

Constituents and Antiulcer Effect of *Alchornea glandulosa*: Activation of Cell Proliferation in Gastric Mucosa during the Healing Process

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Alchornea glandulosa (Euphorbiaceae) is a plant used in folk medicine as an antiulcer agent. Rats pretreated with methanolic extract obtained from the leaves of *A. glandulosa* (AG) showed a dose-dependent effect and significant reduction of gastric ulcers induced by absolute ethanol at the doses of 500 (57%) and 1000 mg/kg (85%) in relation to the control group. Pretreatment of mice with AG (500, 1000 mg/kg, *p.o.*) showed dose-dependent activity and significantly decreased the severity of lesions caused by HCl/ethanol and by non steroidal anti inflammatory drug-induced gastric lesions. Pretreatment with AG also induced antisecretory action *via* local and systemic routes and a significant decrease in the total gastric acid content. The gastroprotective effects of AG involved the participation of nitric oxide and increased levels of endogenous sulfhydryl compounds, which are defensive mechanisms of the gastrointestinal mucosa against aggressive factors. The ability of AG to heal gastric ulcers was evaluated after 14 consecutive days of treatment. The results showed that single oral administrations of AG (250 mg/kg/once daily) potently stimulates gastric epithelial cell proliferation that contributes to the accelerated healing of gastric ulcers induced by acetic acid. In addition, no subacute toxicity (body weight gain, vital organs, and serum biochemical parameters) was observed during treatment with AG. Phytochemical investigation of AG led to the isolation of myricetin-3-*O*- α -L-rhamnopyranoside, quercetin-3-*O*- α -L-arabinopyranoside, quercetin-3-*O*- β -D-galactopyranoside, quercetin, amentoflavone, methyl gallate, gallic acid, and pterogynidine. We also established the phytochemical profile of AG with the quantification of total phenolic compounds. These compounds may contribute to the observed antiulcerogenic effects of AG.

Key words *Alchornea glandulosa*; proliferating cell nuclear antigen (PCNA); antiulcer activity; phenolic compound

Alchornea glandulosa POEPP. & ENDL. (Euphorbiaceae) is a tree of 10–20 m popularly known as tapiá, tanheiro-de-folha redonda, tanheiro, or canela-raposa. It is found in the southern and southwestern regions of Brazil, mainly in the Atlantic pluvial forest.¹⁾ An ethnopharmacologic survey indicated that some species from the genus *Alchornea* are commonly used for the treatment of gastric diseases.²⁾ Despite the common use of this species as a medicinal plant, there are no data on its pharmacologic effects on the gastrointestinal system or its possible toxic properties.

The literature reports that *A. glandulosa* contains sitosterol, stigmasterol, the terpenoid loliolide, the guanidine alkaloid *N*-1,*N*-2,*N*-3-trisopentenylguanidine, the tannin coriagin, gallic acid, ethyl gallate, and the flavonoids quercetin-3-*O*- α -L-rhamnoside, kaempferol-3-*O*- α -L-rhamnoside, and myricetin-3-*O*- α -L-rhamnoside.^{3,4)}

Concerning the pharmacologic effects, crude extracts of *Alchornea cordifolia* demonstrated antimicrobial, antiamoebic, antispasmodic, and antiarrhythmic activities.^{5–8)} Activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Escherichia coli* was found in the methanolic extract of the leaves of *A. cordifolia*, which contain phenolic compounds and terpenoids.⁹⁾ The chloroformic fraction from the methanolic extract of the leaves of *Alchornea sidifolia*

showed the presence of triterpenoids and steroids with antifungal activity.¹⁰⁾ *A. cordifolia* exhibited trypanocidal activity and activity against *Plasmodium falciparum*.^{11,12)} The observed effects were correlated with the presence of ellagic acid.¹³⁾ In addition to flavonoids with muscle-relaxant activity, *Alchornea latifolia* contains pentacyclic triterpenoids with cytotoxic activity against the cancer cell lines Hep-G2 and A-431 and potently inhibits topoisomerase II.¹⁴⁾ Sulphated quercetin derivatives with activity against Gram-positive and Gram-negative bacteria as well as against mushrooms were isolated from *Alchornea laxiflora*.¹⁵⁾

The present study was conducted to evaluate the antiulcer activity of the methanolic extract from the leaves of *A. glandulosa* (AG) using different *in vivo* experimental models in rodents, followed by its phytochemical investigation.

MATERIALS AND METHODS

Drugs Absolute ethanol, hydrochloric acid, acetic acid (Synth, Brazil), *N*-omega-nitro-L-arginine methyl ester (L-NAME), carbenoxolone, cimetidine, *N*-ethylmaleimide (NEM) (Sigma Chemical Co., St. Louis, MO, U.S.A.), lansoprazole and piroxicam (Hexal, Brazil), PCNA mouse monoclonal antibody (Novo Castra NCL-PCNA) (1:100), and

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avidin and biotin complex (ABC) reagents (ABC kit-Vector) were used in this study. All drugs and reagents were prepared immediately before use, and the reagents used were of analytical grade.

Animals Male Swiss albino mice (25–35 g) and male Wistar albino rats (150–250 g) from the UNESP Central Animal House were used. The animals were fed a certified Nuvelab (Nuvital) diet with free access to tap water under standard conditions of 12 h dark–12 h light, humidity ($60 \pm 1.0\%$), and temperature ($21 \pm 1^\circ\text{C}$). Fasting was imposed prior to all assays because standard drugs and extract were always administered orally (by gavage) or intraduodenally using a saline solution (10 ml/kg) as the vehicle. Animals were kept in cages with raised floors of wide mesh to prevent coprophagy. All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care. The UNESP Institutional Animal Care and Use Committee approved all of the protocols employed.¹⁶⁾

Plant Material Leaves of *A. glandulosa* POEPP & ENDL were collected at Piracicaba, São Paulo State, Brazil, in June 2003 and authenticated by Prof. Jorge Tamashiro. A voucher specimen (n°. 132828) was deposited in the Herbarium of the University of Campinas.

Extraction Leaves (500 g) of *A. glandulosa* were air-dried (7 d at 40°C) and powdered. The powdered dried leaves were exhaustively extracted with chloroform and methanol successively at room temperature (three times, 48 h for each solvent). Extracts were concentrated in a vacuum, yielding 21 g (4.2%) and 59 g (11.8%) of residues, respectively.

Phytochemical Analysis An aliquot of the methanol extract from *A. glandulosa* (AG) (3 g) was further dissolved in 10 ml of methanol and centrifuged; the supernatant was submitted to Sephadex LH-20 (Pharmacia) column chromatography (100×5 cm) using methanol as an eluent. Fractions of 8 ml were collected and combined after thin-layer chromatography (TLC) analysis to yield 24 fractions. Silica gel SiF254 (Merck) plates were eluted with chloroform:methanol (8:2, v/v), visualized using UV light (254, 365 nm), and further sprayed with anisaldehyde/sulfuric acid solution.¹⁷⁾

An aliquot of the methanol extract (30 mg) was dissolved in 5 ml of water:methanol (8:2, v/v) and filtered in a Sep-Pak cartridge (Sigma, C₁₈). The cartridge was washed with 5 ml of water:methanol (1:1, v/v) and methanol successively. Fractions were analyzed with TLC using silica gel plates on glass (SiF254, Merck) developed with a solvent mixture composed of chloroform:methanol:*n*-propanol:water (5:6:1:4, v/v/v/v lower phase). The spots on the TLC plates were observed under UV light (254 nm). The methanol fraction contained mainly phenolic compounds, especially flavonoids. The methanolic fraction was filtered in a Millex filter (0.45 μm) and analyzed using a Varian ProStar HPLC system equipped with an RP-18 column (250 $\times$ 4.60 mm i.d., 5 μm , Phenomenex Luna). The mobile phase was water (A) and acetonitrile (B), both with 0.05% trifluoroacetic acid, in linear gradient elution of 30 to 70% B in 60 min at a flow rate of 1.0 ml/min. The effluent was monitored using a ProStar 330 photodiode-array UV detection (UV-PDA) system at 210 nm.

The total phenolic compounds of AG were quantitatively determined using HPLC analysis. A stock solution (1 mg/ml) of rutin was prepared in methanol. The calibration curve was constructed with seven different concentrations (10, 20, 50, 100, 200, 300, 500 $\mu\text{g/ml}$ in rutin) and analyzed in duplicate. The peak areas were correlated with the concentrations according to the calibration curve.

A calibration curve using the external standard rutin was constructed to determine the total concentration of flavonoids. The calibration curve was linear over the range of 10–500 $\mu\text{g/ml}$ with a correlation coefficient of 0.9999. All data presented are mean $\pm$ standard deviation of four independent experiments ($n=4$).

Antioxidant Activity The antioxidant activity of AG was evaluated using TLC under the conditions described above. Plates were visualized with 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent as well as β -carotene solution.¹⁸⁾

Acute Toxicity The acute toxicity studies were performed in mice. In this assay, a single dose of AG was orally administered to groups of 10 animals after a 12 h fast. Another group of animals receiving the vehicle (saline) served as controls. The signs and symptoms associated with AG administration (5 g/kg, *p.o.*) were observed at 0, 30, 60, 120, 180, and 240 min after administration and then once a day for the next 14 d. The mortality, measured body weight, and behavioral screening were recorded at 24 h and for 14 d after treatment. The macroscopic analyses and weight of vital organs such as liver, kidney, heart, and lung were compared between the animals treated with AG and vehicle. The acute toxicologic effects were estimated using the method of Souza-Brito.¹⁹⁾

Intestinal Motility Swiss mice were fasted for 6 h with water *ad libitum*. The experimental group was treated with AG at doses of 250, 500, and 1000 mg/kg; the control group was administered saline (10 ml/kg) and the positive control atropine (5 mg/kg). Thirty minutes after the treatments, animals received a suspension of 10% active charcoal in water. After 30 min, the animals were killed and intestines removed. The total length of the intestine and the distance traveled by the charcoal suspension were measured.²⁰⁾

Antilcerogenic Activity. Ethanol-Induced Ulcer This experiment was performed as described by Morimoto *et al.*²¹⁾ Rats were divided into 5 groups of 4 or 5 animals that were fasted 24 h prior to receiving an oral dose of the vehicle, saline (10 ml/kg), lansoprazole (30 mg/kg), or AG (250, 500, 1000 mg/kg body wt). After 60 min, all groups were orally treated with 1 ml of absolute ethanol for gastric ulcer induction. Animals were killed 1 h after the administration of ethanol. The extent of the lesions was measured and the lesion index expressed as the sum of all lesions.²²⁾

HCl/Ethanol-Induced Ulcer The experiments were performed according to the description of Mizui and Doteuchi with modification.²³⁾ Mice were divided into groups of 5–8 animals that were fasted 24 h prior to receiving an oral dose of the vehicle, saline (10 ml/kg), lansoprazole (30 mg/kg), or AG (250, 500, 1000 mg/kg). After 50 min, all groups were orally treated with 0.2 ml of a 0.3 mol/l HCl/60% ethanol solution (HCl/ethanol) for gastric ulcer induction. Animals were killed 1 h after the administration of HCl/ethanol and the gastric damage was determined as described above.

NSAID Gastric Ulcers in Mice In this model, gastric

lesions were induced with piroxicam (30 mg/kg, s.c.) administered to mice ($n=5$) after a 36 h fast. AG (250, 500, 1000 mg/kg), cimetidine (100 mg/kg), and saline were orally administered 30 min before the induction of gastric lesions. Animals were killed 4 h after treatment with the ulcerogenic agent. The stomachs were removed and gastric lesions determined as described above.²⁴⁾

Shay Ulcer Mice were randomly divided into three groups ($n=7$ or 8) that were fasted for 24 h with free access to water. Thirty minutes or immediately after oral administration of a single dose of AG (1000 mg/kg), cimetidine (100 mg/kg) as a positive control, or vehicle (saline), pylorus ligation was performed as described by Shay *et al.*²⁵⁾ The animals were killed 4 h later, the abdomen opened and another ligature placed around the esophagus close to the diaphragm. The stomach was removed, inspected internally, and its content was drained into a graduated tube and centrifuged at 3000 rpm for 15 min. The supernatant volume and pH were recorded with a digital pH meter. The total acid content of gastric secretion was also determined by titration to pH 7.0 with 0.01 N NaOH using a digital burette.

Determination of Gastric Secretion The assay was performed using the method of Shay *et al.* with a few modifications.²⁵⁾ All groups of mice ($n=5-9$) were fasted for 24 h, with free access to water. Immediately after pylorus ligation, AG (1000 mg/kg), cimetidine (100 mg/kg) as positive control, or vehicle (saline 10 ml/kg) was administered intraduodenally. The animals were killed 4 h later 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 to determine the total amount of gastric juice acid (ml) and pH values. Distilled water was added and the resultant solution centrifuged at 3000 rpm for 10 min. Total acid in the gastric secretion was determined in the supernatant volume by titration to pH 7.0 with 0.01 N NaOH.

Ethanol-Induced Gastric Lesions in NEM-Pretreated Rats²⁶⁾ Rats were divided into groups of 4 or 5 animals that were fasted for 24 h. They had been previously treated intraperitoneally with NEM 10 mg/kg or saline, and 30 min later received an oral dose of vehicle (10 ml/kg), AG (1000 mg/kg), or carbenoxolone (100 mg/kg). After 60 min, all groups were orally treated with 1 ml of absolute ethanol for gastric ulcer induction. Animals were killed 1 h after the administration of ethanol, and the stomachs excised and gastric injury determined as described above.

Ethanol-Induced Gastric Lesions in L-NAME-Pretreated Rats Rats were divided into groups (7 animals) and fasted for 24 h. Animals were treated with L-NAME 70 mg/kg or saline by the i.p. route, and 30 min later received an oral dose of vehicle (10 ml/kg), AG (1000 mg/kg), or carbenoxolone (100 mg/kg). After 60 min, all groups were orally treated with 1 ml of absolute ethanol for gastric ulcer induction. Animals were killed 1 h after the administration of ethanol, and the stomachs excised and gastric lesions determined.²⁶⁾

Healing of Acetic Acid-Induced Gastric Lesions The experiments were performed according to the method described by Takagi *et al.*, with some modifications.²⁷⁾ Three groups of male Wistar rats that had fasted for 24 h were used in this experiment ($n=6-8$). Under anesthesia, a 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 (20 °C) to avoid adherence to the external surface of the ulcerated region. The abdomen was then closed and all animals were fed normally. We selected the lowest dose of AG (250 mg/kg), cimetidine (100 mg/kg) administered or vehicle (10 ml/kg) to determine the healing effects of subacute treatment. All treatments were orally once a day for 14 consecutive days beginning 1 d after surgery. Body weight was recorded daily throughout the experiments and the macroscopic analyses and weight of vital organs (liver, kidney, heart, spleen, and lung) were compared among the different treatments to evaluate the possible subacute toxicity they may have induced. On the day after the last drug administration, the rats were killed and their stomachs removed. The gastric lesions were evaluated by examining the inner gastric surface with a dissecting magnifying glass. The macroscopic ulcer area (mm²) and curative ratio (%) were subsequently determined.²⁷⁾

Biochemical Analysis The blood of rats submitted to different treatments was collected immediately after death and centrifuged (3000 rpm for 10 min). After centrifugation, the serum obtained was kept frozen at -20 °C until biochemical analysis. Serum biochemical parameters such as urea, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured with an automated biochemical analyzer.

Histology Methods The stomach of the rats submitted to gastric ulcers in the acid acetic model with different treatments were isolated and opened at the greater curves and the lesions were located. The lesions were sectioned and fixed in alcohol, chloroform, and acetic acid (ALFAC) for 24 h at 4 °C. Then the samples were routinely processed for embedding in paraffin, and cut into 7- μ m-thick sections. These sections were stained with hematoxylin and eosin and observed under a microscope.

Morphometric Analysis For the morphometric analysis a stomach slice was examined by microscopy, using the method described by Ishihara and Ito.²⁸⁾

Immunohistochemistry Proliferating cell nuclear antigen (PCNA) was employed to determine proliferating cells; for this the representative slices were deparaffinized, rehydrated, and immunostained using the peroxidase anti-peroxidase method. The high-temperature antigen unmasking technique was performed in 0.01 mol/l of citrate buffer, pH 6.0, in a microwave oven, two times for 5 min. Blocking of non-specific reaction was done with 1% normal goat serum, 3% non fat milk, and PCNA mouse monoclonal antibody. After rinsing in phosphate-buffered saline (PBS) 0.01 mol/l, pH 7.4, the sections were incubated in secondary antiserum. They were then washed in PBS, incubated with ABC reagents, and subjected to in peroxidase reaction (3,3'-diaminobenzidine tetrahydrochloride containing 0.01% H₂O₂ in PBS buffer). After immunostaining, the sections were lightly counterstained with Mayer's hematoxylin. The slides were observed under a light microscope.

Statistical Analysis Results are expressed as mean $\pm$ S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's or Tukey's test,

with the level of significance at $p < 0.05$.

RESULTS AND DISCUSSION

Peptic ulcers that affect a considerable number of people globally. After 1900, physicians realized that the incidence of peptic ulcer was more prevalent than previously supposed.²⁹⁾ Stress, smoking, nutritional deficiencies and ingestion of NSAIDs increase gastric ulcer incidence.³⁰⁾ Products on the market for the treatment of gastric ulcers, including antacids, proton pump inhibitors, anticholinergic, and histamine H₂-antagonists, can produce several adverse reactions.³¹⁾ Brazil has abundant biodiversity still underexplored. Based on a previous survey, in this work we investigated the effect of extracts of *A. glandulosa* on gastric lesions induced by several agents.

Fractionation of the methanolic extract of *A. glandulosa* (AG) with GPC followed by purification on HPLC led to the isolation of gallic acid **1**, methyl gallate **2**, the flavonoids myricetin-3-*O*- α -L-rhamnopyranoside **3**, quercetin-3-*O*- β -D-galactopyranoside **4**, quercetin-3-*O*- α -L-arabinopyranoside **5**, quercetin **6**, the biflavonoid amentoflavone **7**, and the alkaloid pterogynidine **8**, identified by comparison of their spectroscopic data with those reported in the literature (Fig. 1).^{32–35)}

Figure 2 presents the chromatogram and UV spectra of a fraction containing mainly phenolic compounds from AG using HPLC-UV-PDA. Identification of the peaks was performed by co injecting the standards available from a collection in our laboratory and by comparison with the UV spectral data obtained from the literature.³⁶⁾ The analysis of the retention time of the compounds compared with the respective standards and UV data (Fig. 2) suggested that peaks **1** ($16.69 \pm 0.09\%$) and **2** ($10.99 \pm 0.18\%$) are gallic acid and methyl gallate, respectively. Peaks **7** ($5.99 \pm 0.08\%$) and **9** ($7.34 \pm 0.04\%$) are amentoflavone and an amentoflavone derivative; peaks **3** ($16.22 \pm 0.12\%$), **4** ($17.25 \pm 0.03\%$), **5** ($21.07 \pm 0.12\%$), and **6** ($3.25 \pm 0.13\%$) are myricetin 3-*O*- α -L-rhamnopyranoside, quercetin 3-*O*- β -D-galactopyranoside, quercetin 3-*O*- α -L-arabinopyranoside, and quercetin, respectively. The amount of total phenolic compounds was 10% (98.81 ± 0.05 mg/g) in the methanolic extract. Peaks **8** and **10** correspond to pterogynidine and a guanidine alkaloid derivative, respectively.

As part of this pharmacologic study, the acute toxicity in AG mice was first investigated. A single oral administration of AG up to a dose of 5000 mg/kg did not produce any signs or symptoms of acute toxicity in the treated animals. During the 14 d following the administration of AG, no animal died and no significant changes in daily body or organ weight were observed until the end of this period (Table 1). At autopsy, no significant changes or lesions were observed in the internal organs of any animal. These findings indicate that acute administration of AG (5000 mg/kg, *p.o.*) in mice is safe.

Ethnopharmacologic information on the posology of *A. glandulosa* used in folk medicine is inconsistent. Souza-Brito reported that active principles from medicinal plants are generally found in low concentrations.³⁷⁾ Amounts higher than 1% are the exception. Since the maximum dose used to perform the acute toxicity assay was 5000 mg/kg of AG, a five fold lower dose (1000 mg/kg) was selected as the maxi-

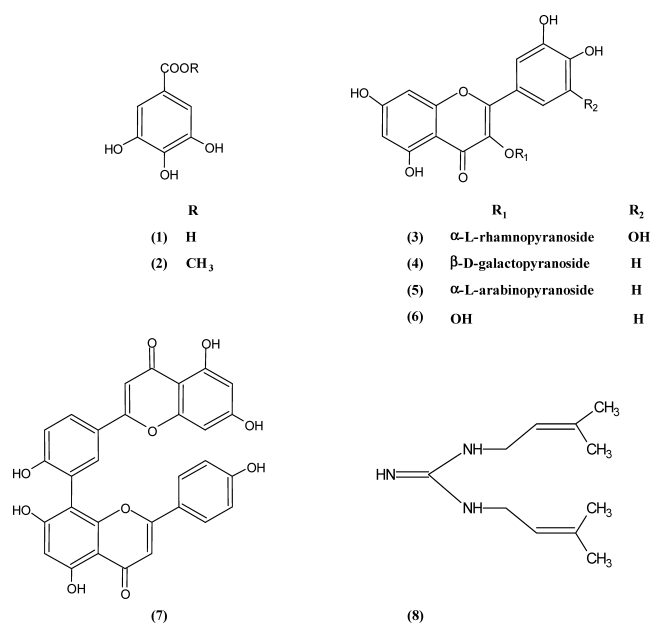


Fig. 1. Chemical Structure of *Alchornea glandulosa* Constituents

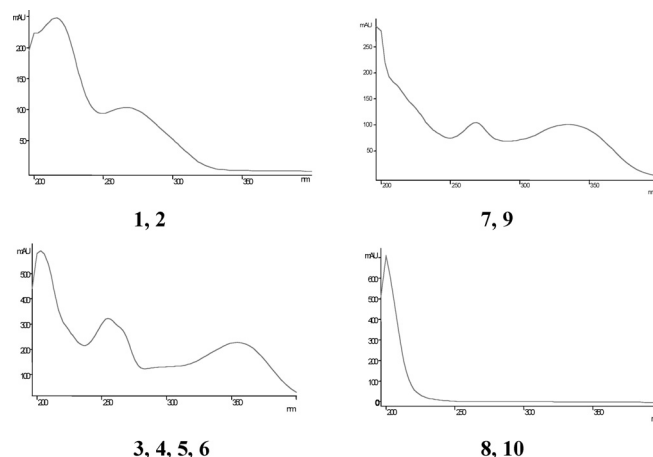


Fig. 2. HPLC Profile and UV Spectra (210 nm) of the Methanolic Extract of *Alchornea glandulosa* (AG)

Gallic acid **1**, methyl gallate **2**, myricetin 3-*O*- α -L-rhamnopyranoside **3**, quercetin 3-*O*- β -D-galactopyranoside **4**, quercetin 3-*O*- α -L-arabinopyranoside **5**, quercetin **6**, amentoflavone **7**, guanidine alkaloid pterogynidine **8**, amentoflavone derivative **9**, and guanidine alkaloid derivative **10**. Chromatographic conditions: see text.

Table 1. Evaluation of the Acute Toxicity of the Methanolic Extract of *Alchornea glandulosa* (AG) (5 g/kg, *p.o.*) in Mice

Weight	Control	AG
Body (g)	41.0 $\pm$ 2.40	41.71 $\pm$ 3.07
Kidney (mg)	514.98 $\pm$ 32.08	582.95 $\pm$ 49.57
Liver (mg)	2187 $\pm$ 146.28	2211.82 $\pm$ 176.12
Heart (mg)	202.08 $\pm$ 12.70	203 $\pm$ 15.03
Spleen (mg)	183.60 $\pm$ 18.95	197.51 $\pm$ 12.46
Mortality	0/7	0/7

Results are mean $\pm$ S.E.M.; $n = 7$. Student's *t* test. Non significant, $p > 0.05$.

imum amount to evaluate the antiulcer activity of AG.

Intestinal motility is one of the factors that may affect the intensity of luminal absorption of drugs administered orally and probably regulates their bioavailability. Motility changes play a role in the development and prevention of gastric le-

sions.³⁸⁾ Under the conditions used in our experiments, pretreatment with different doses of AG was unable to change the propulsion of the charcoal meal ($p>0.05$) when compared with atropine (Table 2).

The antiulcerogenic activity was assayed in distinct acute and subacute models of gastric ulceration in mice and rats. Rats pretreated with AG showed a dose-dependent effect with significant reduction of gastric lesions induced by absolute ethanol at the doses of 500 (57%) and 1000 mg/kg (85%) (Table 3). These lesions were characterized by numerous hemorrhagic red bands of different sizes along the major axis of the glandular stomach. The pathogenesis of ethanol-induced gastric mucosal damage is still unknown but the formation of oxygen-derived free radicals and neutrophil infiltration into the gastric mucosa are considered the main sources of mucosal damage.³⁹⁾ Plants containing substances like quercetin and gallic acid are also effective in preventing this kind of lesion, mainly because of their antioxidant properties.⁴⁰⁾ Kahraman *et al.* revealed that quercetin inhibited the development of mucosal gastric ulcers induced by the administration of ethanol.⁴¹⁾ Products of lipid peroxidation, protein carbonyl compounds, histamine levels, and myeloperoxidase activities were significantly decreased after treatment with quercetin.⁴¹⁾

Hydrochloric acid is a necrotic agent that induces wide hemorrhagic bands in the gastric corpus 60 min after HCl administration.⁴²⁾ Pretreatment with AG 50 min before

HCl/ethanol solution increased the severity of lesions in a dose-dependent manner by 64% (250 mg/kg) and 46% (500 mg/kg), in relation to mice treated with vehicle (Table 3). We also observed that the lowest dose of AG showed the highest activity.

Piroxicam is a preferential COX-1 inhibitor. Cyclooxygenase is constitutively expressed in the gastrointestinal tract in large amounts and has been suggested to maintain mucosal integrity through continuous generation of prostaglandins.⁴³⁾ We observed that pretreatment with AG showed significant ($p<0.01$) gastroprotection at doses of 250 (78%) and 500 mg/kg (33%). Again, we observed a decrease in gastroprotection when we used higher doses of AG. Pretreatment with AG at the highest dose (1000 mg/kg) resulted in greater gastric injury than vehicle pretreatment.

These results suggest that compounds present in AG may be involved with inflammation precursors. Gracioso *et al.* described how increasing amounts of flavonoids may change their properties from antioxidant to prooxidant.⁴⁴⁾ Repetto and Llesuy reported that low concentrations of phenolic compounds stimulated prostaglandin H-synthase, whereas high concentrations inhibited PGHS production.⁴⁵⁾ Thus the diminution of the gastroprotective activity observed with increasing doses of AG was probably due to the chemical profile of this species that induced modulation of endogenous PGs. Our data are in accord with those obtained by Banerjee *et al.*, which showed that amentoflavone and quercetin exerted suppression of PGE₂ biosynthesis *via* downregulation of COX-2/iNOS expression.⁴⁶⁾

Gastric juice obtained from pylorus-ligated mice was used to analyze the gastric biochemical parameters (Table 4). Animals pretreated with AG by either route (oral or intraduodenal) had increased gastric volume and decreased total gastric acid content compared with the control group. This result indicates that the antisecretory property is involved in the antiulcer activity of AG. This effect observed after treatment with AG indicates that compounds present in AG, such as quercetin derivatives, may inhibit histamine release from mast cells in gastric tissues. Kimata *et al.* reported that quercetin suppresses Ca²⁺ influx and protein kinase activ-

Table 2. Effects of the Methanolic Extract of *Alchornea glandulosa* (AG) on Intestinal Propulsion Induced by Activated Charcoal in Mice

Treatment (p.o.)	Dose (mg/kg)	n	Distance moved by charcoal (arc sine)
Control	10	7	57.85±2.57
Atropine	5	6	40.16±2.74*
AG	250	7	56.0±2.47
	500	7	56.57±4.29
	1000	7	60.57±2.95

Results are mean±S.E.M. Dunnett's test, represents significantly different from the control group. Arc sine * $p<0.01$, $F_{(4,29)}=6.84$.

Table 3. Effects of Different Doses of the Methanolic Extract of *Alchornea glandulosa* (AG) on Models of Gastric Lesions Induced in Mice and Rats

Method	Treatment (p.o.)	Dose (mg/kg)	n	U.L.I.	Inhibition (%)
Ethanol (Rats)	Control	—	5	89.0±6.70	—
	Lansoprazole	30	5	35.40±8.26*	60
	AG	250	4	79.25±8.91	11
		500	4	37.75±6.0*	57
		1000	4	13.0±5.84*	85
HCl/Ethanol (Mice)	Control	—	8	71.0±10.03	—
	Lansoprazole	30	6	30.0±3.81**	58
	AG	250	5	25.80±3.86**	64
		500	5	38.40±5.58*	46
		1000	5	48.60±9.88	32
NSAID (Mice)	Control	—	5	50.80±5.67	—
	Cimetidine	100	5	12.80±1.93**	75
	AG	250	5	11.20±1.59**	78
		500	5	33.80±3.38**	34
		1000	5	58.0±2.34	-14

Results are mean±S.E.M. Ethanol: ANOVA, Dunnett's test * $p<0.01$, $F_{(4,27)}=8.63$; HCl/ethanol: Dunnett's test * $p<0.05$, ** $p<0.01$, $F_{(4,24)}=5.9$; $F_{(4,28)}=7.54$; NSAID: Dunnett's test, ** $p<0.01$, $F_{(4,20)}=41.06$ for U.L.I.

Table 4. Effects of a Single Dose of the Methanolic Extract of *Alchornea glandulosa* (AG), Given by the Oral or Intraduodenal Route, on the Parameters of Gastric Juice Obtained from Pylorus-Ligated Mice

Treatment	Route	n	Dose (mg/kg)	Total acid ($\mu\text{Eq/ml/4 h}$)	pH	Gastric volume (ml)
Control	i.d.	7	—	7.46 ± 0.72	2.14 ± 0.26	0.66 ± 0.07
Cimetidine	i.d.	7	100	$4.91 \pm 0.80^*$	3.14 ± 0.34	$0.63 \pm 0.10^*$
AG	i.d.	8	1000	$4.57 \pm 0.47^*$	2.0 ± 0.29	$1.10 \pm 0.07^*$
Control	p.o.	8	—	6.86 ± 0.51	3.33 ± 0.37	0.63 ± 0.08
Cimetidine	p.o.	9	100	$4.28 \pm 0.45^{**}$	3.66 ± 0.28	0.56 ± 0.06
AG	p.o.	5	1000	$4.45 \pm 0.35^{**}$	2.50 ± 0.18	$1.33 \pm 0.23^{**}$

Results are mean $\pm$ S.E.M. i.d. route: ANOVA, Dunnett's test $*p < 0.05$, $F_{(2,21)} = 4.84$ (volume); $*p < 0.05$, $F_{(2,25)} = 5.79$ (total acid); $p > 0.05$, n.s., $F_{(2,22)} = 2.5$ (pH). p.o. route: ANOVA, Dunnett's test $**p < 0.01$, $F_{(2,22)} = 4.93$ (total acid); $**p < 0.01$, $F_{(2,22)} = 12.73$ (volume); $p > 0.05$ n.s., $F_{(2,22)} = 4.2$ (pH).

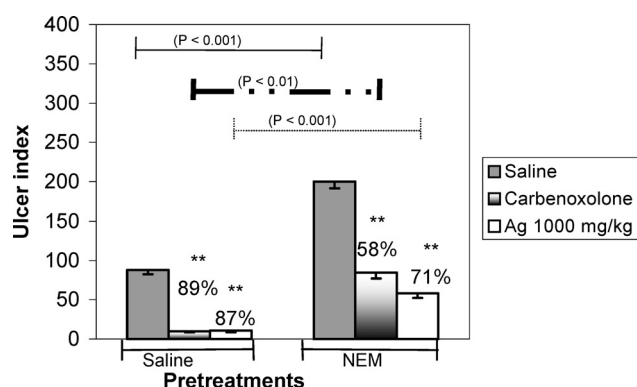


Fig. 3. Effects of the Methanolic Extract of *Alchornea glandulosa* (AG) on Gastric Lesions Induced by Ethanol with Different Combinations of NEM (*N*-Ethylmaleimide) in Pretreated Rats

Results are mean $\pm$ S.E.M. ANOVA followed by Dunnett's test. $**p < 0.01$, significant difference from the control group saline_saline $F_{(2,12)} = 185.4$; and NEM_saline $F_{(2,12)} = 91.78$.

ity.⁴⁷) Furthermore, the increase in gastric volume observed after pretreatment with AG indicates enhancement of mucous-alkaline secretion.

Analysis of the crude methanolic extract and of the substances isolated from AG using TLC followed by visualization with DPPH and β -carotene solutions revealed the presence of typical yellowish spots for all tested compounds, indicating their free radical-scavenging effect, except for the alkaloid pterogynidine.

Szabo reported that sulphhydryl compounds might scavenge free radicals and consequently prevent membrane damage caused by lipid peroxidation.⁴⁸) The role of endogenous SH in mucosal protection has been demonstrated in ethanol-induced gastric injury, where the development of damage was accompanied by a reduced number/total amount of mucosal SH compounds. The SH compounds bind to free radicals that form after tissue injury by noxious agents. These agents may also protect mucus, since mucus subunits are joined by disulfide bridges that, if reduced, render mucus water soluble.⁴⁹)

We investigated the possible involvement of endogenous SH compounds in the gastroprotective effects of AG by pretreatment of animals with NEM, a blocker of SH compounds (Fig. 3). In the ethanol-induced model, animal pretreatment with NEM increased the severity of gastric lesions. Hence animals treated with AG (1000 mg/kg) showed attenuated gastroprotection (11 ± 2.28 with saline vs. 58 ± 11.83 with NEM). This significant ($p < 0.001$) reduction of gastroprotec-

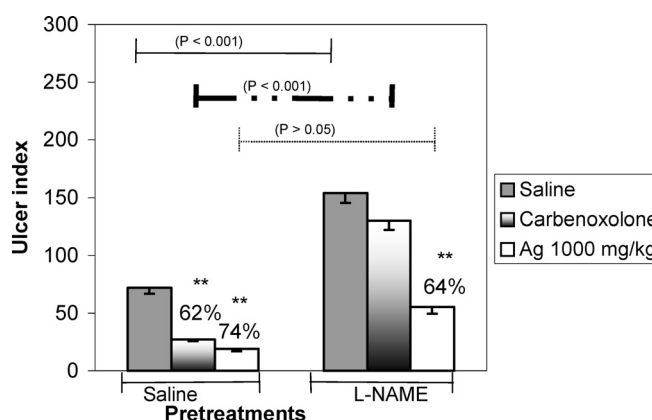


Fig. 4. Effects of the Methanolic Extract of *Alchornea glandulosa* (AG) on Gastric Lesions Induced by Ethanol with Different Combinations of L-NAME (*N*-Nitro-L-arginine Methyl Ester) in Pretreated Rats

Results are mean $\pm$ S.E.M. ANOVA followed by Dunnett's test. $**p < 0.01$, significant difference from the control group saline_saline $F_{(2,18)} = 24.97$; and L-NAME_saline $F_{(2,17)} = 19.97$.

tion observed with AG indicates the involvement of endogenous SH compounds in the gastroprotective effects of this extract.

The next step was to confirm the probable involvement of nitric oxide (NO) in AG gastroprotection. NO is a molecule implicated in mechanisms that control the integrity of the gastric epithelium, regulation of gastric blood flow, and stimulation of gastric mucus secretion due to the activation of the enzyme guanylate cyclase. Therefore, to investigate the role of endogenous NO in cytoprotection, we used the NO synthase (NOS) inhibitor L-NAME to assess the protective effects of AG on ethanol-induced gastric hemorrhagic lesions (Fig. 4). Oral administration of AG to animals pretreated with L-NAME produced an increase in gastric injury compared with L-NAME pretreated with saline. Therefore the gastroprotective effects of AG observed with saline pretreatment (74%) were reduced compared with the protective activity after pretreatment with L-NAME (64%), although this difference was not significant ($p > 0.05$).

The ulcer produced by the injection of acetic acid into the rat stomach wall was assumed to be similar to human chronic ulcer, since it is difficult to treat and it requires a long time to be healed.²⁷) Oral administration of AG for 14 consecutive days accelerated the healing of gastric mucosa in rats, producing a 43% cure rate when compared with the control group treated with vehicle (Table 5). Healing of the gastric ulcer was not observed in macroscopic analysis of injuries but it was evi-

Table 5. Effects of Treatment for 14 Consecutive Days with the Extract of *Alchornea glandulosa* (250 mg/kg/d) on Healing of Ulcer Produced by Acetic Acid Solution in the Stomachs of Rats

Macroscopic analyses						Histologic analyses	
Treatment (p.o)	n	Dose (mg/kg)	External lesion area (mm ²)	Internal lesion area (mm ²)	Curative ratio (%)	Normal (μm)	Regeneration area (μm)
Control	6	—	1.10±0.10	0.44±0.07	—	563.2±34.35	566.07±73.82
Cimetidine	7	100	1.01±0.18	0.37±0.04	16	461.75±38.05	748.25±117.53
AG	8	250	1.01±0.05	0.25±0.04	43	513.97±16.78	931.07±42.52**

Results are mean±S.E.M. ANOVA followed by Dunnett's test. ** $p<0.01$, $F_{(2,15)}=7.2$.

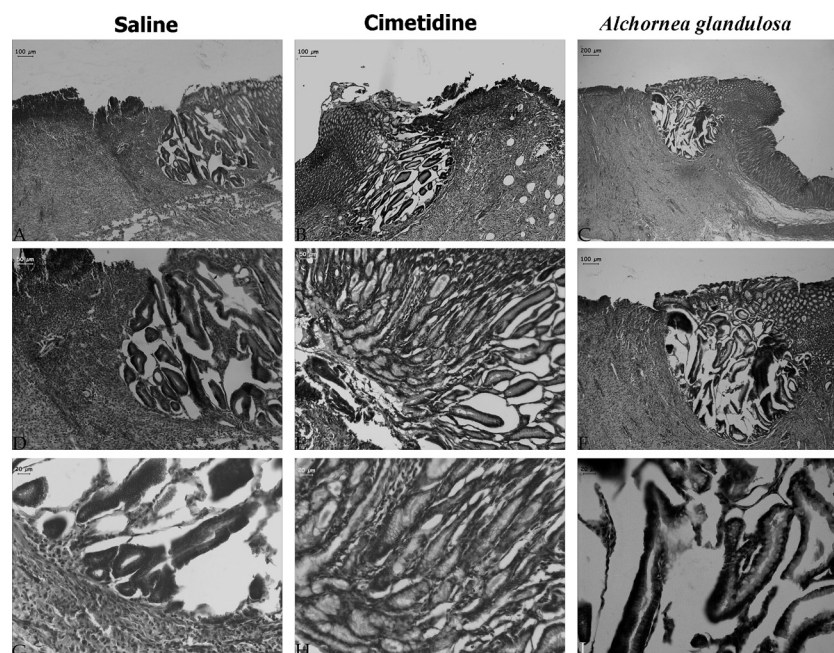


Fig. 5. Slides of Rat Stomach after Treatment with Saline (A, D, G), Cimetidine (B, E, H), and *Alchornea glandulosa* (C, F, I), Hematoxylin and Eosine Staining

The arrows indicate lesion areas. Note the difference in mucus inside the lumen (*).

dent in morphometric analyses. Comparison of the normal and regenerated areas of the gastric mucosa showed an increase in the transition area due to treatment with both cimetidine and AG (250 mg/kg). Subacute treatment with AG increased the regenerated area almost two-fold compared with that in the control group, thus indicating that AG stimulated proliferative factors that led to healing. Organization of the glands is not observed in the stomach mucosa. However, glands apparently show mucous secretion and appearance similar to those treated with cimetidine (Fig. 5).

PCNA is a highly conserved 36 kDa nuclear polypeptide identified as the auxiliary protein of DNA polymerase delta.^{50–52} PCNA is expressed throughout the cell cycle and its concentration is increased further in the S-phase.⁵³ Szabo and Vincze⁵⁴ and Tarnawski *et al.*⁵⁵ have demonstrated that the capacity to accelerate the ulcer healing process depends on many factors, such as platelet-derived growth factor, fibroblast growth factor, and vascular-endothelial growth factor stimulation of angiogenesis and cell proliferation.^{54–56} Kitajima *et al.* demonstrated proliferative activity during the healing of gastric ulcers using the PCNA method. In the morphologic analysis of immunohistochemical PCNA, it was noted that the three treatments promote proliferation of the

cells.⁵⁷ However, only in the cells treated with AG did proliferation occur in the basal region of the glandule (Fig. 6F). Cimetidine significantly inhibited cell proliferation in three of the five cell lines, which may indicate the dependence of the proliferation of these cell lines on stimulation of the H₂ receptor.⁵⁸ This possibly occurs because the stem cell reorganizes from the base of the gland, indicating the unidirectional character of the cell proliferation, which is different from the normal region, which has a bidirectional proliferation.⁵⁶ Cell proliferation also denotes that AG induces the expression of the growth factor in the gastric mucosa, thus leading to gastric ulcer healing activity.

The healing experiment of ulcers induced by acetic acid in rats also supports the monitoring of the body weight evolution of the animals subjected to different treatments. This method also indicates the possible presence of subacute toxicity after 14-d treatment, when parameters such as body weight gain, relative weight of vital organs, and serum biochemical parameters are evaluated (Table 6). No animal mortality due to toxicity and no significant differences in body weight gain were observed at the end of the 14 d of treatment (data not shown). Moreover, the average weights of most vital organs and visceral conditions were normal and compa-

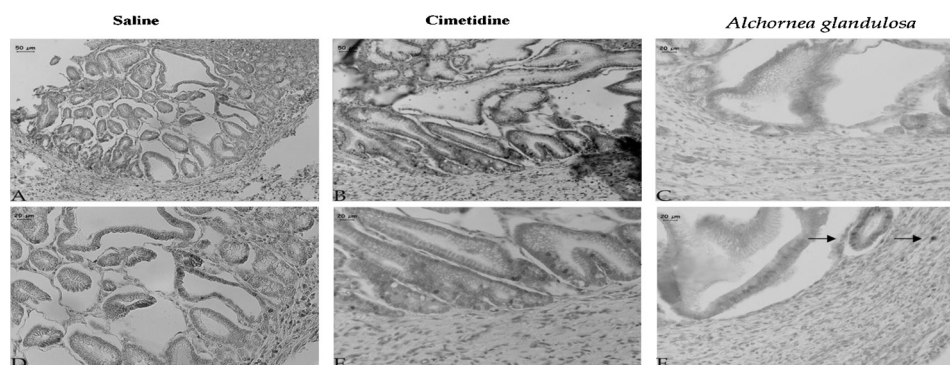


Fig. 6. Slides of rat Stomach after Treatment with Saline (A, D), Cimetidine (B, E), and *Alchornea glandulosa* (C, F) in Immunohistochemistry with the PCNA Method and Peroxidase Immunostaining

Note the organization in the glandule and the PCNA-positive nucleus in the *Alchornea glandulosa* (arrows).

Table 6. Evaluation of the Toxicity of the Methanolic Extract (AG) Obtained from Leaves of *Alchornea glandulosa* in Rats

Treatment (p.o.)		Control	Cimetidine	AG
Weight (g)	Body	237.45 ± 6.67	250.72 ± 7.64	250.36 ± 7.52
	Kidney	1.81 ± 0.08	2.02 ± 0.07	2.04 ± 0.11
	Lungs	1.43 ± 0.04	1.57 ± 0.14	1.73 ± 0.25
	Liver	7.42 ± 0.17	8.18 ± 0.30	8.4 ± 0.48
	Heart	1.23 ± 0.06	1.18 ± 0.06	1.3 ± 0.07
	Spleen	0.69 ± 0.04	0.67 ± 0.04	0.62 ± 0.04
Biochemistry	Urea (mg/dl)	108.5 ± 2.91	115.6 ± 2.89	120 ± 6.16
	Creatinine (mg/dl)	1.86 ± 0.04	1.86 ± 0.09	1.75 ± 0.08
	AST (UI/l)	222.80 ± 7.10	267.00 ± 16.68	202.83 ± 25.38
	ALT (UI/l)	93.76 ± 5.01	87.5 ± 5.44	71.66 ± 7.16

Effects on body organ weight, relative weight of vital organs, and serum biochemical parameters of a single dose (250 mg/kg, p.o.) of AG administered for 14 consecutive days after ulcer formation. Results are mean ± S.E.M. ANOVA followed by Dunnett's test. Non significant, $p > 0.05$.

table to those of the controls (Table 6). The 14-d treatment with oral AG did not elicit changes in serum biochemical parameters related to renal (urea and creatinine) or hepatic toxicity (AST and ALT). Although these results did not suggest any significant adverse effects of AG at the dose used for 14 d, further studies will be carried out to confirm the absence of chronic toxicity.

AG did not show acute toxicity and exhibited antisecretory and gastroprotective activities. The observed effects probably involve the participation of NO as well as an increase in endogenous SH compounds, which are related to the protective mechanisms of the gastrointestinal mucosa against aggressive factors. Oral administration of AG for 14 d accelerated the healing of gastric ulcers in rats by promoting epithelial cell proliferation. Phytochemical evaluation showed that AG contains quercetin and myricetin derivatives, the biflavonoid amentoflavone, gallic acid, methyl gallate, and the alkaloid pterogynidine. These compounds probably contribute to the antiulcerogenic effects of the polar extract of *A. glandulosa* leaves.

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REFERENCES

- 1) Lorenzi H., "Árvores Brasileiras: Manual de Identificação e Cultivo de Plantas Arbóreas Nativas do Brasil," 3rd ed., Plantarum, São Paulo, 1998.
- 2) Osabede P. O., Okoye F. B. C., *J. Ethnopharmacol.*, **89**, 19–24 (2003).
- 3) Conejero L. S., Ide R. M., Nazari A. S., Sarragiotto M. H., *Química Nova*, **26**, 825–827 (2003).
- 4) Urrea-Bulla A., Suárez M., Moreno-Murillo B., *Fitoterapia*, **75**, 392–394 (2004).
- 5) Tona L., Kambu K., Mesia K., Cimanga K., Apers S., De Bruyne T., Pieters L., Totte J., Vlietinck A. J., *Phytomedicine*, **6**, 59–66 (1999).
- 6) Tona L., Kambu K., Ngimbi N., Mesia K., Penge O., Lusakibanza M., Cimanga K., De Bruyne T., Apers S., Totte J., Pieters L., Vlietinck A. J., *Phytomedicine*, **7**, 31–38 (2000).
- 7) Abo K. A., Ashidi J. S., *Afr. J. Med. Med. Sci.*, **28**, 167–169 (1999).
- 8) Ajali U., *Fitoterapia*, **71**, 436–438 (2000).
- 9) Ebi G. C., *Fitoterapia*, **72**, 69–72 (2001).
- 10) Barbo F. E., Meda C. I., Claudia M., Young M., Cordeiro I., Blatt C. T. T., *Biochem. Syst. Ecol.*, **30**, 605–607 (2002).
- 11) Adewunmi C. O., Agbedahunsi J. M., Adebajo A. C., Aladesanmi A. J., Murphy N., Wando J., *J. Ethnopharmacol.*, **77**, 19–24 (2001).
- 12) Mustofa M., Valentin A., Benoit-Vical F., Pelissier Y., Koné-Bamba D., Mallié M., *J. Ethnopharmacol.*, **73**, 145–151 (2000).
- 13) Banzouzi J. T., Prado R., Menan H., Valentin A., Roumestan C., Mallié M., Pelissier Y., Blache Y., *J. Ethnopharmacol.*, **81**, 399–401 (2002).
- 14) Setzer W. N., Shen X., Bates R. B., Burns J. R., McClure K. J., Zhang P., Moriarity D. M., Lawton R. O., *Fitoterapia*, **71**, 195–198 (2000).
- 15) Ogundipe O. O., Moody J. O., Houghton P. J., Odelola H. A., *J. Ethnopharmacol.*, **74**, 275–280 (2001).
- 16) Olfert E. D., Cross B. M., McWilliam A., "Guide to the Care and Use of Experimental Animals," 2nd ed., Vol. I, Canadian Council on Animal Care, Ottawa, Ontario, 1993.
- 17) Wagner H. M., Bladt S., Zgainski E. M., "Plant Drug Analysis," Springer, Berlin, 1986.
- 18) Potterat O., *Curr. Org. Chem.*, **1**, 415–440 (1997).
- 19) Souza-Brito A. R., "Manual de Ensaios Toxicológicos *in Vitro*," 1st

- ed., Editora da UNICAMP, Campinas, São Paulo, 1994.
- 20) Stickney J. C., Northup D. W., *Proc. Soc. Exp. Biol. Med.*, **101**, 582 (1959).
 - 21) Morimoto Y., Shimohara K., Oshima S., Sukamoto T., *Jpn. J. Pharmacol.*, **57**, 495—505 (1991).
 - 22) Szelenyi I., Thieme K., *Arch. Toxicol.*, **41**, 99—105 (1978).
 - 23) Mizui T., Doteuchi M., *Jpn. J. Pharmacol.*, **33**, 939—945 (1983).
 - 24) Puscas I., Puscas C., Coltau M., Pasça R., Torres J., Márquez M., Herrero E., Fillat O., Ortiz J. A., *Arzneim.-Forsch.*, **47**, 568—572 (1997).
 - 25) Shay H., Komarov S. A., Fels S. S., Meranze D., Gruenstein M., Siplet H., *Gastroenterology*, **5**, 43—61 (1945).
 - 26) Arrieta J., Benitez J., Flores E., Castillo C., Navarrete A., *Planta Med.*, **69**, 905—909 (2003).
 - 27) Takagi K., Okabe S., Saziki R., *Jpn. J. Pharmacol.*, **19**, 418—426 (1969).
 - 28) Ishihara M., Ito M., *Eur. J. Pharmacol.*, **31**, 209—215 (2002).
 - 29) Grob G. N., *N. Engl. J. Med.*, **101**, 19—30 (2004).
 - 30) Belaiche J., Burette A., De Vos M., Louis E., Huybrechts M., Deltenre M., *Acta Gastroenterol. (Belg.)*, **65**, 65—73 (2002).
 - 31) Yacyshyn B. R., Thomson A. B. R., *Digestion*, **66**, 67—78 (2002).
 - 32) Harborne J. B., "The Flavonoids: Advances in Research since 1986," Chapman & Hall, New York, 1996.
 - 33) Agrawal P. K., "Carbon 13 NMR of Flavonoids," 1st ed., Vol. XXXIX, Elsevier, Amsterdam, 1989.
 - 34) Binutu O. A., Cordell G. A., *Pharm. Biol.*, **38**, 284—286 (2000).
 - 35) Bolzani V. S., Gunatilaka A. A. L., Kingston D. G. I., *J. Nat. Prod.*, **58**, 1683—1688 (1995).
 - 36) Mabry T. J., Markham K. R., Thomas M. B., "The Systematic Identification of Flavonoids," Springer-Verlag, New York, 1970.
 - 37) Souza-Brito A. R. M., *J. Ethnopharmacol.*, **54**, 131—138 (1996).
 - 38) Tsukimi Y., Okabe S., *Biol. Pharm. Bull.*, **24**, 1—9 (2001).
 - 39) Chow J. Y., Ma L., Cho C. H., *Eur. J. Pharmacol.*, **342**, 253—260 (1998).
 - 40) Gonzalez E., Montenegro M. A., Nazareno M. A., Lopez M. B. A., *Arch. Latinoam. Nutr.*, **51**, 395—399 (2001).
 - 41) Kahraman A., Erkasap N., Koken T., Serteser M., Aktepe F., Erkasa P., *Toxicology*, **183**, 133—142 (2003).
 - 42) Sawaoka H., Tsuji S., Tsujii M., Gunavan E. S., Nakama A., Takei Y., Nagano K., Matsui H., Kawano S., Hori M., *J. Clin. Gastroenterol.*, **25**, S105—S110 (1997).
 - 43) Halter F., Tarnawski A. S., Schmassmann A., Peskar B. M., *Gut*, **49**, 443—453 (2001).
 - 44) Gracioso J. S., Vilegas W., Hiruma-Lima C. A., Souza-Brito A. R. M., *Biol. Pharm. Bull.*, **25**, 470—491 (2002).
 - 45) Repetto M. G., Llesuy S. F., *Braz. J. Med. Biol. Res.*, **35**, 523—534 (2002).
 - 46) Banerjee T., Van Der Vilet A., Ziboh A., *Prostaglandins Leukot. Essent. Fatty Acids*, **66**, 485—492 (2002).
 - 47) Kimata M., Inagaki N., Nagai H., *Planta Med.*, **66**, 225—229 (2000).
 - 48) Szabo S. M. G., "New Pharmacological of Ulcer Disease," Elsevier, New York, 1987.
 - 49) Avila J. R., De La Lastra C. A., Martin M. J., Motilva V., Luque I., Delgado D., Esteban J., Herrerias J., *Inflamm. Res.*, **45**, 83—88 (1996).
 - 50) Bravo R., Flank R., Blundell P. A., MacDonald-Bravo H., *Nature (London)*, **326**, 515—517 (1987).
 - 51) Prelich G., Tan C., Kostura M., Mathews M. B., *Nature (London)*, **326**, 517—520 (1987).
 - 52) Nanji A. A., Tahan S. R., *Toxicol. Appl. Pharmacol.*, **140**, 101—107 (1996).
 - 53) Celis J. E., Celis A., *Embo J.*, **4**, 1187—1192 (1985).
 - 54) Szabo S., Vincze A., *J. Physiol. (Paris)*, **94**, 77—81 (2000).
 - 55) Tarnawski A., Szabo I. L., Husain S. S., Soreghan B., *J. Physiol. (Paris)*, **95**, 337—344 (2001).
 - 56) Tatematsu M., Tsukamoto T., Inada K., *Cancer Sci.*, **94**, 135—141 (2003).
 - 57) Kitajima T., Okuhira M., Tani K., Nakano T., Hiramatsu A., Mizuno T., Inoue K., *J. Clin. Gastroenterol.*, **17**, S116—S120 (1993).
 - 58) Finn P. E., Purnell P., Pilkington G. J., *Neuropathol. Appl. Neurobiol.*, **22**, 559 (1996).