Can a *Strychnos* species be used as antiulcer agent? Ulcer healing action from alkaloid fraction of *Strychnos pseudoquina* St. Hil. (Loganiaceae)

Flávia Bonamin[a], Thiago Mello Moraes[a], Hélio Kushima[a], Marcelo Aparecido Silva[b], Ariane Leite Rozza[c], Cláudia Helena Pellizzon[c], Tais Maria Bauab[d], Lucia Regina Machado Rocha[a], Wagner Vilegas[b], Clélia Akiko Hiruma-Lima[a,*]

[a] UNESP Univ Estadual Paulista, Departamento de Fisiologia, Instituto de Biociências, cp 610, CEP 18618-000, UNESP, Botucatu, SP, Brazil
[b] UNESP Univ Estadual Paulista, Departamento de Química Orgânica, Instituto de Química, cp 355, CEP 14801-970, UNESP, Araraquara, SP, Brazil
[c] UNESP Univ Estadual Paulista, Departamento de Morfologia, Instituto de Biociências, cp 610, CEP 18618-000, UNESP, Botucatu, SP, Brazil
[d] UNESP Univ Estadual Paulista, Departamento de Ciências Biológicas, cp 970, CEP 14801-970, UNESP, Araraquara, SP, Brazil

ARTICLE INFO

ABSTRACT

Ethnopharmacological relevance: Strychnos pseudoquina St. Hil. (Loganiaceae) is one Brazilian native medicinal species described in the first edition of the Brazilian Official Pharmacopoeia in 1929. This medicinal plant, popularly known as "quina-quina", "quina-branca" or "casca aromatica was very commonly used in folk medicine in tea form obtained from the bark and/or leaves as tonic, antipyretic, antimalarial and mainly against diseases of the liver, spleen and stomach.

Aim of the study: Previous study already characterized the gastroprotective action of this species The aim of the present study is to elucidate the mechanism of the healing process mediated by the methanolic extract (ME) and their enriched alkaloid fraction (EAF) from *Strychnos pseudoquina* in chronic gastric ulceration induced by 5% acetic acid in rats, an experimental model that accurately reflects human gastrointestinal disease.

Material and methods: The ME and EAF was administered orally in a single dose (based on previously study of dose–response curve) for 14 days after chronic ulceration was induced in rats. The healing effect of ME and EAF was evaluated by macroscopic and morphometric analyses, immunohistochemical assay (PCNA and SOD) and anti-*Helicobacter pylori* effect was evaluated by *in vitro* assay.

Results: Our results demonstrated that EAF significantly reduced border internal (42%) and external (38%) lesion area (mm^2) by macroscopic analyses ($P < 0.05$). Animals treated with EAF stimulated some proliferative factors by increasing the height of epithelial regenerative area and the expression of PCNA-positive nuclei. The number of vessels in gastric mucosa of rats treated with EAF reveals an expressive increase (4 times more than vehicle treatment) of vessels that stimulate cells proliferation in the healing region. These results suggest that the recovery of vascularization of the ulcerated area is involved in the healing action of alkaloid fraction of *Strychnos pseudoquina*. The MIC (minimum inhibitory concentration) of 75 μg/ml from EAF showed an effective *in vitro* anti-*Helicobacter pylori* action of this fraction. EAF also was quite effective in the process of SOD release that is an important protective factor against bacterial agents. The efficacy of EAF was accomplished safely without presenting any alteration of toxicological parameters during 14 day of treatment.

Conclusions: The expressive gastric healing effect by increasing cellular proliferation together with expression of SOD activity and antibacterial action against *Helicobacter pylori* confirm the efficacy of this species in heal gastric mucosa and these results are a important contribution to the knowledge of a crude drug presents at the Brazilian Official Pharmacopoeia since 1929.

1. Introduction

The genus *Strychnos* includes more than 200 species distributed among tropical areas of the globe (Philippe et al., 2004). Many of these species are known by their medicinal properties and for the powerful toxicity character among their phytocompounds, such as strychnine, one of the poisonous alkaloids produced by the species

* Corresponding author. Tel.: +55 01438116077; fax: +55 1438153744.
E-mail address: hiruma@ibb.unesp.br (C.A. Hiruma-Lima).

from this genus (Thongphasuk et al., 2003). Among the medicinal species, a folk medicine from Cerrado region of Brazil, there is the species named *Strychnos pseudoquina* St. Hil known popularly as "quina-quina", "quina-branca" or "casca aromatica". This plant was very commonly used in folk medicine in tea form obtained from the bark and/or leaves as tonic, antipyretic, antimalarial and mainly against diseases of the liver, spleen and stomach (Pott and Pott, 1994; Almeida et al., 1998; Lorenzi and Matos, 2002; Andrade-Neto et al., 2003). This medicinal plant was one of the Brazilian native species described in the first edition of the Brazilian Official Pharmacopoeia in 1929 as intermittent fevers, liver and spleen disorders and as digestive (Brandão et al., 2008) but even eighty years afterwards few pharmacological studies have been developed with this species. Andrade-Neto et al. (2003) showed that crude ethanol extract from the bark of this species presents weak antiplasmodial activity. Honório-França et al. (2008) demonstrated that aqueous extract from *Strychnos pseudoquina* bark provides hypoglycemic action but did not heal wounds in diabetic rats. Silva et al. (2005) showed the protective action of methanolic extract (ME) and enriched alkaloid fraction (EAF) from leaves of *Strychnos pseudoquina* to the gastric mucosa against injurious agents such as non-steroidal anti-inflammatory drugs (NSAID) and an ethanol acidified solution in mice. The same authors also observed that an *in vivo* assay of ME and EAF presented no toxic effects and no mortality for 14 days. However, studies from Santos et al. (2006) showed that ME from *Strychnos pseudoquina* was mutagenic against strains of *Salmonella*, but the EAF presented absence of mutagenicity in this same assay.

The confirmation of gastroprotective action of ME and EAF from *Strychnos pseudoquina* by Silva et al. (2005) is an important indicative of antiulcer property of this species, corroborating its reputed utility in folk medicine that mainly obtained polar compounds as tea form. However, these actions do not imply that these same preparations also present ulcer healing effect on injured gastric mucosa. So the aim of this study was to evaluate the healing action of ME or EAF of *Strychnos pseudoquina* throughout 14 consecutive days in the chronic ulcer model induced by acetic acid in rats. We also evaluated some toxicological parameters by subacute treatments with *Strychnos pseudoquina* and the anti-*Helicobacter pylori* action of this species.

2. Materials and methods

2.1. Plant material and extraction of ME and EAF

Leaves of *Strychnos pseudoquina* St. Hil. were collected in May 2001 from Porto Nacional city, Tocantins State, Brazil and identified by Prof. E.R. Santos, from the Institute of Biology at Tocantins University. A herbarium specimen voucher (Nr. 3291) was deposited at the Herbarium of Tocantins University (HTINS). The leaves were air-dried and powdered in a mill. Leaves of *Strychnos pseudoquina* (300 g) were extracted in powder form with methanol as solvent during seven days. Solvent was evaporated in a vacuum to yield 17.7 g of extract (ME) (3:10 was the plant:solvent ration). A portion (5.0 g) of the methanolic extract (MeOH) of *Strychnos pseudoquina* was submitted to column chromatography on Sephadex LH-20 (100 cm × 5 cm) with MeOH as the eluent. One hundred fractions (5 ml) were collected, checked by TLC on silica gel plates CHCl$_3$–MeOH–*n*-PrOH–H$_2$O (5:6:1:4, v/v/v/v lower phase) and revealed with Draggendorff, iodoplatinate or NP/PEG (Natural Products/Polyethyleneglycol) reagents. Alkaloids were detected in Fr. 3–29 (denominated "enriched alkaloid fraction—EAF 3 g) and flavonoids were detected in Fr. 35–90 (250 mg). These exactly the same batches from MeOH and the EAF have been used in this study were extensively study and

phytochemical profiles of both were described by Silva et al. (2005).

2.2. Animals

Male Wistar albino rats (150–250 g) from the Central Animal House of the UNESP were used. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark-12 h light, humidity (60 ± 1.0%) and temperature (21 ± 1 °C). Fasting (24 h) was used prior to assays because standard drugs, ME or EAF were always administered by orally using a saline solution (10 ml/kg) as 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 (Olfert et al., 1993). The UNESP Institution for Animal Care approved the employed protocols.

2.3. Healing in acetic acid-induced gastric lesion

The experiment was performed according to the method described by Takagi et al. (1969), with some modifications. Three groups of rats that had fasted for 24 h were used in this experiment (*n* = 7–8). 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 the animals were fed normally. We selected the lowest effective dose of ME or EAF (250 mg/kg body wt.) to evaluate the healing effect of *Strychnos pseudoquina* based on data from Silva et al. (2005) that evaluated the gastroprotective action of ME or EAF on acute gastric lesion model. The other group was treated with 100 mg/kg of pure cimetidine (Sigma Co., USA) or vehicle (10 ml/kg) to determine healing effects by subacute treatment. All treatments were administered orally once a day for 14 consecutive days beginning one day after surgery. Body weight was recorded daily throughout the experiments and the macroscopic analyses and weights of vital organs (liver, kidney, heart, spleen and lung) were compared among the different treatments and the group treated with vehicle to evaluate the possible subacute toxicity induced by ME or EAF. On the day after the last drug administration, the rats were killed and their stomachs were gently removed. The gastric lesions were evaluated by examining the inner gastric surface with a dissecting magnifying glass. The macroscopic ulcer area of the internal border and external border (mm^2) were determined as described by Takagi et al. (1969).

2.3.1. Histological methods

The stomach lesions induced by acetic acid in rats submitted to the different treatments were located, sectioned, and fixed in ALFAC solution (70% ethanol, 4% formaldehyde and 5% glacial acetic acid) for 24 h at 4 °C. Then the samples were routinely processed for embedding in paraplast, and cut into 7 μm-thick sections that were strained with periodic acid–Schiff (PAS) (Vacca, 1985) and hematoxylin-eosin (HE) (Behmer et al., 1976). Paraffin slides were processed for HE straining and imunohistochemical reaction in blood vessels. For both analyses, we used at least 6 fields for each group and the results were submitted to statistical analyses.

2.3.2. Morphometric analyses

For the morphometric analyses a slice of stomach was examined in a Leica microscope coupled with Leica Qwin Software (Leica,

Table 1

Regeneration measures of the gastric lesion borders (epithelial height) and the new blood vessels in the regeneration areas under the mucous membrane in histological analysis of the stomach treated with EAF of *Strychnos pseudoquina* under the model of ulcer induction by acetic acid.

Treatments (p.o.)	Doses (mg/kg)	Epithelial height (μm)	Number of new blood vessels (μm^2)
Saline	–	1462.00 ± 25.27	178.01 ± 20.64
Cimetidine	100	1489.50 ± 17.56	309.17 ± 30.33[*]
EAF	250	1611.70 ± 28.14[**]	633.95 ± 33.97[**]

Results are mean ± S.E.M. Statistical analysis among the groups: ANOVA following by Dunnett's test.
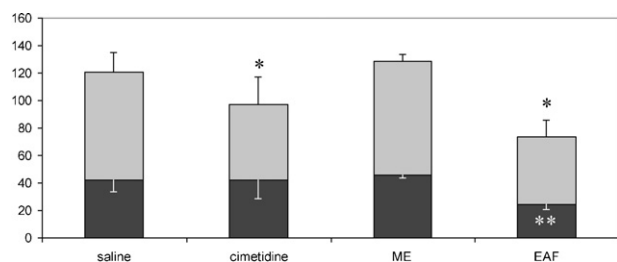
[*] $P < 0.05$.
[**] $P < 0.01$.

Fig. 1. Influence of ulcer healing activity of methanolic extract (ME = 250 mg/kg) and alkaloids fraction (EAF = 250 mg/kg) of the *Strychnos pseudoquina* in acetic acid-induced gastric lesion. Data from black bars represents internal border and gray bars represents external border from lesion.

England), where the height of regenerated mucosa was measured by employing a variation of the method used by Ishihara and Ito (2002).

2.3.3. Immunohistochemical analyses

The antibodies utilized (Novo Castra NCL-PCNA) included anti-PCNA (cell division marker, to evaluate regeneration potential) and anti-SOD (superoxide dismutase, to evaluate antioxidant activity on the superoxide radicals). Slices were deparaffinized, dehydrated and immunostrained with peroxidase by the avidin–biotin method. High temperature antigen unmasking technique in 0.01 M citrate buffer, pH 6, in a microwave oven was performed two times for 5 min total. Blocking of nonspecific reaction was completed with 1% normal goat serum and 3% not-fat milk. After rising in phosphate buffered saline (0.01 mol/L PBS pH 7.4), the sections were incubated in secondary antiserum. They were then washed in PBS and incubated in ABC (avidine and biotine complex) reagents (ABC kit—Vector) and incubated in peroxidase reaction (3,3′-diaminobenzidine tetrahydrochoride containing 0.01% H_2O_2 in PBS buffer. After being immunostrained, the sections were lightly counterstained with Mayer's hematoxylin. The slides were observed under a Leica light microscope. For positive control we used the stem cell region in stomach mucosa and for negative control the primary and secondary antibodies were omitted.

2.4. Anti-Helicobacter pylori activity

The ME and EAF were tested to detect anti-*Helicobacter pylori* activity (Hachem et al., 1996). The strain of *Helicobacter pylori* (ATCC 43504) was isolated from patients with duodenal ulcer disease. Frozen *Helicobacter pylori* isolate was thawed and grown on 5% sheep blood agar plates for 3–4 days at 37 °C in 10% CO_2 and 98% humidity. Each plate was swabbed with a sterile cotton-tipped applicator and the cells were suspended in sterile saline to obtain turbidity equivalent to a 2.0 McFarland standard. Muller–Hinton broth containing 10% horse serum was added to all wells of a 96-well microtiter plate (Corning, USA). Each well was incubated with *Helicobacter pylori* at a final concentration of ∼1 × 105 CFU/ml. The plates were incubated for 5 days in a microaerobic atmosphere at 37 °C. Following incubation, the plates were examined visually and spectrophotometrically and the lowest concentration showing complete inhibition of growth was recorded as the MIC (minimum inhibitory concentration). *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as control organisms for clarithromycin and ampicillin, respectively. The results were considered valid only when the MIC values for the control organisms were within the ranges established by the National Committee for Clinical Laboratory Standards (NCCLS).

2.5. Statistical analysis

Results were expressed as mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's or Tukey's test, with the significance level at $P < 0.05$.

3. Results and discussion

Since the gastric ulcer model induced by acetic acid was established more than 40 years ago (Takagi et al., 1969) this assay has been used for the study of underlying mechanisms of ulcer cicatrisation by subacute and chronic treatment of anti-ulcer drugs that accelerated the lesion-healing effect (Pawlik et al., 2002). Based on data from Okabe and Agamase (2005), this model was the one that most closely approximates human peptic ulcer disease, because the damage caused by the acetic acid penetrates in gastric mucosa not

Fig. 2. Histological analysis of the stomachs of rats treated with saline, cimetidine and *Strychnos pseudoquina* (ME and EAF) during 14 consecutive days. The arrows indicate the area used as morphometric parameter (height) of the regeneration area (#) in gastric lesions caused by acetic acid in rats. Microscopy magnification 2.5×.

only in the mucous membrane and submucous layer but also the muscular layer.

Fig. 1 shows the result of the macroscopic analysis of the gastric lesions induced by acetic acid in rats treated with vehicle, cimetidine, ME or EAF from *Strychnos pseudoquina*. Our results shown that although ME from *Strychnos pseudoquina* induced significant gastroprotective effect against injuries caused by NSAID and ethanol/HCl in mice (Silva et al., 2005), the treatment of rats for 14 consecutive days with this extract did not reveal significant healing action by reducing internal or external border area, when compared macroscopically with vehicle-treated animals ($P > 0.05$). However, the gastric lesions of the animals treated with EAF, at the same dose, induced significant reduction ($P < 0.05$) in internal (42%) and external (38%) border. This macroscopic result was also proven by histological analyses (Table 1) in which the height of the regeneration area (μm) was measured. The animals treat with EAF from *Strychnos pseudoquina* significantly increased epithelial height in regenerative area of gastric mucosa when compared with vehicle-treated ones. These results suggested EAF stimulated some proliferative factors in gastric mucosa that were important factors to regeneration of injured gastric mucosa.

Wallace and Devchand (2005) reported that reconstructing the entire structural architecture of gastric mucosa damage can require several weeks and involves the formation of granulation tissue at the base of the ulcers, formation of new vessels (angiogenesis), and re-establishment of the whole glandular architecture. The quality of mucosal structural restoration may be the most important factor in determining future ulcer recurrence (Tarnawski, 2005). H&E straining of histological cuts (Fig. 2) from EAF-treated animals reveals well structured mucous glands in the highly regenerated area that probably provoked their elongation and alignment. The architectural reorganization of regenerated area in EAF-treated animals (Fig. 2) facilitates the passage of the mucus and of other substances towards the surface of the gastric mucous membrane. Tarnawski et al. (1991) indicated that some gastric mucosa showed re-epithelialization of the mucosal surface but that subepithelial mucosa displayed prominent abnormalities. These abnormalities could interfere with oxygenation, nutrient supply, and mucosal resistance and defense; therefore, they could be a basis for ulcer recurrence.

Cellular proliferation plays an important role in maintaining the integrity of the gastrointestinal system. Cell division in gastrointestinal treatment is limited to a discreet anatomical area, the proliferative compartment (Okamoto and Watanabe, 2004). To investigate the effect of EAF on cell proliferation in the gastric ulcer area, this parameter was determined by immunohistochemical assay (Fig. 3). In the vehicle group, the immunoreactions for PCNA (expressed throughout the cell cycle whereas its concentration increased further in the S-phase) were observed as dark accumulations of DAB (diaminobenzidine) reaction products in the nuclei of the gastric mucosa layer. We observed that the EAF-ulcerated group stimulated the cell proliferation in the regeneration area, mainly at the base of the mucous glands (Fig. 3). So PCNA-positive nuclei from EAF-group was incontestably more intense than in the vehicle-treated or the cimetidine positive control group.

Previous studies also suggested that the quality of epithelial surface reconstitution was guaranteed by angiogenesis (formation of a new vascular network) that ensures an adequate supply of oxygen and nutrients to the healing mucosa (Tarnawski et al., 2001; Tarnawski, 2005). We quantified vessels in gastric mucosa submitted to different treatments (Table 1). The number of vessels in rats treated with cimetidine was double that in those treated only with vehicle. But the number of vessels in gastric mucosa of rats treated with EAF from *Strychnos pseudoquina* reveals an expressive increase (4 times more than vehicle treatment) of vessels. This result indicates that EAF treatment induced healing action in gastric

Table 2
Effects on body and organ weights and serum biochemical parameters of a single dose (250 mg/kg, p.o.)/day of methanolic extract (ME) or alkaloid fraction (EAF) from *Strychnos pseudoquina* administered for 14 consecutive days after ulcer formation ($n = 7$–8).

Treatment (p.o.)	Organ weight						Serum biochemical parameters				
	Kidney	Lungs	Heart	Liver	Spleen		Glucose (UI/L)	ALT (UI/L)	AST (UI/L)	Urea (mg/dl)	Creatinine (mg/dl)
Control	4.93 ± 0.26	4.41. ± 0.23	3.85 ± 0.10	10.75 ± 0.13	3.39 ± 0.12		25.57 ± 3.12	62.57 ± 1.96	408.0 ± 35.38	64.61 ± 3.98	0.63 ± 0.06
Cimetidine	5.03 ± 0.07	4.55 ± 0.18	3.76 ± 0.07	10.30 ± 0.14	3.34 ± 0.12		29.11 ± 2.56	48.55 ± 2.52	346.67 ± 18.67	58.00 ± 1.61	0.49 ± 0.02
ME	5.39 ± 0.11	4.46 ± 0.13	3.80 ± 0.06	10.30 ± 0.10	3.21 ± 0.12		26.33 ± 3.88	69.11 ± 6.58	433.22 ± 26.12	57.11 ± 3.40	0.55 ± 0.03
EAF	5.10 ± 0.08	4.62 ± 0.27	3.85 ± 0.19	10.47 ± 0.14	3.44 ± 0.12		34.00 ± 3.57	64.78 ± 9.16	415.56 ± 39.49	57.22 ± 3.71	0.51 ± 0.01

Results are mean ± S.E.M.; ANOVA followed by Dunnett's test. No significant differences $P > 0.05$.
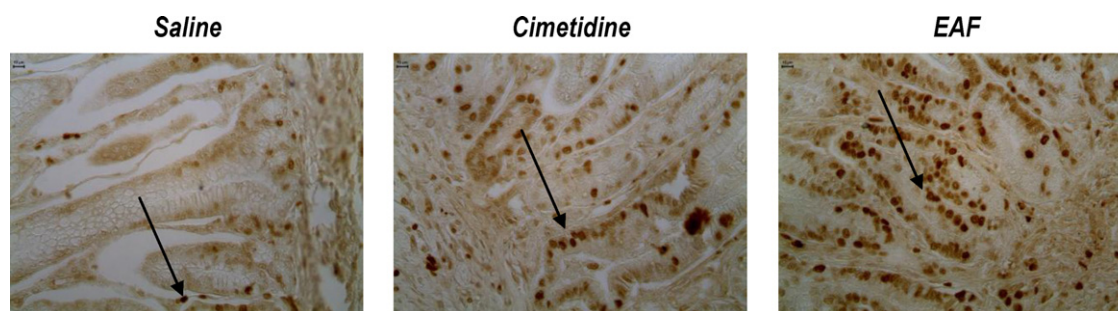
Fig. 3. Coloration immunohistochemistry for PCNA in stomachs of rats treated with saline, cimetidine (100 mg/kg) and *Strychnos pseudoquina* (EAF). (→) The area of reverse-organization of the mucous membrane (EAF) is with a lot of cells positive PCNA (brown color, suitable for the arrow). Microscopy magnification 20×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

mucosa by proliferating vessels in gastric mucosa that stimulated cell proliferation in the healing region.

Leite et al. (2004) observed that replenishment of SOD (superoxide dismutase) led to decrease in constrictive remodeling and enhanced angiogenesis. SOD is the most important enzymes responsible for eliminating free radicals from the cell, given that it transforms $O_2^{\bullet-}$ radicals into H_2O_2 and protects the cells from damage through the removal of those radicals. The SOD activity level represents the intracellular antioxidant ability of the cell. The free radicals affect the structure of the cell membrane as well as the membranes of various organelles including the mitochondria, lysosomes and the endoplasmic reticulum (Wang et al., 2005).

The participation of SOD in the EAF action mechanism was also evaluated, due to its importance as an antioxidant and eliminator of free radicals from the cells (Wang et al., 2005; Fan et al., 2005). The result of this study evidenced a greater number of strained cells (in brown) from the EAF-treated group than in groups treated with vehicle (Fig. 4). We also observed that SOD strained cells were not present in cimetidine-treated group. Konturek et al. (2000) observed that SOD accelerates the healing of ischemia and reperfusion lesions due to suppression of oxygen free radicals and improvement of gastric microcirculation. Based on our result we concluded that the EAF is quite effective in the process of releasing SOD, eliminating free radicals and, thus, healing gastric lesions.

Some new antiulcer drugs, such as rebamipide, were effective in inducing ulcer healing but failed in *Helicobacter pylori* eradication therapy (Terano et al., 2007). As it is highly desirable to identify one drug that produces gastroprotective action, heals injured gastric mucosa and also acts against *Helicobacter pylori*, we evaluated the anti-*Helicobacter pylori* effect of EAF from *Strychnos pseudoquina* against a strain of *Helicobacter pylori* isolated from patients with duodenal ulcer disease. Recently, Castillo-Juárez et al. (2009) evaluated plants used in traditional medicine against *Helicobacter pylori* while considering an MIC value lower than 125 μg/ml, to constitute strong antibacterial action. We obtained a very interesting MIC from EAF of 75 μg/ml, a finding that indicates a highly satisfactory anti-*Helicobacter pylori* action from this fraction.

Over twenty years ago Garner (1986) predicted that the future of antiulcer drug research must address the multifactorial etiologies of gastric ulcer as well as attempt to cure the disease rather than simply induce antisecretory action. But the efficacy of the new drug must also be accompanied by a clear indication for safe human use. Silva et al. (2005) observed that EAF and ME from *Strychnos pseudoquina* did not present acute toxicity given that no mortality was observed up to 5 g/kg (p.o.). Animals submitted to different treatments (EAF, ME, cimetidine and vehicle) in gastric ulcer model induced by acetic acid supply some important toxicity parameters such as evolution of body weight during 14 days(data not show), mortality, and weight of the vital organs (Table 2). The 14-day evolution in body weight alteration under EAF did not differ significantly from the vehicle group while the average weights of vital organs and visceral conditions were normal and comparable to those of the control group ($P > 0.05$). The finding of one death registered in each of the saline, cimetidine and EAF groups, does not necessarily represent a toxicity sign but rather a consequence of the surgical procedure of this model. Table 2 also presents results obtained by biochemical analyses of serum, organized by treatments, among which we observed no alteration of these parameters.

Santos et al. (2006) described that mutagenic effect of methanolic extract from *Strychnos pseudoquina* present in Ames and micronucleus tests. But they also recommend that EAF from *Strychnos pseudoquina* should be explored as a possible source of new antiulcerogenic phytotherapeutic preparation because the absences of mutagenicity but new toxicological studies are necessary to ensure its safety use in human.
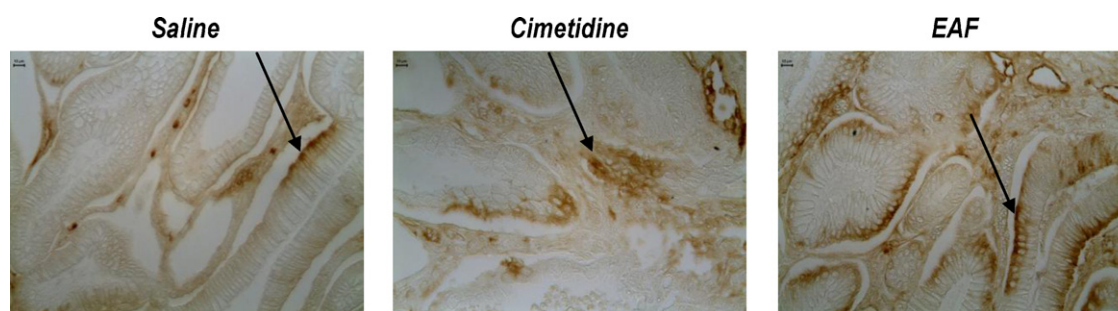

Fig. 4. Coloration immunohistochemistry for SOD in stomachs of rats treated with saline, cimetidine (100 mg/kg) and *Strychnos pseudoquina* (EAF). (→) The area of reverse-organization of the mucous membrane (EAF) is with a lot of cells positive SOD (brown color, suitable for the arrow). Microscopy magnification 20×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Phytochemical studies of EAF, in agreement with Silva et al. (2005), yielded nordiidrofluorcurarine, an alkaloid indole, in addition to rutin and kaempferol 3-O-β-rutenoside. The structure of indolic alkaloid 3-hydroxy-enolate resembles that of omeprazole, a well known antiulcer agent. However, this alkaloid may possess a reversible bond between sulfhydryl groups and does not present adverse side effects from omeprazole related to carcinoma.

4. Conclusions

Based on the results of the present work we concluded that the EAF from *Strychnos pseudoquina* presents expressive healing effect on gastric mucosa through the increase of angiogenesis, cell proliferation, antioxidant activity through expression of SOD activity and antibacterial action against *Helicobacter pylori*. The absence of toxicity throughout the 14 consecutive days of treatment ensures its safety for use by the population.

Acknowledgements

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