Evaluation of *Strychnos pseudoquina* St. Hil. Leaves Extract on Gastrointestinal Activity in Mice

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***Strychnos pseudoquina* St. Hil. (Loganiaceae) was investigated for its ability to protect the gastric mucosa against injuries caused by non-steroidal anti-inflammatory drugs (piroxicam) and a necrotizing agent (HCl/EtOH) in mice. The MeOH extract and enriched alkaloidic fraction (EAF) provided significant protection in experimental models wheer used at doses of 250 and 1000 mg/kg. *In vivo* tests were carried out to evaluate for possible toxic effects and no mortality was observed up to the 5 g/kg dose level. Phytochemical investigation led to the isolation of a new indole alkaloid, which elucidated the observed pharmacological effects.**

Key words *Strychnos pseudoquina*; antiulcer activity; toxic activity; alkaloid

As part of a state collaborative program named BIOTA-Fapesp, our project entitled "Sustainable use of the Brazilian Biodiversity: Chemical and Pharmacological Prospection on Higher Plants" aims to investigate plants with potential activity on the prevention of gastric injuries. This research is based on ethnopharmacological investigation, followed by the chemical and pharmacological investigation of plant extracts. In this work, we investigated the leaves of *Strychnos pseudoquina* St. Hil. (Loganiaceae). *Strychnos pseudoquina* is used in traditional medicine for the treatment of malaria and for the preparation of so-called "aqua inglesa," largely used in Brazil as a tonic and febrifuge and also to relieve stomach pains.[1)] It is popularly known as "quina branca" (white quine). Many plants from the genus *Strychnos* are also called "quina," since they are used against malaria.[2)] Phytochemical investigations of several *Strychnos* species led to the isolation of a number of indole alkaloids as the main compounds. Flavonoids, terpenoids and waxes were also reported to a lesser extent. Previous investigation of *S. pseudoquina* afforded the alkaloids bisnordihydroxytoxiferine, diaboline and 11-methoxydiaboline, as well as the flavonoids isorhamnetine and estricnobiflavone.[1,3)] Despite the well known toxicity of many *Strychnos* species, our ethnopharmacological survey indicated that *S. pseudoquina* is used as a bitter folk medicine against stomach diseases. Since *Strychnos* species are known to contain toxic compounds, it is important to conduct a scientific investigation in order to assure its efficacy and/or to detect the presence of toxic components.[4)]

Results and Discussion

For this phytochemical examination we used an aliquot of the methanolic extract of leaves from *S. pseudoquina*. TLC plates sprayed with Draggendorff, iodoplatinate, and NP/PEG (Natural Products/Polyethyleneglycol) reagents indicated that this polar extract contained alkaloids and flavonoids.[1,3)] This extract was then fractionated by gel permeation chromatography over Sephadex LH-20, and the fractions were purified by adsorption chromatography on silica or alumina, affording compounds **1**—**3**, which were identified by several spectroscopic methods. After analyses, **2** and **3** were identified as rutin and kempferol 3-*O*-β-rutinoside.

Compound **1** was identified as a pale yellow gum. The IR spectrum presented bands at 3300 cm^{-1} (OH) and 1600 cm^{-1} (C=C).

The molecular formula of **1** was determined to be $C_{19}H_{22}N_2O_2$ by the EI-MS, which showed pseudo molecular ion peak sat *m*/*z* 311 $[M+H]^+$ and *m*/*z* 333 $[M+Na]^+$, and the elemental analysis data. The structure of **1** was fully elucidated by 1D and 2D NMR experiments at 500 MHz. The ^{1}H-NMR spectrum in $CDCl_3$ (Table 1) displayed signals at δ 7.05 (d, J=8.0 Hz), 6.71 (dd, J=8.0, 8.0 Hz), 7.00 (dd, J=8.0, 8.0 Hz) and 6.57 (d, J=8.0 Hz), corresponding to an *ortho*-dissubstituted benzene ring. These signals and the signal at δ 4.03 suggested the presence of an indolic nucleus.[5—7)] The signal at δ 6.16 (d, J=1.5 Hz) suggested the presence of a double bond in the structure of **1**. The >C=CH–CH_3 system was deduced from the signal at δ 5.54 (q, J=6.5 Hz, =CH–) coupled to the doublet at δ 1.67 (J=6.5 Hz, –CH_3) in the ^{1}H–^{1}H COSY (gradient correlate spectroscopy) spectrum. Other signals corresponding to the aliphatic chain of **1** were observed between δ 1.97 and 4.03 (Table 1). The ^{13}C-NMR spectrum presented 19 signals, eight of which could be assigned to the indolic nucleus: δ 54.5 (C-7), 65.0 (C-2), 110.6 (C-12), 119.9 (C-10), 122.5 (C-9), 128.8 (C-11), 132.4 (C-8) and 146.3 (C-13). The signals at δ 139.9 (C-16), 124.4 (C-17), 124.1 (C-19) and 130.2 (C-20) confirmed the presence of two double bonds. The presence of the signal at δ 91.8 indicated the presence of an additional OH group at position C-3.[6,7)] The shielding effects on C-5, C-6 and C-21 when compared to the literature also support the presence of an OH-group at C-3.[6,7)] When compared to the literature, the deshielding α-effect over C-17 and β-effect over C-16 suggest the presence of an OH-group at C-17.[6,7)]

The structure of **1** was deduced from a combination of 1D NOESY (nuclear overhauser effect spectroscopy), ^{1}H–^{1}H-

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Table 1. ^{1}H- and ^{13}C-NMR Data of **1** ($CDCl_3$, 500 MHz)

Position	^{1}H (J_{HH} in Hz)	^{13}C	COSY	gHMQC	gHMBC
2	4.03 (s)	65.0	H_2/H_{17}	H_2/C_2	C_2/H_{17}
3	—	91.8	—	—	C_3/H_{21a} C_3/H_{17}
5	H_{5a} 2.88 (m); H_{5b} 3.72 (m)	52.4	H_{5a}/H_{5b} H_{5b}/H_{5a}	H_{5a}/C_5 H_{5b}/C_5	—
6	2.21 (m)	39.0	H_6/H_{5a} H_6/H_{5b}	H_6/C_6	—
7	—	54.5	—	—	—
8	—	132.4	—	—	C_8/H_{10} C_8/H_{12}
9	7.05 (d, J=8.0)	122.5	H_9/H_{10}	H_9/C_9	C_9/H_{11}
10	6.71 (dd, J=8.0, 8.0)	119.9	H_{10}/H_{11} H_{10}/H_9	H_{10}/C_{10}	C_{10}/H_{12}
11	7.00 (dd, J=8.0, 8.0)	128.8	H_{11}/H_{12} H_{11}/H_{10}	H_{11}/C_{11}	C_{11}/H_9
12	6.57 (d, J=8.0)	110.6	H_{12}/H_{11}	H_{12}/C_{12}	C_{12}/H_{10}
13	—	146.3	—	—	C_{13}/H_{11} C_{13}/H_9
14	H_{14a} 2.03 (d, J=14.5) H_{14b} 1.97 (dd, J=14.5, 9.0)	32.2	H_{14a}/H_{14b} H_{14b}/H_{14a} H_{14b}/H_{15} H_{14a}/H_{15}	H_{14a}/C_{14} H_{14b}/C_{14}	—
15	3.43 (d, J=9.0)	31.4	H_{15}/H_{14b} H_{15}/H_{14a}	H_{15}/C_{15}	C_{15}/H_{19} C_{15}/H_{17} C_{15}/H_{18} C_{15}/H_2
16	—	139.6	—	—	C_{16}/H_2
17	6.16 (d, J=1.5)	124.4	H_{17}/H_2	H_{17}/C_{17}	C_{17}/H_{16} C_{17}/H_2
18	1.67 (dd, J=6.0, 1.0)	13.6	H_{18}/H_{19}	H_{18}/C_{18}	—
19	5.54 (q, J=6.0)	124.1	H_{19}/H_{18}	H_{19}/C_{19}	C_{19}/H_{18} C_{19}/H_{21b}
20	—	130.2	—	—	C_{20}/H_{18} C_{20}/H_{21a}
21	H_{21a} 3.80 (m) H_{21b} 3.42 (m)	54.1	H_{21a}/H_{21b} H_{21b}/H_{21a} H_{21b}/H_{18}	H_{21a}/C_{21} H_{21b}/C_{21}	C_{21}/H_{19}

COSY, gHMQC (gradient heteronuclear through multiple quantum coherence) and gHMBC (gradient heteronuclear multiple bond correlations) experiments, as presented in Fig. 1. Correlation in the HMBC spectrum between the proton at δ 3.80 (H_{21a}) and the carbon at δ 91.8 (C-3) supported the presence of an additional OH-group at C-3, whereas the long-range correlation between the proton at δ 6.16 (H_{17}) and the carbons at δ 139.6 (C-16) and at δ 65.0 (C-2) agree with an OH-group at position C-17.

The structure of **1** is the 3-hydroxy-enolate of the known alkaloid nordiidrofluorocurarine, previously isolated from *S. amazonica* and *S. froessi*.[8] We can pose the question of whetter compound **1** has a role as a precursor in the biosynthesis of strychnine,[9] since **1** seems to be originated just before the formation of the Wieland–Gumlich aldehyde (Fig. 2), but without hydroxylation at positions 3 and 18. It was already reported that hydroxylation at position 3 can result from an artifact produced when chlorinated solvents are used in the extractive processes.[10] To check this possibility, we extracted the leaves of *S. pseudoquina* with pure MeOH and with water. TLC analyses using the isolated substance **1** as standard showed the presence of **1** in both extracts, thus suggesting that **1** is not an artifact.

Although much work has been done on the pharmacological properties and chemical composition of species belonging to the genus *Strychnos*, only limited data are available in the literature from the pharmacological and toxicity properties of *S. pseudoquina* that might ensure the safe use of this important medicinal plant.

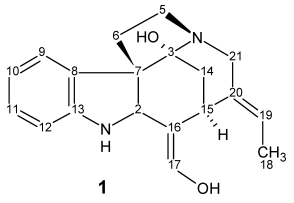

Fig. 1. Indole Alkaloid **1** Isolated from the Leaves of *Strychnos pseudoquina*

Due to the high cytotoxicity of some of the *Strychnos* alkaloids, we first evaluated the crude MeOH extract and the EAF in *in vivo* acute toxicity assays in mice. Animals were treated with a single dose of the MeOH extract and EAF (5000 mg/kg each) and observed during a period of 14 d. Results showed that there are no significant differences between the animals treated with saline and those treated with MeOH or EAF obtained from *S. pseudoquina* (Fig. 3). In agreement with this, no signs or symptoms of acute toxicity were observed. There were no significant differences in organ weight, in water or food intake, or in the amount of faeces

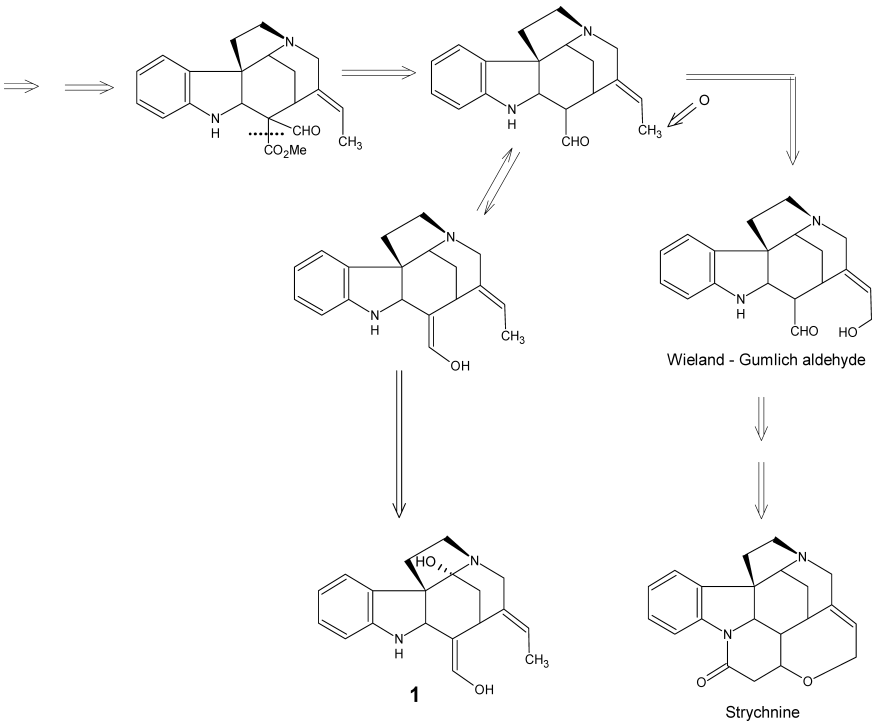

Fig. 2. Proposed Role of **1** on the Biogenetic Pathway to the Formation of the Wieland–Gumlich Aldehyde in the Biosynthesis of Strychnine (Based on Dewick, 1997)

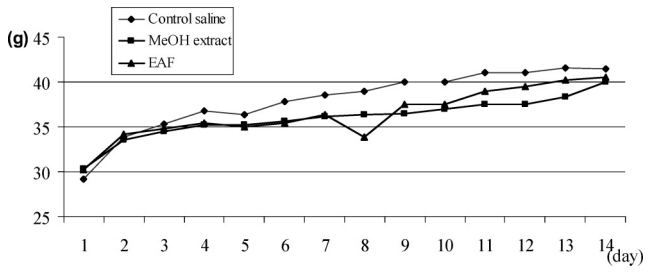

Fig. 3. Body Weight Gain in Mice Treated with Saline, MeOH Extract and Enriched Alkaloidic Fraction (EAF) of *S. pseudoquina* (5 g/kg, *p.o.*) during 14 d

Table 2. Evaluation of the Acute Toxicity of MeOH Extract and Enriched Alkaloidic Fraction (EAF) of *S. pseudoquina* (5 g/kg, *p.o.*) in Mice (Mean±S.D.)[a)]

Weight (g) (n=10)	Control (10 ml/kg)	MeOH (5 g/kg)	EAF (5 g/kg)
Body	41.50±7.09	40.00±3.33	40.50±5.98
Kidney	0.52±0.12	0.51±0.14	0.45±0.06
Liver	2.22±0.53	1.89±0.39	1.94±0.34
Heart	0.20±0.05	0.180±0.036	0.17±0.31
Lungs	0.27±0.04	0.27±0.04	0.22±0.03
Mortality	0/10	0/10	0/10

ANOVA $F_{(2,27)}$=2.17. Dunnett test $*p<0.05$. *a*) Results were obtained 4 d after daily administration of MeOH extract and EAF.

produced by treated and control mice (Table 2). None of the treated mice died during the 14 d of observation after the administration of the MeOH extract or the EAF. These results add more information about the possible therapeutic safety of this species.

The antiulcer activity of the crude MeOH extract and of the EAF of *S. pseudoquina* was then evaluated by two different models. In the model of HCl/EtOH in mice (Table 3), oral administration of the crude MeOH extract (1000 mg/kg) significantly inhibited 58.3% of the ulcerogenic lesions from the HCl/ethanol solution over the gastric surface, whereas the EAF significantly inhibited 57.9 and 43.7% of the gastric ulcers at doses of 250 and 1000 mg/kg, respectively.

Table 3. Effects of the Lansoprazole, MeOH Extract and of the Enriched Alkaloidic Fraction (EAF) of *S. pseudoquina* in the Model of HCl/EtOH in Mice (Mean±S.D.)

Treatments	Dose (mg/kg)	n	pH (mean±S.D.)	ULI (mean±S.D.)	Inhibition (%)
Saline	10	7	3.0±0.7	47.0±27.6	—
Lansoprazole	30	8	6.0±0.5**	5.7±3.2**	87.8
MeOH	250	5	2.4±0.5	25.2±9.7	46.4
	1000	5	2.8±1.1	19.6±16.2*	58.3
Saline	10	8	2.2±0.5	64.6±24.9	—
Lansoprazole	30	8	5.5±1.3**	2.1±2.3**	97.7
EAF	250	5	3.0±0.0	27.2±4.3*	57.9
	1000	5	3.0±0.0	36.4±15.1*	43.7

ANOVA $F_{(4,25)}$ for MeOH=6.54 $p<0.001$. Test of Dunnett $*p<0.05$; $**p<0.01$; ANOVA $F_{(4,25)}$ for EAF=16.22 $p<0.001$. Test of Dunnett $*p<0.05$; $**p<0.01$.

NSAIDs (non-steroidal anti-inflammatory drugs), such as piroxicam, resulted in the production of gastric ulcers, mainly in the glandular portion of the stomach. Nearly 100% of the animals showed gastric ulceration. The majority of ulcers were gastric erosions that were multiple, dotted or elongated superficial haemorrhagic mucosal lesions that did not penetrate the muscularis mucosae.[11)] As shown in Table 4, in the NSAID-induced gastric ulceration model, pretreatment with crude MeOH extract produced a decrease in the gastric ulceration induced by piroxicam. The ulcerogenic lesions caused by piroxicam (Table 4) were significantly

Table 4. Effects of the Cimetidine, MeOH Extract and of the Enriched Alkaloidic Fraction (EAF) of *S. pseudoquina* in the Piroxicam Model in Mice (Mean±S.D.)

Treatments	Dose (mg/kg)	*n*	pH (mean±S.D.)	ULI (mean±S.D.)	Inhibition (%)
Saline	10	7	2.6±0.5	21.1±6.2	—
Cimetidine	100	5	3.0±0.0	6.0±1.6**	68.0
MeOH	250	5	2.6±0.5	8.8±4.8**	55.5
	1000	5	2.8±0.4	6.4±4.4**	63.2
Saline	10	8	3.0±0.5	31.4±9.3	—
Cimetidine	100	8	3.4±0.5	10.0±4.8**	68.1
EAF	250	5	3.0±0.0	9.0±2.5**	71.2
	1000	5	3.0±0.0	8.0±3.4**	74.5

ANOVA $F_{(4,22)}$ for MeOH=7.11 $p<0.001$. Test of Dunnett $^{**}p<0.01$; ANOVA $F_{(4,26)}$ for EAF=20.70 $p<0.001$. Test of Dunnett $^{**}p<0.01$.

inhibited by the crude MeOH extract, by 55.5% (250 mg/kg) and 63.2% (1000 mg/kg). The best results were obtained with the oral administration of EAF, with significant inhibition of the ulcerogenic lesions caused by piroxicam by 71.2% (250 mg/kg) and 74.5% (1000 mg/kg). These results show the effective antiulcer activity of EAF when compared with cimetidine treatment (68%), however no significant differences were observed between the groups treated with EAF and cimetidine ($p>0.05$).

Therefore, our results show that MeOH extract and EAF from *S. pseudoquina* are orally effective against gastric damage induced by cytodestructive agents (HCl/ethanol) and ulcerogenic agents (NSAIDs).

The mechanisms by which *S. pseudoquina* exerts its antiulcer activity is far from clear. However, EAF in particular is involved in the protection of the stomach against ulceration. It is interesting to observe that the structure of **1** roughly resembles that of omeprazole, a well known antiulcer agent. Literature reports that omeprazole can irreversibly bind to H^+/K^+ ATPase through the sulphydril group.[12] However, the irreversible suppression of the gastric pump has been related to some carcinomas.[13] In the case of **1**, there is the possibility of a reversible bond between the alkaloid and the enzyme, thus revealing the promising potential of **1** as an anti-ulcer agent (Fig. 4).

In conclusion, this study has shown that both the polar extract and the enriched alkaloid fraction of *Strychnos pseudoquina* have a significant anti-ulcer and cytoprotective effect on different experimentally induced gastric lesions, with as absence of toxicity by acute treatment. Our results show that the anti-ulcer activity of the MeOH extract is almost as potent as that of the alkaloid-enriched fraction. Therefore, it is possible that other compounds present in the crude extract (*e.g.* flavonoid glycosides) may also contribute to the observed activity. Despite the moderate potency of the extracts and fractions, the inhibition of gastric damage and absence of acute toxicity of *S. pseudoquina* show that the ethnopharmacological approach can be useful in the search for active extracts and compounds. Further studies are in progress to evaluate the chronic toxicity and the modes of action.

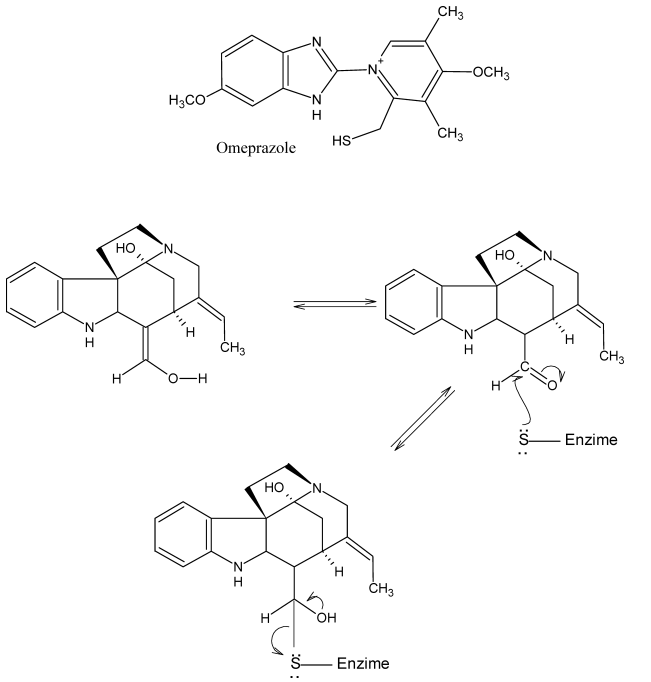

Fig. 4. Possible Mechanism of Action of **1** Showing the Reversibility of the Reaction

Experimental

Biological Material The leaves of *Strychnos pseudoquina* St. Hil. were collected in May 2001, at Porto Nacional, Tocantins State, Brazil, and authenticated by Professor Eduardo Ribeiro dos Santos from Instituto de Biociências (IB), Universidade do Tocantins (UNITINS). A voucher specimen (Nr 3291) was deposited at the Herbarium of the UNITINS.

Apparatus NMR spectra in $CDCl_3$ were obtained using a Varian INOVA-500 spectrometer, operating at 500 MHz for ^1H and at 125 MHz for ^{13}C and 2D-NMR (inverse detection ^1H–^{13}C HSQC and HMBC). ES-MS was performed on a Fisons VG Platform spectrometer in positive (70 V) mode. The sample was dissolved in MeOH and injected directly. IR spectrum was performed in a FT-IR-Nicolet Impact IMACT-400, KBr. UV spectra were obtained on a Beckman DU 670 spectrometer. Elemental analysis was made with a Carlo Erba EA 1110 apparatus. TLC was performed on silica gel SiF254 (Merck). The plates were visualized using UV light (254, 365 nm).

Extraction and Isolation The leaves of *S. pseudoquina* (300 g) were powdered and successively extracted with CH_2Cl_2 and MeOH (1 week for each solvent). The solvents were evaporated in a vacuum to give 13.9 and 17.7 g of each extract, respectively. A portion (5.0 g) of the methylene chloride extract (CH_2Cl_2) was repeatedly fractionated by column chromatography on silica gel eluted with several gradient mixtures of hexane/EtOAc to give lupeol, α-amirin and β-amirin, identified by their NMR data compared to the literature,[14] and also by GC-FID analyses with authentic standards. A portion (5.0 g) of the methanolic extract (MeOH) of *S. pseudoquina* was submitted to column chromatography on Sephadex LH-20 (100×5 cm) with MeOH as the eluent. One hundred fractions (5 ml) were collected and checked by TLC on silica gel plates $CHCl_3$–MeOH–*n*-PrOH–H_2O (5 : 6 : 1 : 4, v/v/v/v lower phase) was revealed with Draggendorff, iodoplatinate or NP/PEG (Natural Products/Polyethyleneglycol) reagents. Alkaloids were detected in Fr. 3—29 (named "enriched alkaloidic fraction (EAF)" *ca.* 3 g) and flavonoids were detected in Fr. 35—90 (250 mg). Fractions 23—25 (69.8 mg) were further purified by column chromatography on neutral alumina using $CHCl_3$–MeOH–NH_4OH (95.0 : 0.5 : 0.05, v/v/v) as an eluent to afford the alkaloid **1** (20 mg). Fractions 35—37 (40 mg) and fractions 40—42 (45 mg) were purified by repeated column chromatography on polyvinylpolypyrrolidone (Sigma) eluted with MeOH, yielding **2** (9.0 mg) and **3** (7.0 mg), respectively. Compounds **2** and **3** were identified by their NMR spectra and by comparison with previous data reported in the literature.[15,16]

Compound 1 IR (KBr): 3300 cm^{-1} (OH), 1600 cm^{-1} (C=C). ES-MS *m/z* (rel. int.) (70 V, positive ion): 311 (100) $[M+H]^+$, 333 (30) $[M+Na]^+$. Elemental analysis: *Anal.* C 73.45%, H 7.24%, N 9.15% Calcd for $C_{19}H_{22}N_2O_2$ C 73.52%, H 7.14%, N 9.03%. ^1H- and ^{13}C-NMR: see Table 1.

Animals Male Swiss albino mice (25—35 g) from the Central Animal House of the Universidade Estadual Julio de Mesquita Filho (UNESP/Botucatu) were used. The animals were fed a certified Nuvilab CR-a® (Nuvital)

diet with free access to tap water under standard conditions of 12 h dark–12 h light and temperature (22±1%). Fasting was done prior to all assays because the standard drugs and extract were always administered orally (by gavage). Moreover, the animals were kept in cages with raised floors of wide wire mesh to prevent coprophagy. The protocols were approved by the UNESP Institutional Animal Care and Use Committee, following the recommendations of the Canadian Council on Animal Care.[17)]

Drugs and Chemicals HCl, EtOH (Nuclear, Brazil), cimetidine (Sigma Chemical Co., St. Louis, MO, U.S.A.), piroxicam (Hexal, Brazil) and lansoprazole (Medley, Brazil) were used in this study. Each extract was dissolved in NaCl solution 0.9% (vehicle). All substances were prepared immediately before use, and the reagents used were of analytical grade.

Hippocratic Screening and Acute Toxicity The signs and symptoms associated with the oral administration of the MeOH extract and of the EAF (5000 mg/kg, each) were monitored in 8 mice per dose. The mice were examined 0, 0.5, 1, 2, 4, 8, 24 and 48 h after administration to assess possible clinical or toxicological symptoms. The mortality rate was monitored for a period of 2 weeks.[18)]

Antiulcerogenic Effect a) HCl/EtOH-Induced Ulcer: The antiulcerogenic activity of the extract obtained from *S. pseudoquina* was studied in HCl/EtOH-induced gastric ulcer. The experiment was performed as described in the literature.[19)] Mice were divided into groups of 7—8 animals which were fasted 24 h prior to receiving an oral dose of the vehicle, saline (10 ml/kg), lanzoprazole (30 mg/kg) or *S. pseudoquina* (250, 1000 mg/kg). After 50 min all groups were orally treated with 0.2 ml of a 0.3 M HCl/60% EtOH solution (HCl/EtOH) for gastric-ulcer induction. Animals were killed 1 h after the administration of HCl/EtOH, and the stomachs excised and inflated by saline injection (2 ml). The extent of the lesions was measured using an ulcerative lesion index (ULI), and the pH of gastric juice determined. This index was expressed as the sum of all lesions, as described in the literature.[20)]

b) Non-steroidal Antiinflammatory Drug (NSAID)-Induced Gastric Ulcers in Mice: In this model,[21)] gastric ulcer was induced using piroxicam (30 mg/kg, s.c.) administered to mice. The crude MeOH extract or EAF from *S. pseudoquina* (250, 1000 mg/kg), cimetidine (100 mg/kg) or saline was administered orally 30 min before induction of the gastric ulcer. The animals were killed by cervical dislocation 4 h after treatment with the ulcerogenic agent; the stomachs were removed and the gastric juice pH and gastric damage were determined by the ULI calculated as described previously.

Statistical Analysis Results ware expressed as the mean±S.D. Statistical significance was determined by one-way analysis of variance followed by Dunnett's test, with the level of significance set at $p<0.05$.

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