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 and the anti-H. pylori proactivity 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+ were 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 known 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 present 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) is a 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 presented acute 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-H. 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, carbamoxolone, N-nitro-L-Arginine methyl-ester (L-NAME) and N-ethyl-maleimide (NEM) 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 cut 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 rotary 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 µL) were carried out on a C18 column (Luna Phenomenex, 250 x 4.5mm; 5.5µm film thickness and 100 A) conditioned at 35°C. The mobile phase consisted of acetonitrile (A) and water (pH 2.5, phosphoric acid) (B) eluted 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 Universidade do Vale do Itajai (UNIVALI), Itajai – 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 coprophagia. The experiments were authorized by the Ethical Committee for Animal Care (301/09a) of the Universidade do Vale do Itajai, Itajai, 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 carbeneoxolone, an antioxidant) was included in every assay.

**Ethanol-induced ulcer gastric**

The experiment was carried out according to the method of Morimoto *et al.*[20] 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 removed and opened along the greater curvature. The stomachs were removed and the gastric contents collected and centrifuged at 3000 rpm (8000g, 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 0.01mol−1NaOH 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 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 (8000g, 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 0.01mol−1NaOH solution, and phenolphthalein as indicator.

**Ethanol-induced gastric mucosal lesion in L-NAME or NEM pretreated rats**

These experiments were based on the method of Matsuoka [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 later, the animals were sacrificed and the stomachs were removed and opened along the greater curvature. The results were expressed as mean ± SD. The statistical analysis was performed by analysis of variance followed by Tukey’s test.

**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 apophysis. 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 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. The area and percent of injured was calculated from the amount of mucus by titration to pH 7.0, using a 0.01mol−1NaOH solution, and phenolphthalein as indicator.
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% sheep’s blood, the 45-50°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°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, three major peaks can be observed. By co-injection and UV spectra comparison, it was possible to suggest the structure 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].

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).

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

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment p.o</th>
<th>Dose (mg/kg)</th>
<th>Total area of lesion (mm²)</th>
<th>% of lesion area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Control</td>
<td>--</td>
<td>361.29±51.32</td>
<td>27.25±3.58</td>
</tr>
<tr>
<td></td>
<td>Omeprazole</td>
<td>30</td>
<td>43.23±10.48&quot;</td>
<td>3.76±1.02&quot;</td>
</tr>
<tr>
<td></td>
<td>FRF</td>
<td>500</td>
<td>4.07±2.75&quot;</td>
<td>0.36±0.16&quot;</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Control</td>
<td>--</td>
<td>21.65±6.14</td>
<td>1.97±0.42</td>
</tr>
<tr>
<td></td>
<td>Cimetidine</td>
<td>100</td>
<td>7.49±2.30&quot;</td>
<td>0.70±0.20&quot;</td>
</tr>
<tr>
<td></td>
<td>Fr</td>
<td>500</td>
<td>2.69±0.58&quot;</td>
<td>0.33±0.05&quot;</td>
</tr>
<tr>
<td>Chronic ulcer (mice)</td>
<td>Control</td>
<td>--</td>
<td>9.19±1.18</td>
<td>3.47±0.47</td>
</tr>
<tr>
<td></td>
<td>Cimetidine</td>
<td>100</td>
<td>4.09±0.94&quot;</td>
<td>1.34±0.41&quot;</td>
</tr>
<tr>
<td></td>
<td>Fr</td>
<td>500</td>
<td>4.54±0.84&quot;</td>
<td>1.58±0.59&quot;</td>
</tr>
</tbody>
</table>

Results as mean ± SEM for six rats or mice. Statistical comparison was performed using ANOVA followed by Dunnett’s post 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 data 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...
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 500 µg/mL (500 ± 13) and 500 µg/mL (500 ± 0.04) 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

<table>
<thead>
<tr>
<th>Treatment (v.o.)</th>
<th>Dose (mg/kg)</th>
<th>Alcian blue bound (mg/wt tissue (g))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1.41 ± 0.04</td>
</tr>
<tr>
<td>Carbenoxolone</td>
<td>250</td>
<td>1.73 ± 0.02</td>
</tr>
<tr>
<td>FRF</td>
<td>500</td>
<td>1.69 ± 0.04</td>
</tr>
</tbody>
</table>

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

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 hydroalcoholic extract obtained from inflorescences present anti-inflammatory and gastroprotective activity[1,15]. The A. satureoides hydroalcoholic extract presents in its composition flavonoids (luteolin, 3-o-methyl-quecritin and quecritin), fatty acids (oleic, palmitic and stearic acids) and steroids (stigmasterol, gamma-sitosterol, and sitosterol) [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 they are luteolin quercetin and 3-o-methyl-quecritin [3]. This results is in accordance with the literature, that reports that A. satureoides presents in its composition mainly quecritin, luteolin and 3-o-methyl-quecritin [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 mucus, 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 extract inhibited 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 mucus.

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 cyclooxygenase-1 (COX-1) in gastric epithelial cells, leading to inhibition of prostaglandins synthesis [27,28]. Prostaglandins such as E2 and I2 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 maintaining the neutral pH at the stomach mucosa surface[14].

Additionally, the acetic acid ulcer model, established by Takagi et al[10] 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 a micro-aerophilic, Gram-negative, spiral-shaped flagellated bacterium that lives in the stomach and duodenum, is an important causal factor in the pathogenesis of gastric 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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