-suspended in Tyrode’s solution with glucose (composition in mL: NaCl 137, KC1 2.7, NaHCO$_3$ 12, NaHPO$_4$ 0.3, MgCl$_2$ 1.0, CaCl$_2$ 1.0, glucose 5.6 and BSA 0.1%). For the determination of the concentration of leukocytes, 50 μL of toluidine blue 0.05% in saline was added to an equal volume of the cell suspension and was adjusted to 1 x 10$^6$ cells/mL for the experiments.

2.6.2. Myeloperoxidase assay

This assay was performed according to the method of Bradley et al. (1982), modified for lecturing in a microplate reader. Following reagents were added in the order stated to wells of a microtitre plate: 50 μL supernatant, 50 μL phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, 50 μL of 0.68 mg/mL o-dianisidine in distilled water and, to start the reaction, 50 μL freshly prepared 0.003% hydrogen peroxide. The optical density at 450 nm was read immediately and thereafter at 5-min intervals. The amount of enzyme in the samples was obtained by comparison of the rate of reaction with that in wells containing supernatants from the control group treated only with the calcium ionophore A23187. The release of MPO was expressed as enzymatic activity compared with the maximum level (control treated with A23187).

2.6.3. Inhibition of lipid peroxidation induced by Fe$^{2+}$/ascorbic acid in rat liver membranes

Lipid peroxidation was assessed by the method described by Gálvez et al. (1995). Rat liver samples were obtained from seven male Wistar rats and diluted 1:10 (w/v) in buffer containing (in mM): Tris 50, NaCl 100, KCl 0.5, CaCl$_2$ 0.5, MgSO$_4$ 1.0, KH$_2$PO$_4$ 0.55 and sucrose 3200 (pH 7.4). The samples were minced, homogenized and centrifuged at 1000 x g for 10 min at 4 °C. The pellet was discarded and the supernatant centrifuged at 20,000 x g for 20 min at 4 °C. The supernatant was removed, and the pellet (membrane-enriched fraction P2) was diluted 1:10 (w/v) in the buffer mentioned above without sucrose.

The products resulting from the thiobarbituric acid reaction, most of which were MDA reaction products, were taken as indicators of lipid peroxidation in membrane fractions. Membrane concentrates were diluted 1:4 (v/v) in the above-mentioned buffer solution but with 20 mM Tris and without sucrose, and divided in several tubes. Buffer (in the test tubes without inhibitor) or different concentrations of extract of Rhizophora mangle were added. Lipid peroxidation was induced via non-enzymatic way with 75 μM of both ferrous sulphate and ascorbic acid (FeAs).

Test tubes were incubated at 37 °C for 45 min while continuously shaken. Blanks, which contained only tissues, were incubated at 4 °C. Subsequently, the reaction was stopped and malondialdehyde (MDA) was analyzed using 0.5% thiobarbituric acid in 20% trichloroacetic acid. The products used in the samples were used in the blanks. After agitation, the samples were incubated at 100 °C for 15 min and then centrifuged at 1000 x g for 15 min at 4 °C. The amount of MDA produced was determined by measuring the absorbance of the supernatant at 532 nm, and interpolating the data in the corresponding control curve. Results were expressed as inhibitory percentages for MDA produced compared with the control tubes without extract.
2.7. Statistical analysis

Data from 8 to 10 experiments were pooled and expressed as arithmetic means ±S.E.M. The data were evaluated using ANOVA's test followed by Tukey's test for paired data and the non-parametric Mann–Whitney U-test (UI determination). P-values were considered significant at P < 0.05.

3. Results

3.1. Effects of diclofenac, Rhizophora mangle and omeprazole on gastric mucosa: macroscopic and microscopic appearances

As shown in Fig. 1, diclofenac caused important damage on the glandular mucosa (15.42 ± 2.74 mm²). In contrast, pretreatment with Rhizophora mangle 62.5 and 125 mg/kg decreased the ulcerated area to 3.21 ± 1.05 and 5.63 ± 0.63 mm², respectively (P < 0.01), which was comparable to the protective effect exerted by omeprazole.

Histopathologic evaluation revealed that diclofenac provoked a deep alteration of glandular epithelium and a loss of the histological structure almost reaching the submucosa. The lesion was characterized by abundant granulation tissue, intense inflammatory reaction and local infiltration of leukocytes. Pretreatment with the extract prevented the deep ulceration and necrosis induced by the NSAID (Fig. 2).
Fig. 3. Effects of pretreatment with Rhizophora mangle (Rm, 62.5 and 125 mg/kg) and omeprazole (Omp, 20 mg/kg), followed by diclofenac (DI, 100 mg/kg) on myeloperoxidase activity ex vivo. *P < 0.05 vs. sham.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated cells</td>
<td>–</td>
</tr>
<tr>
<td>Rhizophora mangle 25 μg/mL</td>
<td>112.73 ± 1.61</td>
</tr>
<tr>
<td>Rhizophora mangle 50 μg/mL</td>
<td>110.43 ± 4.17</td>
</tr>
<tr>
<td>Rhizophora mangle 100 μg/mL</td>
<td>121.42 ± 7.32</td>
</tr>
</tbody>
</table>

Results show mean values for 3 tests per treatment, except A23187 (12 tests).

3.2. Effects of diclofenac, Rhizophora mangle and omeprazole on MPO activity

At the assayed dose, diclofenac did not affect MPO activity in a significant way ex vivo nor in vitro (Fig. 3; Table 1), only the highest dose of Rhizophora mangle (125 mg/kg) induced an increase on this enzymatic activity (*P < 0.05 versus sham).

3.3. Effects of diclofenac, Rhizophora mangle on mucosal PG content

Basal PGE$_2$ content in gastric mucosa was 613.84 ± 124.91 pg/mg protein and diclofenac caused a marked depletion (87.99 ± 12.46 pg/mg protein; *P < 0.001 versus sham). Pretreatment with Rhizophora mangle extract at both doses augmented PG content (*P < 0.05 versus sham) (Fig. 4).

3.4. Effects of diclofenac, Rhizophora mangle and omeprazole on glutathione metabolism and SOD activity

Diclofenac caused an important depletion of GSH-Px, which was reverted by the pretreatment with Rhizophora mangle in a dose-dependent manner. The values were 0.19 ± 0.03 for diclofenac, while the extract elevated the enzymatic activity up to 3.98 ± 0.50 nmol/min/mg protein (*P < 0.001 versus diclofenac), which was similar to the data obtained with omeprazole (Fig. 5).

SOD activity in gastric mucosa of rats that received diclofenac was 8.40 ± 0.86 U/mg protein. Pretreatment with the extract at the highest dose (125 mg/kg) significantly increased the values in the same range as omeprazole (*P < 0.01 versus diclofenac) (Fig. 6).

3.5. Effects of Rhizophora mangle on lipid peroxidation

The extract of the red mangrove inhibited in a concentration-dependent manner, the lipid peroxidation induced in liver membranes by FeAs, with a maximum inhibitory effect of 74.5 ± 5.2% at the concentration of 300 μg/mL (Table 2).
Inhibitory effect of aqueous extract of Rhizophora mangle bark on lipid peroxidation induced by Fe²⁺-ascorbic acid in rat liver membranes

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Inhibition (%) (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>11.9 ± 3.2</td>
</tr>
<tr>
<td>25</td>
<td>18.4 ± 2.7</td>
</tr>
<tr>
<td>50</td>
<td>22.3 ± 4.9</td>
</tr>
<tr>
<td>75</td>
<td>42.5 ± 11.9</td>
</tr>
<tr>
<td>100</td>
<td>54.4 ± 9.3</td>
</tr>
<tr>
<td>200</td>
<td>60.7 ± 8.5</td>
</tr>
<tr>
<td>300</td>
<td>74.5 ± 5.2</td>
</tr>
<tr>
<td>400</td>
<td>70.5 ± 2.9</td>
</tr>
</tbody>
</table>

4. Discussion

The present study showed that pretreatment with the aqueous bark extract of Rhizophora mangle has a beneficial effect on NSAID-induced ulcers in rats as evidenced by the reduction in the ulcerated area and the histological appearance of the gastric mucosae. However, the protective properties of this drug could not be ascribed to anti-inflammatory mechanisms, as no changes in the MPO activity ex vivo or in vitro were observed.

Pretreatment with the red mangrove reflected a clear tendency to increase PGE2 production in spite of NSAID-induced depletion, although at the considerably high dose of diclofenac used (100 mg/kg); a total reversion to sham levels was not reached. These results may be attributed to the polyphenolic compounds found in Rhizophora mangle, as it has been suggested that phenols stimulate PGE2 formation based on their action as substrates for the peroxidase reaction (Alanko et al., 1999).

SOD activity decreased with diclofenac but augmented significantly in the Rhizophora mangle- and omeprazole-treated groups. This enzyme, which owes its antioxidant properties to its elevated capacity to scavenge O₂⁻, plays an important role in protecting the gastrointestinal mucosa. It has been shown that the subcutaneous administration of SOD and catalase significantly reduces the gastric damage induced by ischemia-reperfusion (Yoshikawa et al., 1989) or indomethacin treatment (Yoshikawa et al., 1993; Naito et al., 1998). The gastroprotective effect of some drugs, such as flavonoids, also has been related to their capacity to modulate this enzymatic activity against ulcers induced by ethanol (La Casa et al., 2000).

GSH-Px enzyme plays a marked role in the removal of H₂O₂ and lipid hydroperoxides in gastric mucosal cells and its antioxidant capacity is similar to that of SOD or Vitamin E (Richard et al., 1997). GSH-Px inhibition results in H₂O₂ accumulation and subsequent lipid oxidation and could be related to the gastric damage induced by indomethacin (Yoshikawa et al., 1993; Naito et al., 1998).

The increase in SOD and GSH-Px levels observed in the groups pretreated with Rhizophora mangle clearly point to an antioxidant mechanism underlying its gastroprotective action and, on the other hand, the ability to prevent lipid peroxidation in vitro reinforces its potential use as a therapeutic drug for free radical pathologies. The increased values of SOD and GSH-Px in omeprazole-treated animals also point to an antioxidant mechanism underlying its gastroprotective action, which is supported by a recent study on ethanol-HCl induced ulcers in rats (Natale et al., 2004).

As mentioned above, the major active principles of the red mangrove are polyphenols, represented in their majority by polymeric tannins (80%) and hydrolysable tannins (20%), and special emphasis has been given to the presence of epicatechin, catechin, chlorogenic, gallic and elagic acids, as well as gallotannins, elagittannins and condensed tannins (Sánchez et al., 1998). These substances characterized by their polyphenolic nature, have shown cytoprotective properties (Gonzalez et al., 2000) and have been associated to antiulcerogenic activity in other plants (Konig et al., 1994; Tebhid et al., 1996; Ramirez and Roa, 2003). Tannins or polyphenols have a number of physical and chemical properties in common, which underlie their physiological and pharmacological actions: their antioxidant and radical scavenging activities and their ability to complex with other molecules, such as proteins and polysaccharides (Haslam, 1996). Vegetable polyphenols are known to inhibit lipid peroxidation in vitro and there is evidence about their ability to scavenge radicals, such as hydroxyl, superoxide and peroxyl, which are important in cellular proxidant states. Incidentally, it has been shown that chlorogenic acid has an antioxidant effect as high as α-tocopherol (Fernandez et al., 2002).

On the other hand, tannins may prevent ulcer development due to their protein precipitating and vasoconstricting effects (Agwu and Nwako, 1988). Their astringent action can help precipitating microproteins on the ulcer site, thereby forming an impervious layer over the lining that hinders gut secretions and protects the underlying mucosa from toxins and other irritants (Nwafor et al., 1996, 2000; Al-Rehaily et al., 2002). This propensity to bind to proteins also explains the fact that polyphenols inhibit enzymes tested in vitro.

The topical action of the aqueous extract of Rhizophora mangle in accelerating wound healing has been explained by several mechanisms, such as coating the wound, forming complexes with proteins of microorganism cell wall, chelating free radicals and reactive oxygen species, stimulating the contraction of the wound and increasing the formation of new capillaries and fibroblasts (Fernandez et al., 2002). In our study, a thick coating of Rhizophora mangle extract was found macroscopically adherent to the gastric mucosa, which suggests that in addition to antioxidant mechanisms, the formation of a physical barrier with similar properties as observed in topical wounds may contribute to the gastroprotective action of the drug.

In summary, the present results indicate that the gastroprotective effects of Rhizophora mangle in the experimental ulcers induced by diclofenac could be related with its antioxidant properties, which increase the activity of the antioxidant enzymes GSH-Px and SOD and, on the other side, decrease lipid peroxidation. Furthermore, prostaglandin-dependent mechanisms also might be involved, while no significant changes in the inflammatory response could be observed. Therefore, we suggest that due to both, antioxidant and cytoprotective properties the aqueous extract of the bark of Rhizophora mangle may represent an attractive therapeutic option for protecting against NSAID-induced gastric ulcers.
References


