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The gastroprotective effect of the essential oil of *Croton cajucara* is different in normal rats than in malnourished rats

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It has been shown previously that malnourished rats are resistant to acute gastric lesions but not to subchronic gastric ulceration. It also has been demonstrated that the essential oil obtained from the bark of *Croton cajucara* (Sacaca) has antiulcer properties. In the present study, the ability of this essential oil to prevent the formation of gastric ulcers in rats fed a diet with 17 % protein (normoproteic rats) or 6 % protein (malnourished rats) was investigated. At a dose of 100 mg/kg body weight, orally, the essential oil significantly reduced the gastric injury caused by indomethacin (25 % after 2 h and 70 % after 4 h) only in normoproteic rats. In the pylorus ligature model, the essential oil increased the pH and gastric volume, but decreased the total acid concentration in both groups when compared to the respective control group. The essential oil significantly increased prostaglandin E2 production in glandular cells by 50 % compared to the controls in both groups of rats. In addition, the amount of gastric mucus was two-fold higher in malnourished rats than in normoproteic rats. The present results show that the enhanced protective effect of essential oil in malnourished rats involved an increase in prostaglandin E2 production and mucus secretion, which are both factors that protect the gastric mucosa against damage. In agreement with this, malnourished rats always had a lower number of acute gastric ulcers.

Antiulcerogenic activity: *Croton cajucara*: Cytoprotection: Essential oil malnourished rats

Undernutrition in man and other mammals is associated with metabolic and developmental alterations, and maternal protein restriction during pregnancy and lactation can adversely affect the offspring (Hales & Barker, 1992). Protein restriction during critical periods of development can lead to generalized growth retardation and a permanent reduction in the size and physiology of organs and tissues, including the pancreas and the stomach (Desai *et al.* 1996). In addition, we have recently shown that gastric ulcerogenesis is different in normal rats than in malnourished rats (Paula *et al.* 2005).

Croton cajucara Benth (*Euphorbiaceae*), popularly named Sacaca, is a well-known medicinal plant used in Amazonian folk medicine to treat several illnesses such as diabetes and liver inflammation (Carvalho *et al.* 1996) and to control high cholesterol (Simões *et al.* 1979; Di Stasi *et al.* 1989). Other medicinal uses include a weak tea (prepared with 5 g stem bark in 100 ml water) to be ingested in cases of heartburn, gastritis and peptic ulcers (Souza Brito & Nunes, 1997). The essential oil (up to 1·5 % of the dry bark) contains primarily sesquiterpenes (Hiruma-Lima *et al.* 2000). Oral administration of the essential oil also has significant antiulcerogenic activity, with no toxicological effects (Hiruma-Lima *et al.* 1999*b*).

Since the protective activity of *C. cajucara* extracts against ulcers has been demonstrated in normal rats but not in malnourished rats, in the present work we used biochemical and histological analyses to compare the antiulcerogenic activity of the essential oil from *C. cajucara* in both of these groups of rats.

Material and methods

Animals

Female Wistar rats (90 d old) were obtained from the animal facilities of the State University of Campinas (UNICAMP). After mating, pregnant females were separated at random and maintained on an isoenergetic diet containing 60 g protein/kg (low-protein diet) or 170 g protein/kg (normal protein diet) from the first day of pregnancy until the end of lactation (Reeves *et al.* 1993). The low-protein and normal protein diets (6 % and 17 % protein, respectively) were based on the AIN-93 report of the American Institute of Nutrition (Reeves *et al.* 1993), which stipulates the dietary standards for nutritional studies with laboratory rodents (Table 1). The experimental protocols were approved by the institutional (UNICAMP)

Abbreviations: EO, essential oil (fraction F1); PGE2, prostaglandin E2; p.o., oral route.

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Animal Care and Use Committee, in accordance with the recommendations of the Canadian Council for Animal Care (Zimmermann, 1983).

During the experiments, the dams were fed *ad libitum* their respective diets and had free access to water.

The rats were housed on a 12 h light/dark cycle at 24°C and the food intake was monitored daily. At 25 d, the pups were weaned and maintained on the same diet as their mothers. After 90 d, normal and malnourished male rats were used to assess the antiulcerogenic activity of the *C. cajucara* essential oil.

Extraction, preparation and analysis of the essential oil

The stem bark of *C. cajucara* was collected at Benfica in the State of Pará, Brazil. A voucher specimen (number 247) identified by Dr Nelson A. Rosa was deposited in the IAN Herbarium, in Belém, Pará. The air-dried and milled bark (20 kg) was subjected to steam distillation for 6 h. A first fraction of 163 ml (F1) was collected after 3 h and a second fraction of 42 ml (F2) was collected at the end of the process. Preliminary GC-FID induction decay FID and ^{6}C-MS analyses done with a Hewlett Packard system (New York, NY, USA) using an HP-5 capillary column showed very similar patterns for the F1 and F2 fractions, which consisted mainly of $C_{15}H_{24}$ sesquiterpenes. The main components of F1 were α-copaene (20·9 %) and cyperene (29·0 %) as confirmed by ^{13}C NMR spectra measured with a Varian spectrometer operated at 75·4 MHz and using benzene-d6 as solvent (Hiruma-Lima *et al.* 1999*b*).

Fraction F1, referred to here as essential oil (EO), was used for the pharmacological tests and was emulsified with 12 % Tween 80 before administration to the rats.

Drugs

The drugs used in the present study were cimetidine (Tagamet®; GlaxoSmithKline, middlesex, UK), and Tween 80®, indomethacin and carbenoxolone (Sigma Chemical Co., St Louis, MO, USA). Indomethacin was prepared in 5 % sodium bicarbonate, and EO was dissolved in 12 % Tween 80. All drugs and reagents were prepared immediately before use.

Table 1. Composition of the normal protein and low-protein diets†

Ingredient	Normal protein (170 g protein/kg)	Low protein (60 g protein/kg)
Casein (840 g protein/kg)	202·0	71·5
Maize starch	397·0	480·0
Dextrinized maize starch	130·5	159·0
Sucrose	100·0	121·0
Soyabean oil	70·0	70·0
Fibre	50·0	50·0
Mineral mix (AIN 93)†	35·0	35·0
Vitamin mix (AIN 93)†	10·0	10·0
L-Cystine	3·0	1·0
Choline chlorhydrate	2·5	2·5

† For detailed composition, see Reeves *et al.* (1993).

Antiulcerogenic activity

Shay ulcers. Forty-two rats (normal and malnourished) were randomly allocated into three groups and fasted for 24 h, with free access to water. The pylorus was ligated, as described by Shay *et al.* (1945), 30 min after the intraduodenal administration of EO (100 mg/kg body weight), cimetidine (100 mg/kg body weight, positive control) or 12 % Tween 80 (10 ml/kg body weight, vehicle). The rats were killed 4 h later, the abdomen was opened, and the stomach was removed and its contents centrifuged at 2000 rpm for 10 min. The supernatant volume and pH were recorded with a digital pH meter (PA 200, Marconi S.A., Piracicaba, Brazil). The total acid content of the gastric secretion was determined by titration to pH 7·0 with 0·05 M-NaOH using a digital burette (E.M.; Hirschmann Technicolor, Eberstadt, Germany). Gastric lesions were counted and scored according to Szelenyi & Thiemer (1978).

Indomethacin ulcers. Forty-two normal and malnourished rats were randomly allocated into three groups and fasted for 24 h, with free access to water, before the experiment. Indomethacin (30 mg/kg) was administered subcutaneously to each group (Hayden *et al.* 1978), 1, 2 and 4 h after the oral administration of EO (100 mg/kg), cimetidine (100 mg/kg) or 12 % Tween 80 (10 ml/kg). The rats were killed 4 h later, and the stomachs were removed, opened and the gastric lesions determined as described earlier.

Assessment of prostaglandin synthesis. Normal and malnourished rats were fasted for 24 h prior to the experiment, which was always done between 09.00 and 11.00 hours. Groups of at least seven rats received one of the following solutions: 12 % Tween 80 (vehicle, orally (p.o.)), indomethacin (20 mg/kg, subcutaneously, positive control) or EO (100 mg/kg). A combination of EO and indomethacin in which EO administration was followed 30 min later by indomethacin, was also tested. The rats were killed 30 min after this treatment, and the abdomen was opened and a sample of the corpus (full thickness) was excised, weighed and then placed in 1 ml 10 mM-sodium phosphate buffer, pH 7·4. The tissue was finely minced and then incubated at 37°C for 20 min. Prostaglandin E2 (PGE2) in the buffer was measured by enzyme immunoassay (EIA RPN222; Amersham, Freiburg, Germany), with the absorbance being read at 450 nm (Curtis *et al.* 1995).

Determination of gastric wall mucus. Gastric wall mucus was determined according to Rafatullah *et al.* (1999). Forty-eight rats (normal and malnourished) were randomly divided into three groups and fasted for 48 h, with free access to water. Each group was treated with EO (100 mg/kg, p.o.), carbenoxolone (200 mg/kg, p.o.) or 12 % Tween 80 (10 ml/kg, p.o.). After 1.5 h, the pylorus was ligated as previously described. The rats were subsequently killed and the stomach was removed and opened to reveal glandular tissue that was cut into segments, weighed and immersed for 2 h in 10 ml 0·1 % (w/v) Alcian blue dissolved in 0·16 M-sucrose solution buffered with 0·05 M-sodium acetate, pH 5·8. Excess dye was removed by washing the segments twice with a 0·25 M-sucrose solution for 15 and 45 min, respectively. The mucus–dye complex was extracted using 10 ml 0·5 M-magnesium chloride with intermittent shaking for 1 min at 30 min intervals for 2 h. Blue extract (4 ml) was mixed with an equal volume of diethyl ether and shaken vigorously for 2 min. The emulsion obtained was centrifuged for

10 min at 3600 rpm and the absorbance of the aqueous layer was measured at 580 nm. The amount of Alcian blue extracted per gram of wet glandular tissue was calculated by comparison with a standard curve of dye.

Histopathological analysis. For histopathological analysis, the stomachs of rats with indomethacin-induced ulcers were removed and the samples collected were fixed in Bouin solution for 24 h. After fixation, the tissues were processed by standard histological procedures that included dehydration in an ethanol gradient (70–100 %) followed by clearing in xylol and embedding in Histosec (11 609; Merck São Paulo, Brazil). Sections (5 μm thick) were mounted on glass slides and stained with haematoxylin-eosin (Yoshitake *et al.* 1991). Photomicrographs were obtained with an Axiophot photomicroscope (D-7082; Carl Zeiss).

Statistical analysis

The results are expressed as means and their standard errors. Statistical comparisons were done using one-way ANOVA followed by the Tukey or Scheffé tests, with the level of significance being set at $P<0{\cdot}05$ and $P<0{\cdot}001$. All statistical analyses were done using Systat software (version 5.0 Richmond, CA, USA).

Results

In the pylorus ligature test, the intraduodenal administration of EO (100 mg/kg body weight) produced a significant increase in gastric juice volume and pH ($P<0{\cdot}05$ and $P<0{\cdot}001$, respectively), and a significant ($P<0{\cdot}001$) decrease in gastric acid content in normal and malnourished rats (Table 2). The present results were very similar to those obtained for pretreatment with the positive control (cimetidine) at the same dose.

The oral administration of EO (100 mg/kg body weight) 1, 2 and 4 h before indomethacin inhibited the appearance of gastric lesions induced by this drug (Table 3) in normoproteic rats, although this protection was significant ($P<0{\cdot}05$) only for the latter four time intervals. Malnourished rats had a lower number of ulcers ($P<0{\cdot}05$) than normalproteic rats both without and with this treatment, i.e. EO provided additional protection against gastric lesions.

As shown in Table 4, EO increased the PGE2 production by gastric tissue in normal and malnourished rats, compared to a significant decrease in normal and malnourished rats treated with indomethacin alone. When EO and indomethacin were given together, the increase in PGE2 previously seen with EO was completely abolished in normoproteic rats. In contrast, in malnourished rats, EO still increased the PGE2 production in the presence of indomethacin. The greater protection against indomethacin seen in malnourished rats probably reflected enhanced synthesis of PGE2 and/or mucus.

Table 5 shows that the EO of *C. cajucara* almost doubled the production of free mucus by the gastric mucosa of normal and malnourished rats, although the latter group had a greater production of this defensive factor ($P<0{\cdot}001$). The positive control carbenoxolone, an antiulcerogenic drug obtained from *Glycyrrhiza glabra*, also increased the mucus production of the gastric mucosa. The additional production of gastric wall mucus seen for EO treatment in malnourished rats could be responsible for its complete effect of indomethacin observed in previous experiments (Table 4).

The histopathological analysis of the stomach (Fig. 1) showed that in normoproteic rats the lesions reached the muscularis mucosa layer (Fig. 1(A)), whereas in malnourished rats the injury was restricted to the superficial epithelium (Fig. 1(B)); in both of these cases, the rats were pretreated with Tween 80. Pretreatment with EO (100 mg/kg body weight) protected the gastric mucosa against erosion induced by indomethacin in normoproteic (Fig. 1(C)) and malnourished (Fig. 1(D)) rats, when both were compared with the sham group. The gastric mucosa of normoproteic (Fig. 1(E)) and malnourished (Fig. 1(F)) rats was preserved.

Discussion

Protein malnutrition results when the body's needs for protein cannot be satisfied by the diet. Since all normal metabolic pro-

Table 2. Effects of essential oil (100 mg/kg body weight) given intraduodenally (i.d.) on the biochemical parameters of gastric juice obtained from pylorus-ligated normal and malnourished rats (seven per group)†

(Mean values with their standard errors)

	pH (units)		Gastric juice (ml)		Total gastric juice (mEq/ml per 4 h)	
Treatment (i.d.)	Mean	SEM	Mean	SEM	Mean	SEM
Normal rats						
Control	3·17	0·20	1·18	0·27	7·57	0·27
Cimetidine	6·00**	0·17	4·37**	0·24	4·93**	0·24
Essential oil	5·50*	0·15	3·83*	0·27	5·96**	0·27
Malnourished rats						
Control	2·86	0·17	4·05	0·31	5·00	0·31
Cimetidine	4·17**	0·20	5·21**	0·31	4·40*	0·40
Essential oil	3·94*	0·17	3·91*	0·40	3·36**	0·30

Mean values were significantly different from those of the control group (Tukey test): *$P<0{\cdot}05$, **$P<0{\cdot}001$; following ANOVA ($P<0{\cdot}05$): pH $F_{(5,35)}=6{\cdot}90$, gastric juice volume $F_{(5,40)}=3{\cdot}70$, total acid gastric $F_{(5,39)}=3{\cdot}81$.

† For details of procedures, see p. 310.

Table 3. Effects of an oral dose (100 mg/kg body weight) of *Croton cajucara* essential oil given 1, 2 and 4 h before the induction of gastric ulcers by indomethacin in normoproteic and malnourished rats (seven per group; the ulcerative index of indomethacin gastric lesions was measured in mm)†

(Mean values with their standard errors)

	Tween		Essential oil	
Animals	Mean	SEM	Mean	SEM
Normoproteic (1 h)	15	0·23	13	0·19
Malnourished (1 h)	7*	0·45	6*	0·38
Normoproteic (2 h)	14	0·29	12	0·56
Malnourished (2 h)	6*	0·51	6*	0·67
Normoproteic (4 h)	18	0·68	7*	0·78
Malnourished (4 h)	8*	0·76	8*	0·78

Mean values were significantly different from those of normoproteic rats treated with 12 % Tween 80 (Scheffé test): *$P<0{\cdot}05$; following ANOVA: $F_{(11,72)}=2{\cdot}68$.

† For details of procedures, see p. 310.

Table 4. Effects of the oral administration of *Croton cajucara* essential oil and indomethacin on gastric prostaglandin E2 production (pg/g wet tissue) in malnourished and normoproteic rats (seven per group)†

(Mean values with their standard errors)

	Normoproteic		Malnourished	
	Mean	SEM	Mean	SEM
Tween	40	0·31	100	0·36
Sham	38·8	0·42	99·8	0·42
Essential oil	100**	0·44	140·2**	0·21
Indomethacin	26*	0·16	29*	0·17
Essential oil + indomethacin	28*	0·18	58*	0·22

Mean values were significantly different from those of the normoproteic group (Tukey test): *$P<0·05$, **$P<0·001$; following ANOVA ($P<0·05$): $F_{(4,30)} = 27·4$.
† For details of procedures, see p. 310.

Table 5. Effects of *Croton cajucara* essential oil and carbenoxolone (positive control) on the production of gastric wall mucus (g) in malnourished and normoproteic rats (eight per group)†

(Mean values with their standard errors)

	Tween		Carbenoxolone		Essential oil	
Animals	Mean	SEM	Mean	SEM	Mean	SEM
Normoproteic	0·8	0·31	1·8*	0·52	1·6*	0·22
Malnourished	1·8*	0·18	3·2**	0·81	2·7**	0·32

Mean values were significantly different from those of the normoproteic group (Tukey test): *$P<0·05$, **$P<0·001$; following ANOVA ($P<0·05$): $F_{(7,16)} = 6·58$ for normoproteic rats and $F_{(7,16)}$ for malnourished rats.
† For details of procedures, see p. 310.

cesses require proteins, all tissues will be affected by a state of protein deprivation. The first tissues to be affected in such circumstances are those with a high rate of cellular turnover, such as the intestinal mucosa, whereas the last tissues to suffer alterations are those with a low rate of cellular renewal, such as the nervous system. In organs such as the stomach and pancreas, there is a significant loss in weight and size (Sant'Ana *et al.* 1997).

We previously examined the effects of perinatal protein restriction on the gastrointestinal tract. After weaning, body weight gain was significantly higher in normal rats than in malnourished rats, indicating that maternal protein restriction during pregnancy and lactation adversely affected the offspring. Malnourishment affects the digestive system in early life and may exert long-term effects on body weight, and on organ weight in early life, compared to normoproteic rats; the ulcerogenic process is also different in normal rats than in malnourished rats (Paula *et al.* 2005).

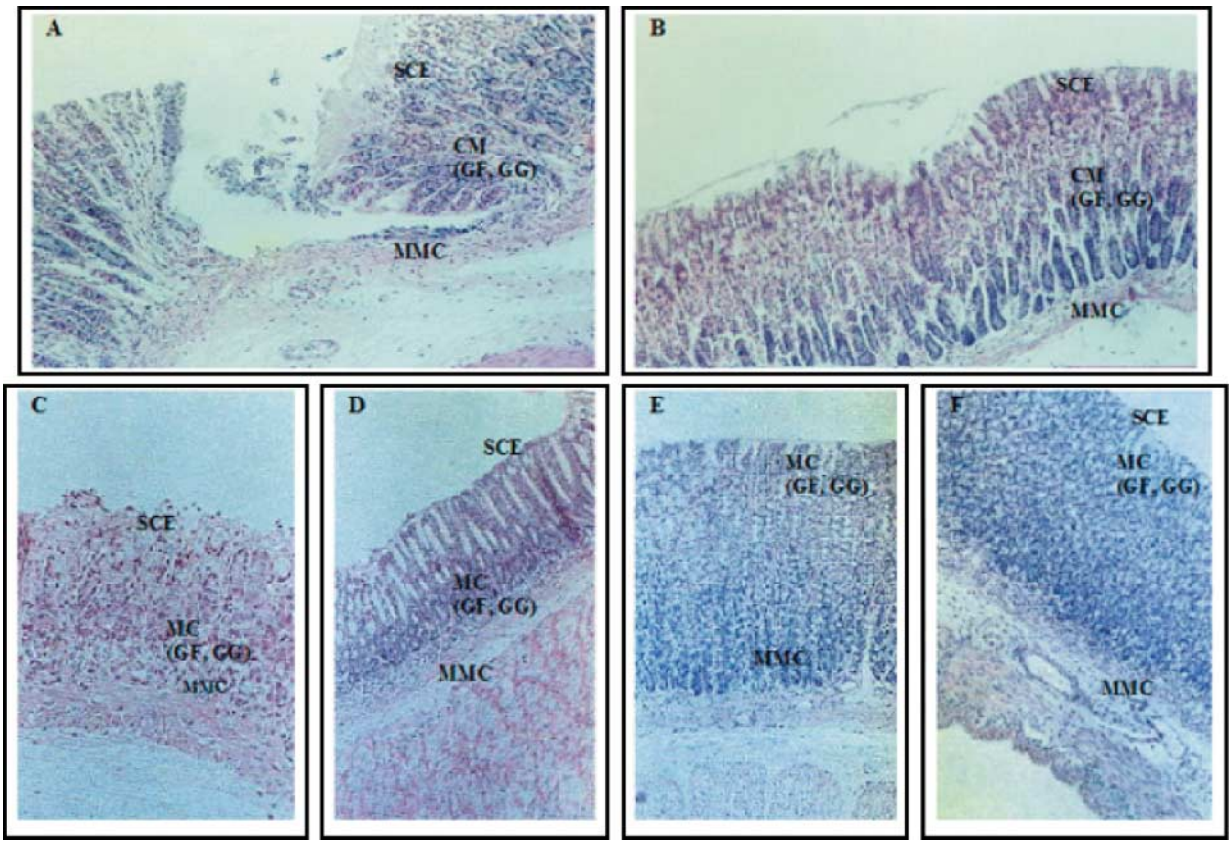

Fig. 1. Photomicrographs (haematoxylin-eosin, 400 ×) showing erosion of the gastric mucosa (pyloric region) induced by acute treatment (indomethacin) in normal (A) and malnourished (B) rats pretreated with Tween 80 (negative control); in normal (C) and malnourished (D) rats pretreated with *Croton cajucara* essential oil (100 mg/kg body weight, p.o.); and in normal (E) and malnourished (F) rats of the sham group. For details of procedures, see p. 310. The superficial coating epithelium (SCE), the mucosal coat (CM) and muscularis mucosal (MMC) coat were destroyed in normoproteic rats. In malnourished rats only the SCE was destroyed. GF, gastric fossets; GG, gastric gland.

In healthy human stomach and duodenum, there is an effective balance between the potential for gastric acid and pepsin to damage gastric mucosal cells and the ability of these cells to protect themselves from injury (Bagchi *et al.* 1999). Disruption of this balance results from a breakdown in the normal mucosal defence mechanisms (Schubert, 2004). A severe decrease in gastric mucosal blood flow after treatment with indomethacin, a typical non-steroidal anti-inflammatory that inhibits cyclooxygenase, results in reduced mucosal prostaglandin levels and a decrease in mucosal blood circulation (Takeeda *et al.* 2003).

Since ulcerogenesis is different in normoproteic rats than in malnourished rats, we sought to determine whether there were also differences in the antiulcerogenic effects of a well-studied agent in these two groups of rats. To address this question, we initially studied the antiulcer properties of EO obtained from *C. cajucara* bark that we had used before in different models of gastric ulcers in rats (Hiruma-Lima *et al.* 1999*a*,*b*, 2000, 2002).

There is evidence of an involvement of prostanoids in the accumulation of fluid in the gastric lumen, with PGE2 significantly increasing the volume flow in the stomach (Wallace, 1992). During experimental ulceration, a fully developed inflammatory response leads to cellular infiltration, extracellular matrix proliferation and, possibly, the establishment of a new microvascular supply. Takeeda *et al.* (2004) suggested that endogenous prostaglandins derived from COX-1 and COX-2 were involved in the mucosal defence of the inflamed stomach, partly by decreasing acid secretion, which helped to maintain the mucosal integrity under such conditions. Hence, the effect of EO after pylorus ligature in normal and malnourished rats could be explained by increased PGE2 production.

Filaretova *et al.* (2004) demonstrated a gastroprotective action of glucocorticoids in gastric lesions induced by indomethacin, and concluded that glucocorticoids may provide protection by multiple actions, including maintenance of the gastric mucosal blood flow and mucus production, attenuation of the enhanced gastric motility and microvascular permeability, and a beneficial effect on healing. Our previous study also showed that malnourished rats had elevated plasma corticosterone levels when compared to normal rats, probably as a result of stress during pregnancy and lactation (Paula *et al.* 2005). These elevated plasma corticosterone levels could explain why malnourished rats had a lower number of ulcers than normal rats.

Prostaglandins play an important physiological role in maintaining the integrity of the gastric mucosa. Non-steroidal anti-inflammatories that inhibit cyclooxygenase, the key enzyme in prostaglandin formation, induce gastric mucosal injury in rodents and man (Singh & Majumdar, 1999). Exogenous prostaglandins protect the gastric mucosa against various types of damage, including gastric ulcers caused by necrotizing agents. In addition, prostaglandins play an important role in the healing of gastric ulcers. Prostaglandins of the E series have an inhibitory action on acid output and maintain the integrity of the gastric mucosa (Selye & Szabo, 1973; Rainsford, 1978). Hence, the protection by EO against indomethacin-induced injury in the gastric mucosa of normoproteic rats may be mediated by increased prostaglandin synthesis and/or release.

In conclusion, the present results indicate that EO protects against gastric ulcers, mainly by increasing PGE2 and mucus production. This protection was seen in normoproteic and malnourished rats. However, a direct comparison of the effects of EO in normal and malnourished rats is not possible because the level of gastric ulcers in malnourished rats is significantly higher than in normal rats.

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