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Gastroprotective and anti-inflammatory effects of resin from *Protium heptaphyllum* in mice and rats

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Abstract

The natural resin collected from the trunk wood of *Protium heptaphyllum* is used in folk medicine to treat inflammatory conditions and to hasten wound repair. In the search of new potential anti-inflammatory agents with gastroprotective property, the present study evaluated its effects in experimental models of gastric ulcer and inflammation. In mice, the resin (200 and 400 mg/kg) significantly attenuated the gastric damage induced by ethanol or acidified ethanol (HCl/ethanol), in a manner similar to *N*-acetyl-L-cysteine (NAC), a replenisher of sulfhydryls. Unlike NAC the resin failed to restore the ethanol-induced depletion of non-protein sulfhydryl content, indicating a different mechanism of gastroprotection. However, in 4-h pylorus-ligated rats, the resin significantly reduced the total acidity without much change in gastric secretory volume. In rats, at similar doses the resin did not modify the hind-paw edema induced by carrageenan, but effectively reduced the formation of cotton pellet-induced granuloma, suggesting its inhibitory effect on collagen formation but not on acute edema. Furthermore, the vascular permeability increase induced by acetic acid was significantly reduced in mice that received 400 mg/kg resin. The resin demonstrated no overt toxicity in mice up to an oral dose of 5 g/kg. Phytochemical analysis revealed the presence of α- and β-amyrins as principal triterpenoid constituents of resin, which were previously described to have anti-ulcer property. These findings indicate the potential gastroprotective and anti-inflammatory property of *P. heptaphyllum* resin and further support its popular use in gastrointestinal disorders.

Keywords: *Protium heptaphyllum*; Resin; α-, β-Amyrins; Gastroprotection; Anti-inflammatory activity

1. Introduction

Resinous exudates from various plants have potential industrial applications such as in perfumery industries and varnish confections and besides possess medicinal value as desenfectants and parasiticides [1–4]. *Protium heptaphyllum* March (Burseraceae), popularly known as almécega is a medicinal plant that grows abundantly in Amazon region and in various other parts of Brazil. The resinous exudate collected from the trunk wood of this plant in its natural form is a reputed folk remedy with anti-inflammatory, analgesic, insect repellant, expectorant and wound healing actions [5–7]. Phytochemical studies on this folk remedy revealed the presence of several monoterpenes and some pentacyclic triterpenes including α- and β-amyrins [8,9]. In general, pentacyclic triterpenes have been reported to possess anti-inflammatory, anti-ulcer, antinociceptive and anti-tumoural properties [10–13]. Previous studies described the anti-inflammatory-related and anti-tumour activities of the essential oils obtained from the leaves and resin of *P. heptaphyllum* [7]. Despite its widespread use in popular medicine against various ailments, there were no scientific reports in literature supporting the use of crude resin as an anti-ulcer and anti-inflammatory agent. In vitro anti-oxidant effects of several naturally occurring resins and substances like amyrin, oleonolic acid, ursolic acid, lupeol and glycyrrhetinic acid have been reported [14]. Since the resin presents a much higher proportion of α- and β-amyrins as major constituents, this study verified the possible gastroprotective and anti-inflammatory potential of *P. heptaphyllum* resin in experimental models of ulcer and inflammation.

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2. Materials and methods

2.1. Plant material

The resinous exudate from the trunk wood of *P. heptaphyllum* was collected during August 2001 from the municipal areas of Timon, Maranhão State of Brazil, after its identification by a botanist Roseli Farias de Melo Barros. A voucher sample (#18247) has been deposited at the Herbarium Graziela Barroso of the Federal University of Piauí.

2.2. Phytochemical analysis

The crude resin (410 g) was dissolved in methanol/dichloromethane (4:1), filtered and the solvent evaporated in a rotavapor to obtain 408 g (99.5%) of amorphous white resin. Phytochemical analysis on resin revealed the presence of pentacyclic triterpenoids (56%), identified by ^{1}H and ^{13}C NMR and mass spectroscopy. These were the mixtures of α- and β-amyrins (45.25%), brein and maniladol (9.5%) and a small quantity of lupeone. Besides, it yielded an essential oil content of 0.7% [15].

2.3. Animals

Male Wistar rats (150–200 g) and male Swiss mice (20–25 g) were used. Experimental groups consisted of 6–10 animals per group. They were housed at 22 ± 2 °C under a 12 h light/12 h dark cycle and had free access to standard pellet diet (purina chow) and tap water. The animals were deprived of food for 15–24 h before experimentation, but had free access to drinking water. The experimental protocols were approved by the Animal Care and Use Committee of our Institute in accordance with the guidelines formulated by Federal University of Ceará on the Care and Use of Laboratory Animals for experimentation.

2.4. Gastric damage induced by ethanol

Groups of 24-h fasted mice ($n = 8$) were treated with resin (200 and 400 mg/kg, p.o.), *N*-acetyl-L-cysteine (NAC) (750 mg/kg, i.p.), or vehicle (3% Tween 80 in 0.9% saline in a volume of 10 ml/kg). One hour after, each animal was given orally 0.2 ml of ethanol (96%) and they were killed 30 min later [16]. The stomachs were excised, opened along the greater curvature, rinsed with saline (0.9%) and the mucosal lesion area (mm^2) was measured by planimetry using a transparent grid (area: 1 mm^2) placed on the glandular mucosal surface [17] and was expressed in percentage (%) in relation to total area of corpus.

2.5. Gastric damage induced by acidified ethanol (HCl/ethanol)

Mice deprived of food for a 24-h period were treated as above with resin, vehicle or NAC and 1 h later each rat received orally 0.2 ml of a 0.3 M HCl/60% ethanol solution [18]. Animals were killed 1 h after the administration of HCl/ethanol, stomachs were excised, opened along the greater curvature and fixed in 5% formalin for 30 min and the gastric mucosal lesions were quantified [19].

2.6. Gastric secretion in 4-h pylorus-ligated rats

Rats ($n = 6$ per group) fasted for a 24-h period but had free access to water and glucose (5%) were anaesthetised with ether, the abdomen was incised and the pylorus-ligated rats [20]. Vehicle, resin (200 and 400 mg/kg), or cimetidine (100 mg/kg) were administered by intra-duodenal route immediately after pylorus ligation to respective groups of animals in a volume of 2.5 ml/kg. Four hours after pyloric ligation, the rats were killed, stomachs were excised, and the gastric contents were collected and centrifuged at 3500 rpm for 30 min. The volume of gastric juice was measured (in ml) and the total acidity was determined by titration with 0.1 N NaOH using 2% phenolphthalein as acid–base indicator [21] and expressed as μEq./h.

2.7. Estimation of non-protein sulfhydryl content in stomach tissues

Groups of mice treated as in item 2.4 were utilised to estimate the reduced glutathione (GSH) content in stomach tissues as non-protein sulfhydryls according to the method described by Sedlak and Lindsay [22]. Glandular segment from each stomach was homogenised in 5 ml ice-cold 0.02 M EDTA solution (0.02 M). Aliquots (4 ml) of tissue homogenate were mixed with 3.2 ml of distilled water and 0.8 ml of 50% (w/v) trichloroacetic acid (50%) in glass tubes and centrifuged at 3000 rpm for 15 min supernatants (2 ml) were mixed with 4 ml Tris buffer (0.4 M, pH 8.9) and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; 0.01 M) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm within 5 min of the addition of DTNB against blank with no homogenate. The absorbance values were extrapolated from a glutathione standard curve and expressed in μg GSH/g of wet tissue.

2.8. Carrageenan-induced paw edema

Paw edema was induced in the right hind paw of rats by sub-plantar injection of 0.1 ml of 1% carrageenan in 0.9% (w/v) saline. The volume of injected paw was measured before and 3 h after carrageenan injection using a plethysmometer (Ugo Basile), and the edema was expressed as an increase in paw volume [23]. Different groups ($n = 6$) of animals were treated orally with resin (200 and 400 mg/kg), the reference compound indomethacin (10 mg/kg) or vehicle (2% Tween 80) in a volume of 10 ml/kg, 1 h before carrageenan injection.

2.9. Cotton pellets-induced granulomas

Each rat received two subcutaneous implants of sterilised cotton pellets weighing 50 mg each in the dorsum (one on each side) under ether anesthesia [24]. Four groups ($n = 8$) of animals were used. They were treated orally with the resin (200 and 400 mg/kg), ibuprofen (300 mg/kg), the reference compound or the vehicle, once daily for 7 days. On Day 8 the rats were killed and the pellets with the surrounding granulation tissues were collected, and their wet and dry weights established. The granulomas were dried at 60 °C for 24 h to obtain the dry weights.

2.10. Acetic acid-induced increase in vascular permeability

Groups of mice ($n = 8$) were treated orally with the resin (200 and 400 mg/kg), acetylsalicylic acid (250 mg/kg) or vehicle (3% Tween 80) in a volume of 10 ml/kg. One hour after these treatments, the mice were given an intravenous injection of Evan's blue solution (25 mg/kg). Five minutes later, each mouse received intraperitoneally 10 ml/kg of 1% acetic acid solution. Thirty minutes after acetic acid injection, the animals were killed, the peritoneal fluid was collected and the concentration of Evan's blue was measured by absorbance at 610 nm in a spectrophotometer [25]. The dye extravasation was quantified from a standard curve and expressed in μg.

2.11. Acute toxicity

Groups of mice ($n = 10$) were treated orally with 0.5, 0.75, 1.5, 3 and 5 g/kg of resin and observed for gross behavioural changes, if any, in the first few hours and for 72 h mortality. A separate group of controls received the vehicle only (10 ml/kg).

2.12. Statistical analysis

All values are expressed as the mean ± S.E.M. ANOVA and Student–Newman–Keuls tests were used to verify the statistical significance of the differences between groups. Differences were considered to be significant when $P \leq 0.05$.

3. Results

3.1. Effect on ethanol and HCl/ethanol-induced gastric lesions

The effects of resin and NAC on ethanol or HCl/ethanol-induced gastric lesions are shown in Table 1. Vehicle-treated control rats showed extensive gastric mucosal lesions in the form of haemorrhagic erosions in glandular segments only. The resin (200 and 400 mg/kg) treatment greatly reduced the gastric injury in both models and the observed effect is dose-related. At the respective doses, the resin significantly reduced the gastric lesions by 68 and 89%, in the ethanol model, and by 45 and 66% in HCl/ethanol model. NAC (750 mg/kg), the reference compound included in the study also caused significant inhibitions (49 and 69%, respectively) in these test models of gastric ulcer.

Table 1
Protective effect of resin from *Protium heptaphyllum* against gastric lesions induced by ethanol or acidified ethanol (0.3 M HCl/60% ethanol) in mice

Treatment	Dosage (mg/kg, p.o.)	Gastric lesions in mice treated with	
		Ethanol (lesion area) (mm^2)	0.3 M HCl/60% ethanol (lesion area) (mm^2)
Control	–	23.15 ± 1.82	16.14 ± 1.95
Resin	200	7.38 ± 0.61***	8.91 ± 0.96**
	400	2.50 ± 0.26***	6.53 ± 1.15**
N-Acetyl-L-cysteine	750	11.78 ± 1.59***	4.89 ± 0.05***

The results are expressed as mean ± S.E.M. ($n = 8$). Asterisks indicate significance from corresponding control. ** $P < 0.01$, *** $P < 0.001$ (ANOVA followed by Student–Newman–Keuls test).

3.2. Effects on gastric secretion in 4-h pylorus-ligated rats

In 4-h pylorus-ligated rats, intra-duodenal application of resin caused no significant influence on gastric secretory volume but significantly inhibited the total acid output, in a manner similar to cimetidine, a known H_2-receptor antagonist (Fig. 1). The extent of inhibitions on total acidity with resin (200 and 400 mg/kg) and cimetidine were in the order of 70, 75 and 88%, respectively.

3.3. Effect on non-protein sulfhydryl (GSH) content in stomach tissues

Fig. 2 shows the influence of different treatments on GSH content of stomach tissues. When compared to normal controls, ethanol controls that received vehicle alone showed significantly lowered levels of GSH. While pre-treatment with NAC restored, the resin treatment at either dose (200 and 400 mg/kg) failed to replenish the GSH to the levels seen in normal controls.

3.4. Effect on acute paw edema induced by carrageenan

At the doses tested, the resin pretreatment did not significantly affect the rat paw edema response to sub-plantar injection of carrageenan. At 3 h after subplantar carrageenan, the paw edema values in vehicle, resin (200 and 400 mg/kg) and indomethacin (10 mg/kg)-treated groups were in the order of 0.625 ± 0.03 ml, 0.663 ± 0.062 ml, 0.510 ± 0.052 ml and 0.392 ml, respectively. Indomethacin caused significant ($P < 0.01$) inhibition of paw edema by about 37% when compared to vehicle-treated control.

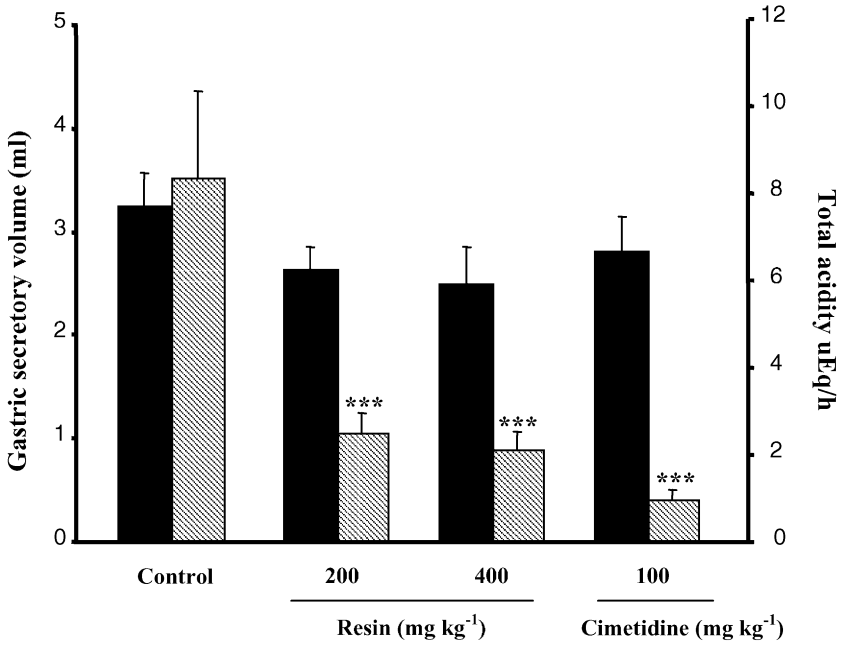

Fig. 1. Effect of resin from *Protium heptaphyllum* and cimetidine on gastric secretory volume (filled bars) and total acidity (hatched bars) in 4-h pylorus-ligated rats. Each column represents the mean ± S.E.M. ($n = 6$). Asterisks indicate significant difference from corresponding control. *** $P < 0.001$ (ANOVA followed by Student–Newman–Keuls test).

3.5. Effect on cotton pellets-induced granuloma

Fig. 3 shows that the resin (200 and 400 mg/kg) and ibuprofen (300 mg/kg) inhibited the development of cotton pellets-induced granulomas in rats. Unlike ibuprofen that showed inhibitions in both wet and dry granuloma weights, the resin at either dose caused significant reductions only in dry weights. Treatment with resin (200 and 400 mg/kg) and ibuprofen (300 mg/kg) resulted in dry weight reductions of 13, 14 and 38%, respectively.

3.6. Effect on acetic acid-induced increase in vascular permeability

Resin (400 mg/kg) and acetylsalicylic acid (250 mg/kg) produced significant inhibitions (52 and 80%, respec-

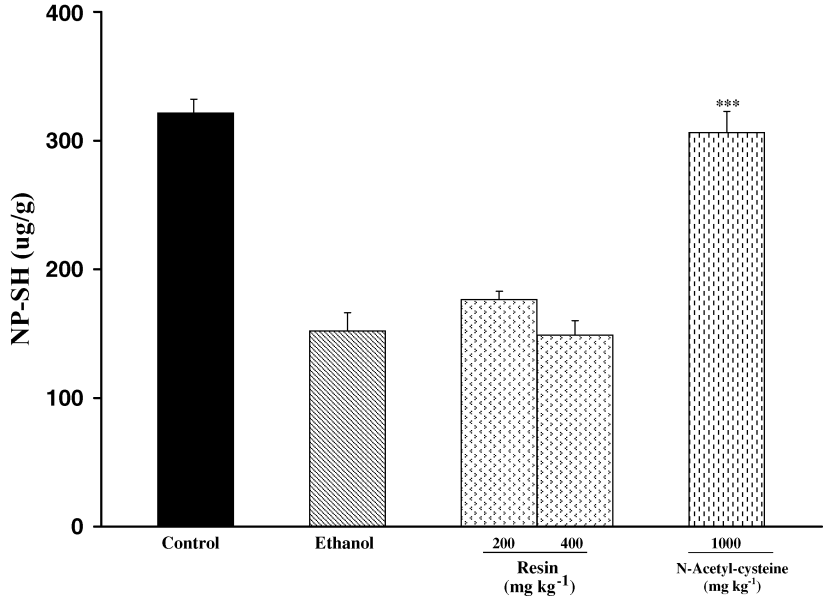

Fig. 2. Effect of resin from *Protium heptaphyllum* and *N*-acetyl-L-cysteine (NAC) on non-protein sulfhydryl (NP-SH) content of gastric tissue in mice. Each column represents the mean ± S.E.M. ($n = 8$). Asterisks indicate significant difference from corresponding control. *** $P < 0.001$ (ANOVA followed by Student–Newman–Keuls test).

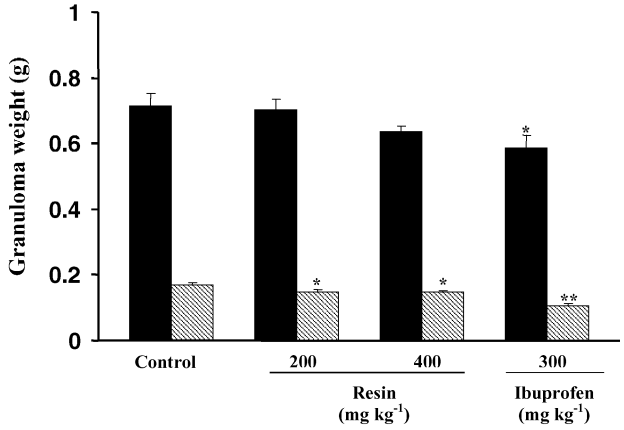

Fig. 3. Effect of resin from *Protium heptaphyllum* and ibuprofen on cotton pellet-induced granuloma wet (filled bars) and dry (hatched bars) weights in mice. Each column represents the mean ± S.E.M. ($n = 8$). Asterisks indicate significant difference from corresponding control. $^{*}P < 0.05$, $^{**}P < 0.01$ (ANOVA followed by Student–Newman–Keuls test).

tively) on the vascular permeability increase induced by intra-peritoneal acetic acid in mice (Fig. 4). However, the resin at 200 mg/kg showed no significant influence on the increase in vascular permeability.

3.7. Acute toxicity

Mice that received the resin at oral doses of 0.5, 0.75 and 1.5 g/kg, did not manifest any clinical signs of toxicity. However, at 3 and 5 g/kg, a mild transient decrease in motor activity starting 10 min after the administration of the test drug was noted. None of the doses tested could produce mortality in mice during the observation period of 72 h.

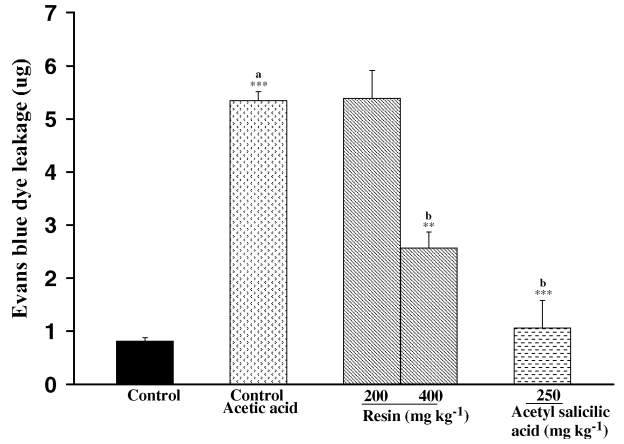

Fig. 4. Effect of resin from *Protium heptaphyllum* and acetylsalicylic acid on acetic acid-induced increase in vascular permeability. Each column represents the mean ± S.E.M. ($n = 8$). Asterisks indicate significant difference from corresponding control. $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA followed by Student–Newman–Keuls test).

4. Discussion

The results of this study showed that the oral application of *P. heptaphyllum* resin prevents gastric damage caused by ethanol or HCl/ethanol, the most commonly employed tests in the evaluation of anti-ulcer/cytoprotective activity [14,26]. It is suggested that oxygen radicals may contribute to the formation of ethanol or acidified ethanol-induced gastric mucosal lesions [27,28] and anti-oxidants are protective against the damage caused by oxidants [18,29,30]. Ethanol has been shown to deplete the level of non-protein sulfhydryl content in stomach tissues and restoration of it appear to be important in gastroprotection since they provide a substrate for hydroxylated and other free radicals to replenish GSH stores [27]. In the present experiments, only NAC but not resin at either dose could restore the ethanol-associated depletion of non-protein sulfhydryls, leaving the possibility of some other different mechanism in its gastroprotection. In the present study, in 4-h pylorus-ligated rats, the resin failed to modify the gastric secretory volume but significantly lowered the total acidity in the stomach, indicating that this effect could be involved in the gastroprotection. It is unlikely that the resin acts as a direct neutralising agent on stomach acidity since it is able to decrease the acid secretion upon intraduodenal administration in pylorus-ligated rats. However, further studies are required to determine its exact mode of gastric acid inhibition. Phytochemical analysis showed the presence of pentacyclic triterpenes like α- and β-amyrins, substances that possess a hydroxyl group at position C-3 that would appear to confer an anti-ulcer activity [12,28]. Past studies have reported the anti-oxidant properties of the natural resins and several triterpenes that belong to α- and β-amyrins and related ones like oleanolic acid, ursolic acid, lupeol and glycyrrhetinic acid [14]. Therefore, we opine that the resin prevents the ethanol-induced gastric damage at least, in part, by this anti-oxidant mechanism.

Inflammation is a very complex process consisting of several unit reactions involving the vascular permeability increase, migration of granulocytes and mononuclear cells, and proliferation of granulation tissue etc. Various types of experimental inflammations are used for evaluating anti-inflammatory potency of drugs. In the present study, we analysed the anti-inflammatory effect of resin in three most widely used experimental models: (i) acute carrageenan-induced foot paw edema; (ii) the vascular permeability increase induced by intraperitoneal acetic acid; and (iii) the cotton pellet-induced granuloma. The results show that the resin is inactive in reducing the carrageenan-induced acute edema but it could effectively inhibit the sub-acute inflammation, as indicated by a significant reductions of granuloma dry weights but not the wet weights. It indicates a more pronounced effectiveness of resin on the chronic inflammatory process rather than on acute inflammatory process and suggests that it should be tested in other animal models of chronic inflammation. Unlike the resin, indomethacin and ibuprofen, the known

anti-inflammatory agents tested showed inhibitory effects not only on the acute 3 h swelling in carrageenan test but also on the development of granuloma in cotton pellet test.

The resin compound did not effectively prevent the edema formation induced by carrageenan but significantly suppressed the increase of vascular permeabilty in response to acetic acid. The development of the increase in vascular permeability induced by acetic acid is known to correspond to the early exudative stage of inflammation. Histamine and serotonin are presumed to play an important roles in the first stage of the acetic acid-induced increase in vascular permeabilty whereas in carrageenan edema, these mediators play a less role [23,25]. Possibly the resin inhibits more potently the mast cell mediators and affect the vascular permeability increase caused by intraperitoneal acetic acid.

The combination of the anti-inflammatory and gastroprotective effects of resin is favourable when taken to account the serious limitations of a large number of anti-inflammatory agents that show a tendency to produce gastric irritation, bleeding and mucosal cellular damage [31,32]. One important feature of resin from *P. heptaphyllum* is its low toxicity. In tests on mice, we found that doses of resin up to 5 g/kg were non-toxic and we were unable to establish its oral LD_{50} value.

In conclusion, although the mechanism of gastroprotection by resin remains to be established, the results may help to provide a scientific basis for its popular use as an anti-infammatory and anti-ulcer agent.

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