ORIGINAL ARTICLE

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Rec. Nat. Prod. 7:1 (2013) 35-44

records of natural products

Gastroprotective Effect of the Ethanolic Extract and Fractions obtained from *Syngonanthus bisulcatus* Rul.

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(Received June 22, 2012; Revised October 9, 2012, Accepted October 30, 2012)

Abstract: *Syngonanthus bisulcatus* Rul., popularly known in Brazil as "*sempre-vivas chapadeira*", is a plant of the family Eriocaulaceae, it is found in the states of Minas Gerais and Bahia. In this work, the ethanolic extract (EtOHE), flavonoid-rich (FRF), and flavonoid-deficient (FDF) fractions obtained from scapes of *S. bisulcatus* were investigated for gastroprotection in both rats and mice. The activity was evaluated in models for induced gastric ulcer (absolute ethanol, stress, non-steroidal anti-inflammatory drugs, and pylorus ligation). The participation of mucus and prostaglandin E_2 were also investigated. Sb-EtOHE (50, 100, and 250 mg/kg, p.o.), Sb-FRF (100 mg/kg, p.o.), and Sb-FDF (100 mg/kg, p.o.) significantly reduced gastric injuries in all models. Sb-FRF altered gastric juice parameters after pylorus ligation. Sb-FRF and Sb-FDF (100 mg/kg each, p.o.) significantly increased the amount of adherent mucus in the gastric mucosa. Sb-FRF maintained the mucosal levels of prostaglandin after the administration of indomethacin. The results indicate that Sb-EtOHE, Sb-FRF and Sb-FDF have significant gastroprotective activity. The observed gastroprotective effects of *S. bisulcatus* probably involve the participation of both mucus and prostaglandins, integral parts of the gastrointestinal mucosa's cytoprotective mechanisms against aggressive factors.

Keywords: Medicinal plants; *e*riocaulaceae; *syngonanthus bisulcatus*; gastric ulcer; gastroprotective activity.

1. Introduction

Gastric ulcer, a gastrointestinal tract problem affects a large number of people worldwide and many studies are being conducted with the goal of producing a general cure. Among the etiological factors related to this disorder are stress, smoking, alcohol, nutritional deficiencies, infections (*Helicobacter pylori*), and frequent or indiscriminate use of non-steroidal anti-inflammatory drugs (NSAIDs) [1, 2].

Gastric ulcer is generally caused by an imbalance between aggressive and protective factors in the stomach. Major aggressive factors are acid/pepsin secretion, *H. pylori* infection, and bile salts.

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Defensive factors involve the mucosal barrier, mucus secretion, prostaglandin (PG), blood flow, cellular regeneration, epidermal growth factors, and motility [3-5].

There are two main approaches to treat peptic ulcer. The first is to reduce gastric acid production; the second is to enhance gastric mucosa protection [3,6]. Therapeutic agents such as antacids, proton pump inhibitors, and histamine receptor antagonists are used to inhibit gastric acid secretion or to boost mucosal defense mechanisms [7,8]. Antimicrobial therapies, in combination with anti-secretory drugs are being successfully used to treat peptic ulcers caused by infection with *H. pylori* [9]. Yet there have been reports of adverse effects and relapses [10, 11].

Several natural drugs have been reported to possess antiulcer activity that strengthens gastric mucosal defense factors [12]. Yet many medicinal plants are able to reduce offensive factors, and have proven to be safe and effective, and show better patient tolerance. They are also less expensive, and therefore globally competitive. These plants among others are currently being evaluated for their gastroprotective activity in animal studies [13-15]. As an example, a data survey conducted by Falcão *et al*. [16] recently referenced American plants with antiulcer activity.

The Eriocaulaceae family comprises about 1200 species, which are distributed in 10 genera. It has a pan tropical distribution, but most species occur in near tropical regions, such as in the mountains of Venezuela and Brazil [17-19]. The family is typical to the vegetation of Brazil's *'campo rupestre*', and is especially frequent in the *Espinhaço* mountain chain of Minas Gerais, and Bahia [19].

The plants have inflorescences and scapes that give them a "living" appearance even after being dried. Some of these plants in Brazil are popularly called "evergreens". The S*yngonanthus* genus is of economic importance, and phytochemicals studies have revealed the rich presence of flavonoids [20, 21]. Flavonoids have been noted in the literature for their gastroprotective effects [22].

Syngonanthus bisulcatus Rul. popularly known in Brazil as "*sempre-vivas chapadeira*" is of the Eriocaulaceae family, and is easily found in the states of Minas Gerais and Bahia. The plant exhibits inflorescences which are dried, and exported. Our group has carried out preliminary studies of five different types of flavonoids from capitula and scape extracts of *S. bisulcatus*, each with demonstrated gastroprotective activity in pharmacological trials using an acidified ethanol-induced ulcer model [23].

The present study evaluates gastroprotective activity facing a variety of experimental induced-gastric ulcer models for ethanolic extract (Sb-EtOHE), flavonoid-rich (Sb-FRF), and flavonoid-deficient (Sb-FDF) fractions obtained from the scapes of *S. bisulcatus* Rul.

2. Materials and Methods

2.1. Animals

The experimental protocols were approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) with register number 502-1. Male Swiss albino mice (30–40 g) or male Wistar albino rats (180–250 g) from the Central Animal House of the State University of Campinas (CEMIB/UNICAMP) were used. The animals were fed a certified Nuvilab CR-diet, and had free access to water under fixed conditions of illumination (12/12 h light/dark cycle), humidity (60 ± 1.0%), and a temperature of (21,5º ± 1.0). Fasting was used prior to all assays because standard drugs, Sb-EtOHE, Sb-FRF and Sb-FDF were administered orally (by gavage), or by intra-duodenal route using a 0.9 % saline solution (10 mL/kg) as the vehicle (negative control). The animals were kept in cages with raised wide mesh floors to prevent coprophagy.

2.2. Drugs

The drugs and reagents were prepared immediately before use. The following drugs were used: carbenoxolone (Sigma Chemical Co, U.S.A), cimetidine (Sigma Chemical Co, U.S.A), lansoprazole (Aché, Brazil), indomethacin (Sigma Chemical Co, U.S.A) and bethanechol chloride (Sigma Chemical Co, U.S.A). The Sb-EtOHE, Sb-FRF and Sb-FDF were obtained from scapes of *S.*

bisulcatus as described below. The Sb-EtOHE was administered at doses of 50, 100, and 250 mg/kg and the Sb-FRF or Sb-FDF at a dose of 100 mg/kg.

2.3. Plant material

S. bisulcatus was collected in the Serra do Cipó mountain range in Minas Gerais state, Brazil, in April 2002. A voucher specimen of number SPF77735 was deposited in the herbarium of the Department of Botany at the Institute of Biosciences, University of São Paulo.

2.4. Preparation of the ethanolic extract and purified fractions

Scapes (500 g) of *S. bisulcatus* were collected, dried in an oven at 60°C for 4 days, and then powdered. The resulting material was macerated sequentially in methylene chloride, absolute ethanol, and 70 % ethanol, at room temperature for one week with each solvent. The extracts were then filtered and concentrated under vacuum. The two ethanolic extracts were analyzed by TLC on silica gel plates using n-BuOH/HOAc/H_2O (6:1:2 v/v/v). The TLC spots were detected using UV light, and NP/PEG reagent, which yielded the yellow or orange spots characteristic of flavonoids. Since these extracts contained material with similar R_f values, they were combined and weighed. An aliquot (3.5 g) of the ethanolic extract was dissolved in 10 mL of MeOH, and fractionated on Sephadex LH-20 CC (3 cm x 100 cm) with elution in MeOH at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and combined based on their migration in the above-described TLC system. Fractions 1-50 did not contain (FDF) flavonoids; fractions 51-70 were intermediate fractions, and fractions 71-126 contained (FRF) flavonoids.

2.5. Gastroprotective activity

Several experimental induced-ulcer models were used to evaluate the gastroprotective effects of the Sb-EtOHE, Sb-FRF and Sb-FDF of *S. bisulcatus*. An appropriate positive control group (lansoprazole, cimetidine or carbenoxolone) was included in every assay.

2.5.1. Ethanol-induced gastric ulcer

Twenty-eight rats were randomly divided into four groups, and were fasted 24 h prior to receiving an oral dose of 0.9% saline solution (10 mL/kg), lansoprazole (30 mg/kg), and Sb-FRF or Sb-FDF (100 mg/kg). After 60 min, all groups were orally treated with 4 mL/kg of absolute ethanol for gastric-ulcer induction. The animals were euthanized 1 h after the administration of ethanol and the stomachs were then removed and opened along the greater curvature to quantify ulcerative lesions [24]. The ulcerative lesion index (ULI) was calculated [25].

2.5.2. Hypothermic restraint-stress ulcer

The gastroprotective activity of Sb-EtOHE, Sb-FRF and Sb-FDF was assessed using the hypothermic restraint stress-induced gastric ulcer model [26]. After fasting for 24 h, the animals received a single oral administration of Sb-EtOHE (at the doses of 50, 100, and 250 mg/kg body weight), Sb-FRF (100 mg/kg), Sb-FDF (100 mg/kg), cimetidine (100 mg/kg), or 0.9% saline solution (10 mL/kg). At 30 min pos-treatment, gastric ulceration was induced by immobilizing the animals in a closed cylindrical cage, maintained at 4 ºC. After 4 h the animals were euthanized, the stomachs removed and examined for ulcers as described previously. The ULI was calculated.

2.5.3. Indomethacin/bethanecol-induced gastric ulcer

Gastric lesions were induced with indomethacin (30 mg/kg, s.c.), and bethanechol (5 mg/kg, i.p.) in mice. After 24 h of fasting, either Sb-EtOHE (50, 100, 250 mg/kg), Sb-FRF (100 mg/kg), Sb-FDF (100 mg/kg), cimetidine (100 mg/kg), or 0.9 % saline solution (10 mL/kg) were orally

administered 30 min before the induction of gastric lesions. At 4 h after treatment with the ulcerogenic agents, the mice were euthanized by cervical dislocation [27]. The stomachs were removed and examined for ulcers as described previously. The ULI was calculated.

2.5.4. Determination of biochemical parameters from gastric juice after pylorus ligature

In brief, male Swiss albino mice (30-40g) were fasted for 24 h yet with free access to water before the start of experimentation and the animals were randomly divided into four groups. At 30 min after intraduodenal administration of a single dose of either Sb-FRF (100 mg/kg), Sb-FDF (100 mg/kg), cimetidine (100 mg/kg), or 0.9 % saline solution (10 mL/kg) pylorus ligature was performed. Parameters such as gastric juice volume, pH, and total gastric secretion acid content were determined. The stomach was removed, inspected internally, and its contents drained into a graduated centrifuge tube and centrifuged at 3000 g for 10 min. The supernatant volume and pH were recorded with a digital pH meter. The total acid content of the gastric secretion was also determined by titration to pH 7.0 with 0.01N NaOH [28].

2.5.5. Determination of mucus in the gastric wall

After 24 h of fasting, the rats under anesthesia (50 mg/kg of ketamine and 10 mg/kg of xylazine, i.m.) were submitted to a longitudinal incision slightly below the xiphoid apophysis for the pylorus ligature. We then administered (i.d.) either 0,9 % saline solution, carbenoxolone (200 mg/kg), Sb-FRF (100 mg/kg), or Sb-FDF (100 mg/kg). After 4 h, the glandular portion of the stomach was separated, weighed and immersed in Alcian Blue solution for mucus quantification. The absorbencies were measured by spectrometer at 598 nm, and the results expressed as µg of Alcian Blue/g of tissue [29].

2.5.6 Determination of prostaglandin synthesis

Thirty minutes after treatment with either 0.9 % saline, 0.9 % saline and indomethacin (20 mg/kg, s.c.), Sb-FRF or Sb-FDF (100 mg/kg), Sb-FRF or Sb-FDF (100 mg/kg) with indomethacin (20 mg/kg s.c.) the rats were euthanized by cervical dislocation and the abdomen opened. Samples of the corpus (full thickness) were excised, weighed and suspended in 1 mL of 10 mM sodium phosphate buffer, pH 7.4. The tissue was minced finely with scissors then incubated at 37 °C for 20 min [30]. The prostaglandin content of the buffer was measured using an enzyme immunoassay kit (RPN222, Amersham).

2.6 Statistical analysis

Results were expressed as the mean ± S.D. Statistical significance between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett´s or Tukey-Kramer's tests, with $p<0.05$ considered significant. The statistical software program utilized was GraphPad Prism® version 4 (U.S.A., 2003).

3. Results and Discussion

This study evaluated the ability of the extract and fractions obtained from *S. bisulcatus* to promote gastroprotection facing varied models of induced gastric ulcer. It is well known that ethanol, stress, and continuous use of nonsteroidal anti-inflammatory drugs are important factors for the development of gastric ulcers. Ethanol however, is widely used in research to induced ulcers in animals. Ethanol easily and rapidly penetrates the gastric mucosa leading to increased permeability, and release of vasoactive factors. It reduces the level of NO in the gastric mucosa, causes vascular damage, and the formation of oxygen free radicals. It increases Na^+ and K^+ flow, increases pepsin secretion, promotes loss of H^+ ions into the lumen, and finally causes cell necrosis, all of which are responsible for ulcer formation [31-35].

Our results showed that Sb-FRF (100 mg/kg p.o.), Sb-FDF (100 mg/kg p.o.), and lansoprazole (30 mg/kg) significantly reduced the ULI to 42, 41, and 67% respectively, when compared to the negative control (Table 1). Similar results were observed in the pharmacological screening performed for the Sb-EtOHE at doses of 50, 100, and 250 mg/kg [23]. The results suggest that Sb-FRF and Sb-FDF also display gastroprotective activity at the doses evaluated.

Because these models involve the vagus nerve, as well as mucosal cytoprotective factors, we are continuing to investigate whether these plant extracts are able to provide protection against gastric ulcerative lesions as induced by acute stress, and NSAIDs.

Gastric ulcer disease remains widespread, and a stressful lifestyle makes significant contributions to this pathology [36]. Recently, research has suggested that stress causes a hypoxic-ischemic gastric mucosa. This enables the rapid and continuing formation of reactive oxygen species (ROS) leading to the development of gastric ulceration [37-41]. Stress-induced ulcer is mediated by the release of histamine, which increases acid secretion and reduces mucus production. Other factors such as increased vagal stimulation, gastric motility, and decreased prostaglandin synthesis are also involved in stress-induced ulcers [42, 39].

In this model, the results demonstrate that Sb-EtOHE (100 and 250 mg/kg), and cimetidine (100 mg/kg) significantly inhibited ulcerative lesions by 73, 65, and 86% respectively when compared with the negative control group (Table 1). A similar result occurred for Sb-FRF and Sb-FDF at 100 mg/kg showing a significant reduction of ulcerative lesions (63%, and 59%) respectively, and 69% for cimetidine (Table 1). The results suggest that extracts of the plant *S. bisulcatus* protected the gastric mucosa of mice from injuries related to stress.

Indomethacin is an indol derivative, non-steroidal antiinflammatory drug with complementary analgesic, and antipyretic effects. This drug was chosen to induced ulcer because it has a higher ulcerogenic potential than other NSAIDs [43, 44]. Indomethacin potently reduces PG synthesis by inhibiting both COX-1 and COX-2 enzymes, making the gastric mucosa more susceptible to injury [44-46]. NSAIDs also down-regulate survivin, an apoptosis inhibitor, and thus permit apoptosis in epithelial mucosal cells [47]. In our experimental model, indomethacin associated with bethanechol results in synergistic promotion of gastric lesions (bleeding and lesions in the glandular part of the stomach) thru increased pepsin and acid secretion in the stomach, and a consequently greater ulceration index result [48-50].

We observed that Sb-EtOHE (50, 100, and 250 mg/kg), and cimetidine (100 mg/kg) significantly reduced the ulcerative lesion index by 51, 52, 49, and 63% respectively, when compared with the negative control (Table 1). We also found that Sb-FRF, and Sb-FDF (100 mg/kg, p.o), and cimetidine (100 mg/kg), reduced the ulcerative lesion index by 46, 31, and 55% when compared with their respective negative controls (Table 1). The promising results led us to investigate yet another model to corroborate.

Since overabundant acid secretion can cause severe mucosal damage and may result in death from intestinal bleeding or perforation (e.g. Zollinger – Ellison syndrome), gastric acid production must be regulated [51]. Most of the anti-ulcer drugs presently used aim to reduce gastric acid secretion. Treatment of gastric ulcers by either antacid, anticholinergic, H_2 receptor antagonists or proton pump inhibitors results in gastric acid inhibition [44, 52]. Our study evaluated the effects of *S. bisulcatus* plant samples on gastric secretion using the pylorus ligature model, an important procedure that reveals changes in biochemical parameters (pH, $[H^+]$, and gastric volume relative to the gastric content).

The results obtained for gastric secretion restraint using the pylorus ligature model in mice showed reductions in acidity, and a significant increase in pH in animals treated with cimetidine (100 mg/kg, i.d.), and Sb-FRF (100 mg/kg, i.d.). However, Sb-FDF (100 mg/kg, i.d.) did not promote changes in these biochemical parameters. The results confirm the anti-secretory activity of gastric acid by Sb-FRF (Table 2).

Table 1. Effects of ethanolic extract and flavonoid-rich and flavonoid-deficient fractions obtained from the scapes of *S. bisulcatus* in different models of acute gastric lesion induced in rats or mice.

Gastric ulcer models	Treatment (p.o.)	Dose (mg/kg)	ULI (mm)	Inhibition (%)
Ethanol in rats	0.9% saline	-	81 ± 14	-
	Lansoprazole	30	27± 6.8*	67
	Sb-FRF	100	47 ± 9.5*	42
	Sb-FDF	100	48 ± 13*	41
Stress in mice	0.9% saline	-	27 ± 5.4	-
	Cimetidine	100	3.8 ± 1.7*	86
	Sb-EtOHE	50	31 ± 18	-
	Sb-EtOHE	100	7.3 ± 2.3*	73
	Sb-EtOHE	250	9.5 ± 6.3*	65
	0.9% saline	-	27 ± 6.6	-
	Cimetidine	100	8.4 ± 3.0**	69
	Sb-FRF	100	10 ± 3.4**	63
	Sb-FDF	100	11 ± 4.8**	59
NSAIDs in mice	0.9% saline	-	13.7 ± 3.0	-
	Cimetidine	100	5.0 ± 3.0**	63
	Sb-EtOHE	50	6.7 ± 2.3**	51
	Sb-EtOHE	100	6.7 ± 1.6**	52
	Sb-EtOHE	250	7.0 ± 2.1**	49
	0.9% saline	-	17 ± 6.0	-
	Cimetidine	100	7.8 ± 4.8**	55
	Sb-FRF	100	9.3 ± 1.9*	46
	Sb-FDF	100	12 ± 2.6*	31

ANOVA followed by Dunnett's test.* $p < 0.05$, ** $p < 0.01$. Data are presented as mean ± S.D (n=5-7).

Table 2. Effects of ethanolic extract, flavonoid-rich, and flavonoid-deficient fractions obtained from the scapes of *S. bisulcatus* on gastric juice parameters for mice submitted to pylorus ligature.

Treatments	Dose (mg/kg)	pH (units)	$[H^+]$ (mEq/mL/4h)	Gastric juice volume (mg)
0.9% saline	-	2.3 ± 0.48	12 ± 4.9	284 ± 50
Cimetidine	100	4.0 ± 0.81**	6.6 ± 1.3*	198 ± 58*
Sb-FRF	100	3.7 ± 0.83**	7.8 ± 2.1*	239 ± 86
Sb-FDF	100	2.3 ± 0.5	13 ± 2.6	255 ± 99

ANOVA followed by Dunnett's test.* $p < 0.05$, ** $p < 0.01$. Data are presented as mean ± S.D (n=5-7).

Given the results, and to better understand the mechanisms involved in the gastroprotective action of *S. bisulcatus* we carried out experimental protocols to assess the involvement of mucus and prostaglandins (PGs) in the gastroprotection promoted by Sb-FRF or Sb-FDF.

Gastric mucus is the first line of defense against acid, and it adheres to bicarbonate secreted by the epithelium to serve as a barrier against self-digestion [53]. Mucus provides a physical barrier to bacteria and acts as a lubricant to reduce physical abrasion with the mucosa. It also protects the mucosa from damage induced by acid, pepsin and luminal toxins [54]. The results showed that Sb-FRF (100 mg/kg), and Sb-FDF (100 mg/kg) promote significant increases in mucus, compared to the negative control (Fig. 1). The results suggest that the gastroprotective effects of Sb-FRF and Sb-FDF are related to increased mucus adherent on the gastric mucosa.

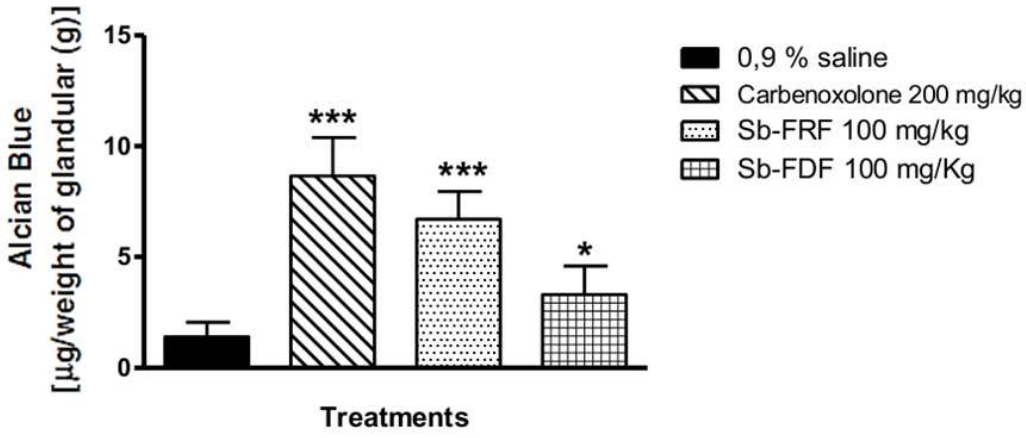

Figure 1. Effects of flavonoid-rich (FRF) and flavonoid-deficient (FDF) fractions from *Syngonanthus bisulcatus*, and carbenoxolone on adherent gastric mucus after pylorus-ligature in rats.
ANOVA: $F(3,24) = 45$ with Dunnett's test. *$p<0.05$ and **$p<0.001$ compared to the saline control.

Another part of the study was to investigate the role of prostaglandins in the gastroprotective effect promoted *S. bisulcatus*. PGs are a class of mediators produced by cyclooxygenase enzymes 1 and 2 (COX-1 and COX-2) [55]. Classic drugs used in peptic ulcer treatment are thought to increase the production of gastro-cytoprotective PGs [56,44]. Prostaglandins are found to produce a gastroprotective effect not only by decreasing acid secretion, but also by increasing gastric mucus levels, and gastric bicarbonate content [57, 58]. Prostaglandins are believed to play a pivotal role in the ulcer healing process as they are involved in improving gastric mucin synthesis, triggering mucosal cell proliferation, promoting angiogenesis, and inducing several other functions which modulate and regulate gastric mucosal integrity and gastric acid secretion [59]. Drugs that arrest ulcer progression by acting as antioxidants, increasing the synthesis and secretion of gastric mucus, and promoting PG synthesis should also accelerate ulcer healing [60].

The results showed that indomethacin significantly reduces PGE_2 production. Yet treatment with indomethacin did not reduce prostaglandin levels in animals receiving Sb-FRF. However, the combination of both Sb-FDF along with indomethacin significantly reduced the PGE_2 levels compared to the Sb-FDF alone group (fig. 2). These results suggest that flavonoids maintain PGE_2 levels and thus promote gastroprotection.

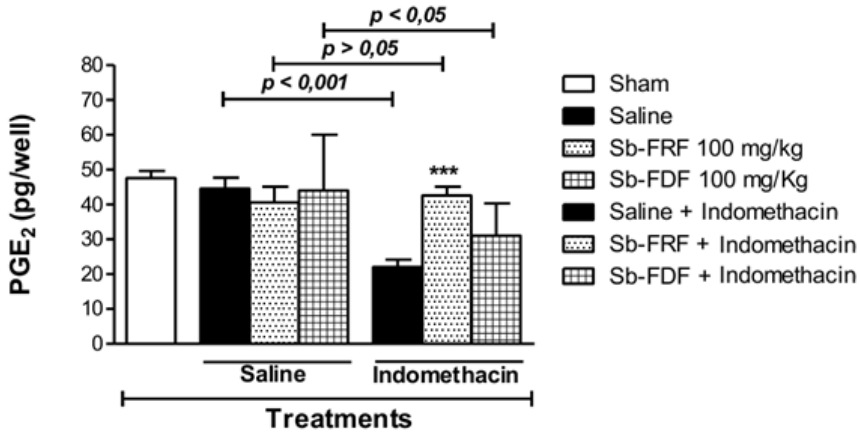

Figure 1. Effects of flavonoid-rich (FRF) and flavonoid-deficient (FDF) fractions from *Syngonanthus bisulcatus* on gastric prostaglandin E_2 (PGE_2) synthesis after administration of indomethacin in rats.
ANOVA: $F(6,42) = 10.5$ with Tukey-Kramer's test. ***$p<0.001$ compared to the saline + indomethacin control.

In conclusion, our results demonstrate the potential of *S. bisulcatus* for possible use and development of anti-gastric ulcer drug with demonstrated gastroprotective activity. The observed gastroprotective effects probably involve the participation of mucus as well protanglandins, cytoprotective mechanisms of the gastrointestinal mucosa against aggressive factors.

Acknowledgments

This work was supported by the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) and the *Fundação de Apoio a Pesquisas do Estado de São Paulo* (FAPESP).

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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