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Original Article

GASTRO PROTECTIVE AND ANTI-*HELICOBACTER PYLORI* EFFECTS OF A FLAVONOID RICH FRACTION OBTAINED FROM *ACHYROCLINE SATUREOIDES* (LAM) D.C.

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ABSTRACT

Objectives: The study aimed to investigate the gastroprotective effects of a flavonoid rich fraction (FRF) obtained from *Achyrocline satureoides*.

Methods: The following protocols were employed: ethanol and NSAID-induced ulcer, ligature pylorus model, and free mucus quantification. Nitric oxide (NO) and sulfhydryl group participation were observed by pretreatment with L-NAME or NEM. Besides, it was assayed the acetic acid-induced chronic ulcer andthe anti-*Helicobacter pylori*activity *in vitro*.

Results: The phytochemical profile of FRF showed three main flavonoids, luteolin, quercetin and 3-*O*-methyl-quercetin. The administration of FRF was able to prevent the damage evoked by ethanol and NSAID-induced ulcer models. The pH and concentration of H^+ in the stomach were not modified by FRF treatment. However, the FRF treatment induces mucus secretion. The effect presented by FRF was mediated by nitric oxide (NO). In chronic ulcer model FRF reduced significantly the lesion area, promoting a cure ratio of 65.42±13.00, a similar data presented by cimetidine treated animals (61.35±11.88). Using an *in vitro* assay was observed that FRF at 500 µg/mL was able to inhibit bacterial growth.

Conclusions: The results show that FRF provided a significant gastroprotective and ulcer healing activity, mainly due to their capacity to enhance mucus secretion.

Keywords: *Achyrocline satureoides*, Gastroprotective, *Helicobacter pylori*, Flavonoids.

INTRODUCTION

Gastric ulcer is the most prevalent gastrointestinal tract disease, being chronic and often recurring[1,2].Although the etiology of gastric ulcer is not completely understood, it is know that pathogenesis of gastric ulcers is influenced by several factors as, genetic predisposition, altered acid secretion, aged, rapid gastric emptying, defective mucosal defense mechanisms, psychological or physical stress conditions, inadequate diet, excessive consumption of alcohol and non-steroidal anti-inflammatory drugs[3,4].Other important aspect of ulcer pathogenesis is the infection with *Helicobacter pylori*, a spiral-shaped flagellated bacterium that live in the duodenum and stomach, where promote the generation of reactive oxygen species leading a highly inflammatory response[5].

The main currently gastric ulcer treatment involves oral administration of synthetic histamine receptor antagonists, proton pump inhibitors or anticholinergic drugs,and in case of *H. pylori* infection is necessary use of antibiotics[6,3].However, these treatments presents high cost and cause many adverse effects. Therefore, this search for new therapies is now important and natural products are ideal by presenting better protection, low cost and lower toxicity [7,8].

Achyrocline satureoides(Lam.) DC. (Asteraceae) isa medicinal plant popularly known as "marcela". The infusion obtained from inflorescences is widely used to treat stomach disorders such as gastric ulcers, as well as to reduce pain and inflammation [9,10,11,12,13].In previous studies, our group described that*in vivo* treatment with*A. satureoides* hydroalcoholic extract promote gastroprotective and anti-inflammatory effects [14,15],without presentedacute toxic effects[15].Additionally, the phytochemical profile showed that of *A. satureoides* extract presents steroids, fatty acids and mainly flavonoids, as quercetin and luteolin, which are the major components of the hydroalcoholic extract. Based on this, we show here in the present the gastroprotective and anti-*Helicobacter pylori* activity evoked by a flavonoid rich fraction (FRF) in different models of gastric ulcer in animals.

MATERIAL AND METHODS

Drugs, reagents and solvents

Indomethacin, cimetidine, omeprazole, carbenoxolone, N_G-nitro-L-Arginine methyl-ester (L-NAME) and N-ethyl-maleimide (NEN) were purchased from Sigma Aldrich (St. Louis, MD, USA). All the other reagents and solvents used were of analytical grade.

Plant material

The inflorescences of *A. satureoides* were collected in Fraiburgo, in the state of Santa Catarina, Brazil (Latitude 27°01'34"S, longitude 50°55'17"W). The material was identified and a voucher specimen was deposited at the herbarium of the State University of Maringá(UEM) with the code HUEM-23568.

Preparation of the hydroalcoholic extract and flavonoid rich fraction

Air-dried plant material was cute into small pieces and macerated with 70% (v/v) aqueous ethanol at room temperature for 7 days. The macerate was filtered and the solvent removed by rotatory evaporator under reduced pressure. The FRF was obtained by liquid-liquid partition of extract, as follow: extract (20 g) was dissolved in methanol:water proportion (9:1), and the liquid-liquid extraction process was carried out using hexane (5x300 mL) as solvent extractor. After, to obtain the FRF the aqueous residue was portioned by liquid-liquid extraction process with ethyl-acetate which after drying, resulted in a yield 8.83 g of FRF.

Apparatus and chromatographic conditions

A Shimadzu LC-20AT LC system (Shimadzu, Tokyo, Japan), consisting of a SPD-M20A photo diode array detector, SIL-20AHT

autosampler and software LC-Solution (Shimadzu, Tokyo, Japan) was used. The sample and standard were diluted in methanol at 1 mg/mL and filtered through a 0.45-mm PTFE membrane filter. The injections of sample and standard (20□l) were carried out on a C18 column (Luna Phenomenex, 250 x 4,5mm; 0.5µm film thickness and 100 A) conditioned at 35°C. The mobile phase consisted of acetonitrile (A) and water (pH 2.5, phosphoric acid) (B) eluded in a gradient system, starting with 10% A (0–4 min), 10–30% A (4–15 min), 30% A (15–25 min) and 30–10% A (25–30 min). This was followed by a 10 min equilibrium period prior to the injection of next sample. The analyses were monitored at 350 nm. All solvents used were HPLC grade and were degassed in an ultrasonic bath.

Animals

Wistar rats male (250-350 g) or Swiss mice male (25-35 g) were provided by the Central Animal House of the Universidadeof Vale do Itajaí (UNIVALI), Itajaí – SC. The animals were housed in standard cages, at room temperature (25±3°C), with 12 h dark/12 h light cycles, and supplemented with food and water *ad libitum*. They were transferred to the laboratory 12 hours prior to the experiments and were given water *ad libitum*. In all experiments, the animals were kept in cages with raised floors constructed from wide mesh, to prevent coprophagy. The experiments were authorized by the Ethical Committee for Animal Care (301/09a) of the Universidade of Vale do Itajaí, Itajaí, Santa Catarina, Brazil.

Doses

The dose used in this study was based in a previous study published by our group [14], which demonstrated that 500 mg/kg presents significant gastroprotector effect. Besides, this data allows reduction in the number of animals used. Thus following the 3Rs program to 1) reduction; 2) refinement; 3) replacement, which aims to use fewer animals in experiments.

Gastroprotective activity

Several methods to evaluate the gastroprotective activity of the FRF obtained from *A. satureoides* were employed. An appropriate positive control (omeprazole, a proton pump inhibitor, cimetidine, a histamine receptor antagonist or carbenoxolone, an antioxidant) was included in every assay.

Ethanol-induced ulcer gastric

The experiment was carried out according to the method of Morimoto *et al.*[16] After 12 h of fasting, the animals were orally treated with vehicle (1% Tween-80 aqueous solution), omeprazole (30 mg/kg) or FRF (500 mg/kg). One hour after treatment was administered 1 mL of ethanol 99.5% to induce the lesion in the gastric tissue. One hour later, the animals were sacrificed, and the stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. The images obtained were analyzed using specific "EARP" software measure each lesion point.

Non-steroidal anti-inflammatory drugs-induced gastric ulcer

The experiment was carried out according to the method of Nwafor*et al.*[17] with a few modifications. After 12 h of fasting, the animals were orally treated with vehicle (1% Tween-80 aqueous solution), cimetidine (100 mg/kg) or FRF (500 mg/kg). One hour after treatment was administered indomethacin (100 mg/kg) to induce the lesion in the gastric tissue. Four hours after the animals were sacrificed and the stomachs were removed, and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. Was performed determination the total lesion area and percentage of injured.

Acetic acid-induced chronic ulcer

The methodology described by Takagi *et al.*[18] was used, with some modifications. The mice were anesthetized and subjected to a longitudinal incision below the xiphoid process aphophysis. After exposure of the stomach, 50µL of 20% acetic acid solution was injected into the sub-serosal layer, the site was held down for 30 seconds to prevent leakage of the injected fluid. The stomach was carefully washed with saline 0,9 %and the abdominal wall was sutured. Two days after surgery, when the animals had recovered, treatment was carried out which lasted seven days. The animals were orally treated once a day with vehicle (1% Tween-80 aqueous solution), omeprazole (30 mg/kg) or FRF (500 mg/kg). After seven days, the animals were sacrificed and the stomachs removed and opened along the greater curvature. They were then stretched and scanned, to capture images, which were analyzed by image analysis software to determine whether regression of the lesion had occurred in the treatments, compared with the positive and negative controls. Was performed the total lesion area and percentage of injured.

Pyloric ligation-induced gastric ulcer

The assay was performed using the method of Shay *et al.*[19], with a few modifications. After 24 h of fasting, the animals were anesthetized with a mixture of xylazine and ketamine (7.6 mg/kg and 77.3 mg/kg, intraperitoneally), the abdomen was incised and the pylorus ligated. Immediately after pylorus ligature, the treatments were intraduodenally administered vehicle (1% Tween-80 aqueous solution), omeprazole (30 mg/kg) or FRF (500 mg/kg). Four hours later, the animals were sacrificed and the abdomen was opened, and another ligature placed at the esophageal end. The stomachs were removed and the gastric contents collected and centrifuged at 3000 rpm (8000×g, 25◦C, 10 min). The amount of gastric-juice acid (mL) and the pH values were determined. Total acid secretion in the gastric lesion was determined in the supernatant volume by titration to pH 7.0, using a $0.01 mol^{-1}$NaOH solution, and phenolphthalein as indicator.

Determination of mucus in gastric content

This assay was performed according to the methodology described previously by Sun *et al.*[20] with few modifications. After 24 h of fasting, under anesthesia, the abdomen was incised and the pylorus ligated. Immediately after pylorus ligature, the treatments vehicle, carbenoxolone (250 mg/kg) or FRF were intraduodenally administered, and the animals were sacrificed 4 h after the drug treatments. The stomach content was immersed in 10 mL of 0.02% Alcian blue 0.16 M sucrose/0.05 M sodium acetate solution, pH 5.8, and incubated for 24 h at 20 ºC. Alcian blue binding extract was centrifuged at 3000×*g* for 10 min. The absorbency of the supernatant was measured by spectrophotometry at 615 nm. The free mucus in the gastric content was calculated from the amount of Alcian blue binding (mg/wt tissue (g)).

Ethanol-induced gastric mucosal lesion in L-NAME or NEM pre-treated rats

These experiments were based on the method of Matsuda *et al.*[21] with some modifications. Male Wistar rats, after fasting for 24 h, were treated or not with 70 mg/kg of NO synthase inhibitor (L-NAME) or 10 mg/kg of sulfhydryl depleter (NEM). Thirty min after the pretreatment, the animals were orally treated with vehicle (1% Tween-80 aqueous solution) or FRF (500 mg/kg). One hour later1 mL ethanol99.5% was given to each rodent, and the animals were sacrificed after 1 h. The stomachs were removed to determine the gastric lesion.

Anti-Helicobacter pylori activity

Test materials

The FRFwas tested to detect the anti-*H. pylori* activity. The strain of *H. pylori* ATCC 43504 was given by the microorganism reference laboratory of Fiocruz-RJ. It was kept frozen at 70^0C in the immunology laboratory at FURB. For activation, it was grown in *Brucella*broth and incubated at 35^0C for 24-48 hours. After this period, it was inoculated on *Brucella*agar supplemented with 10% sheep blood and incubated 48-72 hours at 35 ^{0}C in microaerophilic conditions (85% N_2, 10% CO_2 and 5% O_2) and high humidity. The bacterial identification was performed by characteristic morphology in Gram staining and biochemical tests of positive catalase, oxidase and urease[22].

Determining the Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined by the agar dilution solid method, according to the recommendations of the Clinical Laboratory Standards Institute (CLSI)[23].From stock FRF solution of 40 mg/mL, were serially diluted. In individual glasses, 50 µL of each dilution was added to 950 µL of *Brucella* agar supplemented with 10% sheeps' blood, the 45-50^{0}C of fluid, reaching concentrations of 2000; 1000; 500; 250; 125; 62,5; 31, 25 and 15,625 µg/mL. The bacterial inoculums were prepared based on a scale of 0.5 MacFarland turbidity. After the medium solidification, 1 µL of bacterial suspension was seeded in each glass with the diluted extract agar. It was incubated in humidity and microaerophilic optimal conditions, 35^{0}C for 48-72 hours. The MIC was defined as the lowest the concentration of fractions capable of completely inhibiting bacterial growth. All the experiments were performed in triplicate.

Statistical analysis

Results were expressed as mean ± SEM. Statistical significance between groups was determined by one-way analysis variance (ANOVA) followed by Dunnett's tests, with $p<0.05$ considered significant. The statistical software program utilized was Graph Pad Prism version 6.

RESULTS

Chemical profile of FRF

In the chemical profile of FRF in the Figure 1,threemajor peaks can be observed. By co-injection and UV spectra comparison, it was possible to suggest the structure of three of these peaks as luteolin, quercetin and 3-*O*-methyl-quercetin. This chemical profile is in agreement to what is described in the literature[24].

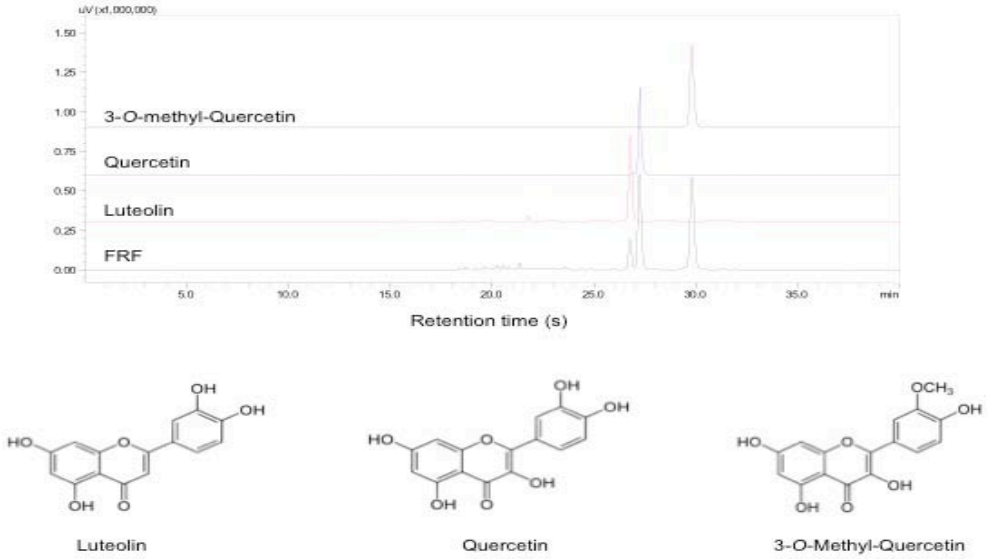

Fig. 1: HPLC overlap chromatogram profile of FRF.

***In vivo* FRF treatment protects Ethanol-induced gastric tissue damage**

Oral administration of ethanol solution caused lesions in the gastric tissue, which were prevented by FRF (500 mg/kg, p.o.) pre-treatment. It is noteworthy that tissue protection seen with 500 mg/kg of FRF was better that observed with 30 mg/kg of omeprazole (Table 1).

***In vivo* FRF treatment protects NSAIDs-induced gastric tissue damage**

The data obtained in this model, we observed that the FRF (500 mg/kg, p.o.) and cimetidine (100 mg/kg, p.o.) significantly reduced the percentage of lesion area to 0.70 and 0.33 when compared to vehicle group (Table 1), suggesting a gastroprotective effect against NSAIDs-induced gastric ulcer.

Table 1: Effects of FRF, cimetine and omeprazole on the ulcer model induced by ethanol and indomethacin, and chronic ulcer induced by acetic acid

Assay	Treatment p.o	Dose (mg/kg)	Total area of lesion (mm^2)	% of lesion area
Ethanol	Control	--	361.20±51.32	27.25±3.58
	Omeprazole	30	43.23±10.48**	3.76±1.02**
	FRF	500	4.07±2.75**	0.36±0.16**
Indomethacin	Control	-	21.65±6.14	1.97±0.42
	Cimetidine	100	7.49±2.30**	0.70±0.20**
	FR	500	2.69±0.58**	0.33±0.05**
Chronic ulcer	Control	-	9.19±1.18	3.47±0.47
(mice)	Cimetidine	100	4.09±0.94**	1.34±0.41**
	FRF	500	4.54±0.84**	1.58±0.59**

Results as mean ± SEM for six rats or mice. Statistical comparison was performed using ANOVA followed by Dunnett´spost test. **p< 0.01 compared with the control group

FRF does not impair acid gastric secretion but induce mucus secretion

The results obtained in this model showed that the FRF (500 mg/kg) administered intraduodenally did not promote changes in the biochemical parameters of the stomach content, such as pH, concentration of H^+ ions and the volume of gastric juice after administration (Table 2).

This date suggest that the gastroprotective effect exerted is not related to reduction of gastric secretion. On the other hand, in the mucus determination model, the FRF (500 mg/kg) administered

intraduodenally promote anincrease in mucus production when compared to vehicle and cimetidine treated animals (Table 3). This date suggest that the gastroprotective effect could be mediated by an increase in mucus production in the stomach.

Table 2: Effects of FRF and cimetidine, administered intraduodenally, on the biochemical parameters of gastric juice obtained from pylorus-ligature in rats.

Treatment (p.o.)	Dose (mg/kg)	Volume (mL)	pH	[H+] mEq/l/4h
Control	-	0.77 ± 0.15	2.76 ± 0.13	65.87 ± 2.23
Cimetidine	100	0.70 ± 0.12	3.92 ± 0.20*	46.91 ± 4.38
FRF	500	0.86 ± 0.04	3.03 ± 0.11	76.51 ± 3.55

Results as mean ± SEM for six rats. Statistical comparison was performed using ANOVA followed by the Dunnett'spost test. *p<0.05 compared with the control group

Table 3: Effects of FRF and carbenoxolone on Alcian blue binding to free gastric mucus from pylorus ligature in rats

Treatment (v.o)	Dose (mg/kg)	Alcian blue bound (mg/wt tissue (g))
Control	-	1,41 ± 0,04
Carbenoxolone	250	1,73 ± 0,02**
FRF	500	1,69 ± 0.04**

Results as mean ± SEM for six rats. Statistical comparison was performed using ANOVA followed by the Dunnett'spos-test.**p<0.01 compared with the control group.

FRF gastroprotective effect is mediated by NO

We observed that the animals pretreated with L-NAME and subsequently treated with FRF display exacerbation on lesion area when compared to the vehicle group (Figure 2). This data suggest that NO is related to the gastroprotective activity promoted by FRF. However, we observed that when the animals were pretreated with NEM (blocker of sulfhydryl groups) and after treated with FRF did not display exacerbation of lesion area, suggesting that the sulfhydryl compounds pathway is not involved in the gastroprotective effect promoted by FRF.

FRF treatment promotes healing of chronic ulcer *in vivo* and presents anti-*Helicobacter pylori* activity*in vitro*

Oral administration of FRF (500 mg/kg, p.o.) once a day during seven days reduced the chronic gastric ulceration induced by acetic acid when compared to vehicle treated animals. The FRF treatment promotes a cure rate of 65.42±13.00, a similar data presented by cimetidine treated animals (61.35±11.88) (Table 1). Regarding the anti-*H.pylori*potential of FRF was estimated using the *in vitro* method of solid agar dilution. It was observed that the FRF at 500 µg/mL was able to inhibit bacterial growth.

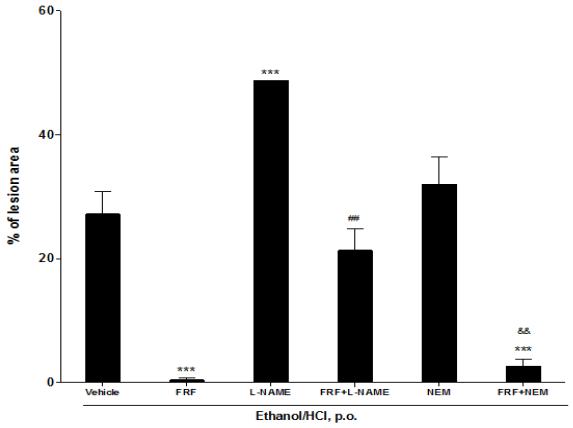

Fig. 2: Effects of FRF on ethanol-induced gastric lesions in rats pre-treated with L-NAME or NEM. Results are mean ± SEM for six rats.

Statistical comparison was performed using ANOVA followed by Dunnett's test. ***p<0.01 vs vehicle; ## p<vs L-NAME; &&p< NEM

DISCUSSION

The literature has reported that medicinal plants have a broad spectrum of biological activities. Previous studies published by our research group showed that *A. satureoides* hidroalcoholic extract obtained from inflorescences present anti-inflammatory and gastroprotective activity[14,15]. The *A. satureoides* hydroalcoholic extract presents in its composition flavonoids (luteolin, 3-*O*-methyl-quercetin and quercetin), fatty acids (oleic, palmitic and stearic acids) and steroids (stigmasterol, gamma-sitosterol, and sitosterone)[15]. The literature reports that gastroprotective activity is attributed to the presence of chemical constituents such as tannins and flavonoids. Based on this, we have reported the gastroprotective activity and some mechanisms involved in the gastroprotection elicited by a FRF obtained from *A. satureoides* inflorescences and the anti-*H. pylori* effect.

The chemical profile of FRF showed that three main flavonoids may be detected by direct comparison with authentic samples, it is possible to propose that are luteolin quercetin and 3-*O*-methyl-quercetin (3).This results is in accordance with the literature, that reports that *A. satureoides* presents in its composition mainly quercetin, luteolin and 3-*O*-methyl-quercetin[24].

Based on chemical composition of FRF, it is expected that it may present gastroprotective effect. In fact, here we show that FRF evoke a gastroprotection on ethanol-induced ulcer model. Ethanol rapidly penetrates the gastric mucosa, and it causes membrane damage, cell exfoliation, erosion and ulcer formation. Multiple action mechanisms including depletion of non-protein sulfhydryl concentration, modulation of the nitric oxide system and reduction of gastric mucosal blood flow are involved in the pathogenic process[25,26].Recently, Santin *et al.*[4] showed that ethanol administration evoked a rapidly influx of neutrophils into the injured tissue and showed that *in vivo* neutrophil depletion significantly reduced the injured area. Newly, Barioni*et al.*[15] showed that *A. satureoides* hydroalcoholic extractinhibit neutrophil influx into the inflammatory area by change the adhesion molecules profile. Together, these data show that the gastroprotection evoked by FRF could be mediated by an inhibition on neutrophil influx, inhibition of gastric secretion or an increase in protective substances release by the mucosa.

Another model largely used is the NSAID-induced ulcer protocol. It is known that indomethacin or other NSAIDs display ulcerogenic effects associated with the blockade of cyclooxigenase-1 (COX-1) in gastric epithelial cells, leading to inhibition of prostaglandins synthesis[27,28].Prostaglandins such as E_2 and I_2 enhance the synthesis of mucus and bicarbonate, regulate the acid secretion and maintain the integrity of the gastric blood flow in the stomach[29].In our hands, FRF 500 mg/kg also showed a gastroprotective effect in this model, it reduced the size of the gastric ulcer area in mice treated with the NSAID drug indomethacin.

Many experiments of our study were dedicated to the elucidation of the mechanism(s) involved in the gastroprotective effect of FRF. One simple mechanism of action of FRF might be that the fraction interferes with gastric secretion, but our experiments based on the model of pylorus ligature clearly show that this mechanism does not play a role with respect to FRF. However, FRF treatment evoked a increase in mucus secretion, an important mechanism of defense

that leads to forming a gel, which is composed of phospholipids and water, and acts as an antioxidant agent, also being responsible for maintaining the neutral pH at the stomach mucosa surface[14].

Other important factor to analyze is the NO, which regulates the inflammatory and vascular process in the stomach, because NO control the integrity of the gastric mucosa and the gastric acid secretion[30]. On the other hand, sulfhydryl groups are responsible for protecting the gastric mucosa against oxidative damage[31]. Intending to evaluated the participation of NO and sulfhydryl groups in the protective activity of FRF were employed the pre-treatment with L-NAME, a NOS inhibitor, and NEM, a thiol blocker. Our data show that the gastroprotective effect is mediated by nitric oxide. Additionally, the acetic acid ulcer model, established by Takagi *et al.*[18] was the one that reflects human peptic ulcer disease from the view of macroscopic and microscopic observation. For this reason, this model has been widely used to study the mechanism of ulcer healing and to evaluate the anti-ulcer effect of several compounds. In this model, FRF treatment during seven days significantly reduced the size of the injury produced by acetic acid when compared to the control group.

Another parameter evaluated was the possible anti-*H. pylori*activity of FRF. *H. pylori*is a micro-aerophilic, Gram-negative, spiral-shaped flagellated bacterium that lives in the stomach and duodenum. It is an important causal factor in the pathogenesis of gastritis and ulcer disease, inducing gastric inflammation, oxidative stress, DNA damage, apoptosis of epithelial cells and inducing cell cycle dysregulation[32]. Some abnormalities are linked with *H.pylori* infection, including increased basal and stimulated acid output, reduced inhibitory effect of somatostatin on gastrin release, and defective inhibition of acid secretion in response to antral distension. Our data support that FRF, even at a concentration of 500 µg/mL, was able to inhibit bacterial growth.

Taken together, the results show that FRF provided a significant gastroprotective and ulcer healing activity, mainly due to their capacity to enhance mucus secretion. Moreover, FRF demonstrated this activity in a dependent manner of NO, without gastroprotection depending on the antioxidant properties of the sulfhydryl groups.

CONFLICT OF INTERESTS

Declared None

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