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The antiulcer and antioxidant mechanisms of the butanolic fraction extract obtained from *Bauhinia forficata* leaves: A medicinal plant frequently used in Brazilian folk medicine

Bárbara Gomes Del Rey[1], Luciana Lopes Guimarães[1], Marcos Sergio de Toledo[2], Hélio Kiyoshi Takahashi[2], Anita Hilda Straus[2], Monike Silva de Freitas[1], Bruna Hattebberger[3], Cláudia Maria Moraes Silva[3], Matheus Roberti de Carvalho[3] and Walber Toma[1*]

[1]Natural Products Laboratory Research, Universidade Santa Cecília, CEP 11045-907, Santos-SP, Brazil.
[2]Laboratory of Glycoconjugate Immunochemistry, Department of Biochemistry, Escola Paulista de Medicina, Universidade Federal de São Paulo, CEP 04023-900, São Paulo-SP, Brazil.
[3]Pharmacy Course, Centro Universitário São Camilo, Campus Ipiranga, CEP 04263-200, São Paulo-SP, Brazil.

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The objectives of this work were to evaluate the antiulcer activity, antioxidant mechanisms, phytochemical analysis and ecotoxicological risk of the butanolic fraction (ButFr) obtained from the leaf extracts of *Bauhinia forficata*. In an ischemia-reperfusion (IR), gastric ulcer model with doses 12.5 and 6.25 mg kg^{-1} promoted significant decreases in the ulcerative lesion area (ULA) by 50% ($p<0.001$) and 46% ($p<0.001$), respectively. Regarding the antioxidant mechanisms, the dose of 6.25 mg kg^{-1} promoted a significant increase in SOD (41%), GPx (62.7%) and GR (54.5%) activities ($p <0.001$) when compared to the negative control. 38% reduction in Myeloperoxidase activity (MPO) activity was also observed as well as 35.5% reduction in the LPO index when compared to the negative control ($p <0.001$). Phytochemical analysis demonstrated the presence of flavonoids (kaempferitrin and rutin) in ButFr, compounds responsible for the pharmacological activities observed. Conclusively the ButFr has antiulcer activity via antioxidant mechanisms.

Key words: *B. forficata*, Pata-de-Vaca, antiulcerogenic, phytochemistry.

INTRODUCTION

Peptic ulcers are a chronic condition responsible for high health care costs around the world. Epidemiologic data show that peptic ulcer disease affects 4 million people around the world every year (Zelickson et al., 2011). Several factors are responsible for peptic ulcer development. Among these are *Helicobacter pylori* infection, the use of non-steroidal anti-inflammatory drugs (NSAID), stress, alcohol consumption and smoking

*Corresponding author. E-mail: walbertoma@unisanta.br. Tel: +55 13 981252286.

(Malfertheiner et al., 2009). Among the main etiologic factors, oxidative stress is a key factor (Bhattacharyya et al., 2014) as it can initiate gastric ulcers and result in the overproduction of reactive oxygen species (ROS) (Abate et al., 1990). ROS include radical compounds such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$), lipid hydroperoxides and reactive non-radical compounds including singlet oxygen (O_2), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), chloramines (RNHCl) and ozone (O_3) (Bedard and Krause, 2007). These molecules contain unpaired valence-shell electrons, making them unstable and reactive with proteins, carbohydrates, lipids and nucleic acids. These interactions may result in the irreversible inactivation of biomolecules. Modifications to the balance between ROS production and the capacity to rapidly detoxify reactive intermediate compounds can be caused by oxidative stress (Bhattacharyya et al., 2014).

Ischemia-reperfusion (IR) is known to induce gastric ulcers due to increases in the formation of free radicals and the adhesion of neutrophils to endothelial cells. Ischemia impairs the gastric mucosal barrier and promotes an increase in gastric acid, promoting damage to gastric tissue. After reperfusion, ROS are generated from the xanthine oxidase system and potentiate neutrophils, leading to tissue lipid peroxidation (LPO), which in association with gastric acid secretions results in cellular damage and death (Rao and Vijayakumar, 2007). Some antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) protect gastric tissue against IR injury by inhibiting the expression of ROS and decreases the levels of the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Ogino et al., 1988; Stein et al., 1990).

Brazil has a high degree of plant biodiversity; studies have shown that Brazil has more than 56,000 species, and nearly 19% of the world's flora (Giulietti et al., 2005). The presence of distinct ecosystems means that Brazil has the greatest biodiversity on the planet (Bolson et al., 2015). Moreover, government policies in Brazil stimulates the use of medicinal plants as a strategy to extend through standardized clinical protocols, the use of the Brazilian biodiversity and public access to herbal medicines (Brasil, 2006).

Among the wide range of plant species we investigated *B. forficata* Link (Caesalpinioideae) for the evaluation of gastric antiulcer activity, popularly known in Brazil as “Unha-de-Vaca”, “Pata-de-Vaca” and “Casco-de-Vaca”, *B. forficata* is a small tree, native to the tropical areas of Asia, Paraguay and Argentina; this vegetal species is well-adapted to the Brazilian climate (Miceli et al., 2016). *B. forficata* is used in popular medicine in Brazil. Some studies have shown that the leaf and stem bark preparations of this plant can be used in traditional medicine for the treatment of rheumatism, local pain, uric acid, uterine problems, diuretic, tonic, blood depurative and elephantiasis (Ferreres et al., 2012) and also, popularly used for the treatment of gastrointestinal diseases, including gastric pain (Peroza et al., 2013; Bieski et al., 2015; Bolson et al., 2015).

The aims of this work were to perform a phytochemical analysis of butanolic fraction extract (ButFr) obtained from the leaves of *B. forficata* which evaluate the antiulcer activity of the extract through antioxidant mechanisms.

MATERIALS AND METHODS

Plant specimen and extraction

B. forficata Link leaves were obtained from Peruíbe, São Paulo, Brazil (-24.267948 latitude, -46.959276 longitude) in March 2007 and were identified by Botanical Paulo Salles Penteado; a voucher specimen with number 4651 deposited in the herbarium of the Universidade Santa Cecília (HUSC).

The leaves of *B. forficata* were dried for seven days at 45°C±3°C, the powdered (3 mm; 100 g of dried leaves) were subjected to extraction by exhaustive maceration for seven days with 1 L different solvents with an increasing polarity: hexane, chloroform and n-butanol, successively. The n-butanol fraction (ButFr) was dried in a rotary evaporator (45°C± 1°C) and used in the experimental protocols.

Animals

Male Wistar rats (180 to 220 g) were obtained from the breeding facility of the Santa Cecília University (UNISANTA). The animals were fed with a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of 12 h dark/12 h light, humidity (60±1.0%) and temperature (21±1°C). The animals were stored in cages with raised ground of wide mesh to restrain coprophagy. The assays were approved by the Santa Cecília University Institutional Animal Care and Use Committee (CEUA-UNISANTA) under code number 53/07.

Gastric ulcer induced by ischemia-reperfusion (IR)

IR gastric ulcers were induced in rats by a method proposed by Ueda et al. (1989). For this purpose, rats (n=8) received saline solution by oral route, (NaCl 0.9%) (10 mL kg^{-1}) (negative control group), lansoprazole (30 mg kg^{-1}) (positive control group) or ButFr (12.5; 6.25; 3.125 and 1.562 mg kg^{-1}). After 30 min, the animals were anaesthetized by an intramuscular administration of ketamine (40 mg kg^{-1}) and xylazine (5 mg kg^{-1}). The celiac artery was dissected and clamped for 30 min. Re-oxygenation was allowed to take place by, removing the clamp for 60 min.

At that point, the animals were culled and the stomachs were removed and opened along with great curvature. The ulcerated area in the stomach corpus was measured using Bioview 4 AvSoft (Brazil). The mucosa of each stomach was scraped, solubilized in phosphate buffer (0.1 M, pH 7.4) and frozen at -80°C until biochemical assays. The protein concentration of the samples was evaluated using the method described by Bradford (1976).

Superoxide dismutase activity (SOD)

We performed a colorimetric assay to assess SOD activity. This protocol is based on the SOD-mediated increase in the rate of auto-oxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield the estimation of red cell superoxide dismutase activity. The results are expressed as units per gram of protein (U/mg) (Winterbourn et al., 1975).

Glutathione peroxidase activity (GPX)

The activity of glutathione peroxidase (GPx) in the gastric mucosa was performed spectrophotometrically. This protocol is based on the oxidation of reduced glutathione by glutathione peroxidase coupled to the oxidation of NADPH by glutathione reductase. The rate of NADPH oxidation was measured photometrically.

After the IR protocols, the stomachs was perfused intraluminally with 5% sulfosalicylic acid and then homogenized in 10 vol/g of the same solution. The tissue homogenate was centrifuged for five min at 10 000 g, and the supernatant was used for GPx assays (Yoshikawa et al., 1993).

Glutathione reductase activity (GR)

The activity of glutathione reductase (GR) was assessed according to Carlberg and Mannervick (1985) using oxidized gluthathione after the reaction with NADPH in phosphate buffer (pH 7.8). The absorbance was measured at 340 nm during the first 10 min.

Myeloperoxidase activity (MPO)

MPO activity in the gastric tissues was evaluated by the method described by Krawisz et al. (1984). The gastric tissues were centrifuged at 3000 ×g for 15 min at 4°C; thereafter aliquots of the supernatant were mixed with 50 mM phosphate buffer (pH 6.8) containing 0.005% H_2O_2 and 1.25 mg mL^{-1} o-dianisidine dihydrochloride. The absorbance was measured at 460 nm.

Estimation of lipid peroxidation (LPO)

The gastric tissue was diluted in 0.15 M KCl and 0.5 mL of this homogenate and added to 0.2 mL of dodecyl sulfate (8.1%), 1.5 mL of acetic acid 20% (adjusted with sodium hydroxide solution to pH 3.5), 1.5 mL of thiobarbituric acid 0.8% (w/v) and 0.3 mL of deionized water. The samples were left in a water bath with a thermostat set at 95°C for 1 h. Then, samples were cooled and added to 1 mL of deionized water and 5 mL of a mixture of n-butanol + pyridine (15 : 1, v/v), shaken on a vortexer for 1 min and centrifuged at 1400x g for 10 min.

The absorbance of the organic layer was measured at 532 nm. TEPP (1, 1, 3, 3-tetraethoxypropane) diluted in ethanol was used as a standard. The data are provided as picomoles of substances which react with thiobarbituric acid (TBARS) per mg of protein (nmol TBARS mg $protein^{-1}$) (Ohkawa et al., 1979).

Phytochemical analysis

Prior to phytochemical analysis, 15 mg of ButFr was re-dissolved in 1 mL of MeOH/H_2O (1:1) and the sample was sonicated and centrifuged (1800 rpm). Supernatants were then purified by successive filtration through 0.45 µm and 0.20 µm filters (Millipore). Mass spectrometry phytochemical analysis was performed using a Varian 310 triple-quadrupole mass spectrometer (Varian Inc., Walnut Creek, CA) with an ESI source (ESI-MS), by direct infusion.

Data acquisition was controlled with a Varian MS Workstation version 6.9 (Varian Inc.). Sample analysis was carried out in positive ESI mode with a needle voltage of 5 kv. The capillary temperature was 200°C, the drying gas pressure was 20 psi and the nebulizing gas pressure was 40 psi. Full scan mass analysis ranged from 200 up to 1000 m/z. Collision-induced fragmentation (CID) protocols were carried out with voltage ranging from 5 to 25 V. All CID-MS experiments were performed using argon at 2 mTorr. Different compounds in ButFr were identified by comparison of their fragmentation patterns with molecules, previously described in the literature of *B. forficata* (Ferreres et al., 2012; Farias and Mendez, 2014).

Statistical analysis

Statistical significance was performed by one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's post hoc tests, with minimum level of significance set at $^*p<0.05$.

RESULTS AND DISCUSSION

B. forficata plant extracts are used in Brazilian traditional medicine for several diseases associated with oxidative stress; some studies have attributed significant antioxidant activity to this plant (Khalil et al., 2008). Ethnopharmacological studies have shown that, this plant is often popularly used to the treatment of gastrointestinal diseases (Bieski et al., 2015; Bolson et al., 2015). Moreover, in a previous study, we demonstrated that the aqueous extract obtained from *B. forficata* leaves displayed preventive anti-ulcerogenic activity in in three mouse ulcer models. Mucus secretion is involved in the gastroprotection exerted by this species, probably due to the flavonoids (flavonols) present in the plant (Mazzeo et al., 2015). Considering this information in the present study, we evaluated the gastroprotective effect of ButFr obtained from the leaves of *B. forficata* against gastric mucosa damage induced by IR.

IR in the gastrointestinal system is known to cause alterations in the tissue due to a reduction in the oxygen supply, which inhibits aerobic metabolism and promotes tissue injury (Stefanutti et al., 2005). The re-introduction of oxygen exacerbates the injury caused by ischemia with the release of pro-inflammatory substances and formation of oxygen-derived free radicals (ROS) (Cuzzocrea et al., 2002).

In IR protocol, we observed that the pre-treatment with lansoprazole (30 mg kg^{-1}) and the ButFr obtained from *B. forficata* leaves (12.5 and 6.25 mg kg^{-1}) reduced the ulcerative lesion area (ULA) by 52.5, 50 and 46%, respectively, compared to the negative control group (0.9% NaCl) (Figure 1). Doses of 12.5 and 6.25 mg kg^{-1} showed no significant differences (p>0.05).

Based on the significant anti-ulcer activity demonstrated by ButFr, assays to elucidate the possible antioxidant mechanisms, using the gastric tissues of animals pre-treated with dose 6.25 mg kg^{-1} was performed. For this purpose, the activities of the enzymes involved in oxidative stress using the mucosa of each stomach compared to the IR-induced gastric ulcers was evaluated. The pathogenesis of gastric mucosal damage includes ROS, because of their high chemical reactivity, due to the presence of uncoupled electrons within the

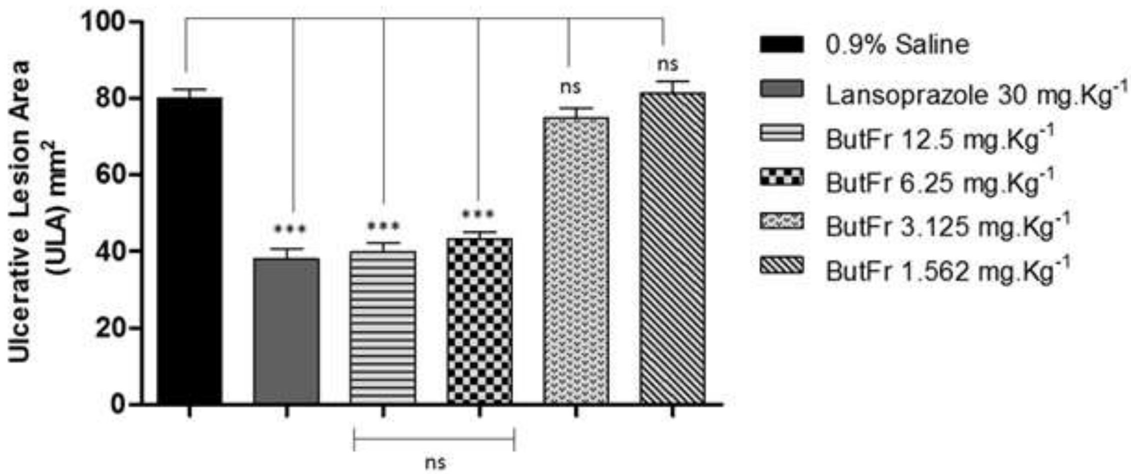

Figure 1. Evaluation of ULA (mm^2) after IR protocols. Results are expressed as mean ± S.D. (n = 8) with statistical significance determined by ANOVA, followed by Tukey's test with level of significance set at ***p<0.001.

molecules. These compounds cause tissue damage, mainly due to enhanced lipid peroxidation. Lipid peroxides are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). A local increase in MDA and 4-HNE concentration indicates ROS-induced tissue damage (Kwiecien et al., 2014).

The enzymes which catalyze ROS-generating chemical reactions are peroxidases, NADPH oxidase, NADPH oxidase isoforms (NOX), xanthine oxidase (XO), lipoxygenases (LOXs), glucose oxidase, myeloperoxidase (MPO), nitric oxide synthase and cyclooxygenases (COXs) (Kulkarni et al., 2007; Swindle and Metcalfe, 2007). Oxidation reactions are crucial for aerobic life, but uncontrolled ROS generation is damaging. Although, free radicals are continuously generated, the body is equipped to defend against the harmful effects of ROS with the help of antioxidants, collectively called the antioxidant defense system. This comprises both enzymatic and non-enzymatic mechanisms. The major enzymatic antioxidants are superoxide dismutases (SOD), glutathione peroxidase (GPx) and glutathione-reductase (GR) (Bhattacharyya et al., 2014).

In this research it was observed that, pretreatment with ButFr at dose 6.25 mg kg^{-1} decreased the LPO index by 35.5% (***p<0.001) and 38% of the MPO activity (***p<0.001) when compared to the negative control group. Moreover, we observed that pretreatment with ButFr increased the activities of superoxide dismutase (SOD) (***p<0.001), glutathione peroxidase (GPx) (***p<0.001) and glutathione reductase (GR) (***p<0.001) (Figure 2).

Studies have shown that, in gastric ulcers induced by IR after reperfusion process, ROS are generated from xanthine oxidase and the activation of neutrophils, leading to gastric mucosa lipid peroxidation (LPO), in combination with acid-gastric secretion process, results in extremely harmful ulcerogenic injury and cell death (Mahmoud-Awny et al., 2007). Thus, it is fundamental to assess lipid peroxidation (LPO) in gastric ulcers induced by IR experimental protocol. Figure 2 shows the measure of lipid peroxidation (LPO) in the gastric mucosa of rats subjected to gastric ulcers by IR and previously treated with ButFr obtained from *B. forficata* leaves. Data indicate a reduction in the lipid peroxidation index around 35% (***p<0.001), when compared to the negative control (0.9% NaCl), supporting the proposed antioxidant mechanism of the plant extract.

The MPO assay is commonly used as an index of neutrophil-mediated infiltration in various experimental models of colitis and gastric ulcers. The development of gastric mucosal lesions was found to occur with an increase in gastric mucosal MPO activity in rats, subjected to oxidative stress (Nishida et al., 1998). Thus, the reduction in MPO levels seen in Figure 2, after treatment with ButFr in rats submitted to the IR process supports an antioxidant mechanism in the gastric mucosa.

According to Bhattacharyya et al. (2014), SOD is the first antioxidant enzyme of gastric mucosa capable of catalyzing the dismutation of O_2, which makes extremely reactive species (H_2O_2), less aggressive to gastric mucosa and also, its metabolism depends on the activity of GPx. The reduction of H_2O_2 by GPx in water is accompanied by, converting glutathione in the reduced form (GSH) to the oxidized form (GSSG), which is then converted to GSH by GR. Thus, increased levels of SOD, GPx and GR after ButFr administration in rats, subjected to IR process indicates a classic antioxidant mechanism induced by the fraction used and thus supports the notion that antioxidant pathways are a central mechanism of antiulcer activity.

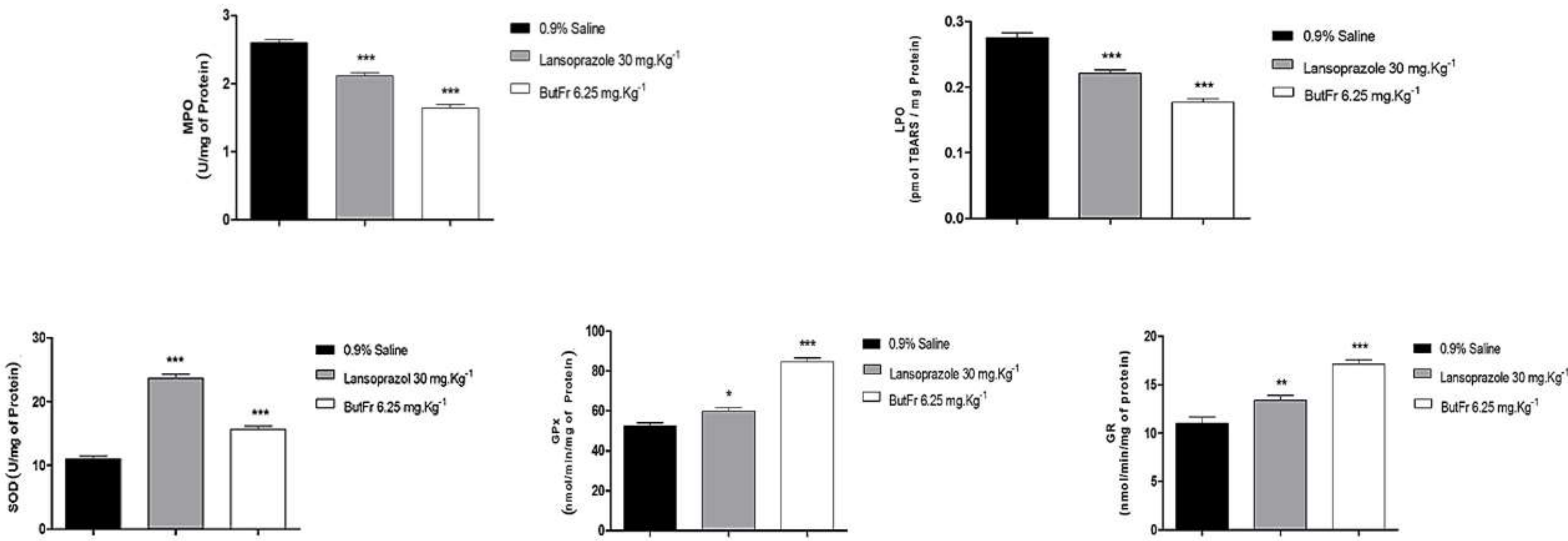

Figure 2. Evaluation of myeloperoxidase (MPO), lipid peroxidation (LPO), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) levels. The results are expressed as mean ± S.D. (n = 8) for each evaluation, and statistical significance was determined by ANOVA followed by Dunnett's test with the level of significance set at *p<0.05, ** p<0.01 and ***p<0.001.

In order to correlate antiulcer activity via antioxidant mechanisms observed after ButFr administration, phytochemical analysis was performed. The ButFr (15 mg) was re-dissolved in 1 mL of $MeOH/H_2O$ (1:1) and submitted to mass spectrometry analysis with an ESI source (ESI-MS/MS) (Figures 3 and 4). After the mass spectrometry analysis, presence of two major flavonoids (kaempferitrin and rutin) was observed.

Flavonoids have been reported to act in gastrointestinal tract, with antispasmodic (Lima et al., 2005), antisecretory, antidiarrheal (Di Carlo et al., 1993), antiulcer and antioxidant properties (La Casa et al., 2000; Martín et al., 1998). According to Sousa et al. (2004), protection against lipid peroxidation in the endoplasmic reticulum was observed following incubation with a butanolic fraction from *B. forficata* leaves. In this study, peroxidation was induced by ascorbyl and hydroxyl radicals. The butanolic fraction possessed strong antioxidant potential, preventing *in vitro* lipid peroxidation in different lipid bilayers, induced by hydroxyl and ascorbyl radicals, as well as acting as a free radical scavenger and inhibitor of prooxidant enzymes. The main compound, present in this fraction was kaempferitrin.

Rutin, a widely occurring flavonoid is known for plethora of pharmacological effects. Several studies have shown that rutin promotes free radical scavenging, suppresses cellular immunity, and has anti-inflammatory effects as well as anti-carcinogenic and antimicrobial activity (Kandaswami and Middleton, 1994; Middleton et al., 2000; Rotelli et al., 2003; Deschner et al., 1991). Moreover, Hussain et al. (2009) showed that, rutin has significant ulcer protective activity via scavenging the reactive oxygen species produced by gastric damage.

Conclusions

Conclusively, the ButFr obtained from the leaves of *B. forficata* displays significant antiulcer and antioxidant activity when administered at a dose

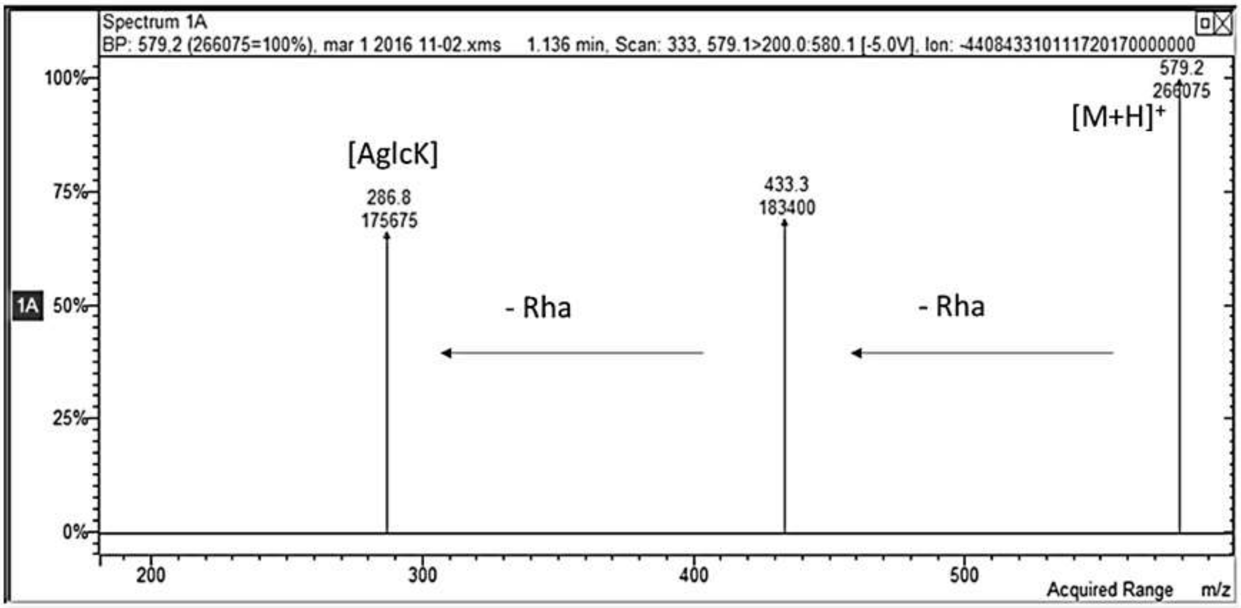

Figure 3. ESI-MS^2 spectra of the compound identified as kaempferitrin (kaempferol -3, 7- di-O-rhamnoside) at m/z 579.2 $[M+H]^+$. Fragments at 433.3 and 286.8 m/z correspond to ions arising from subsequent loss of 146 a.m.u. (rhamnosyl radical), as previously described by Ferreres et al. (2012) for the most abundant phenolic compound identified in *B. forficate* Link. AglcK, aglycone, kaempferol, Rha and rhamnosyl radical.

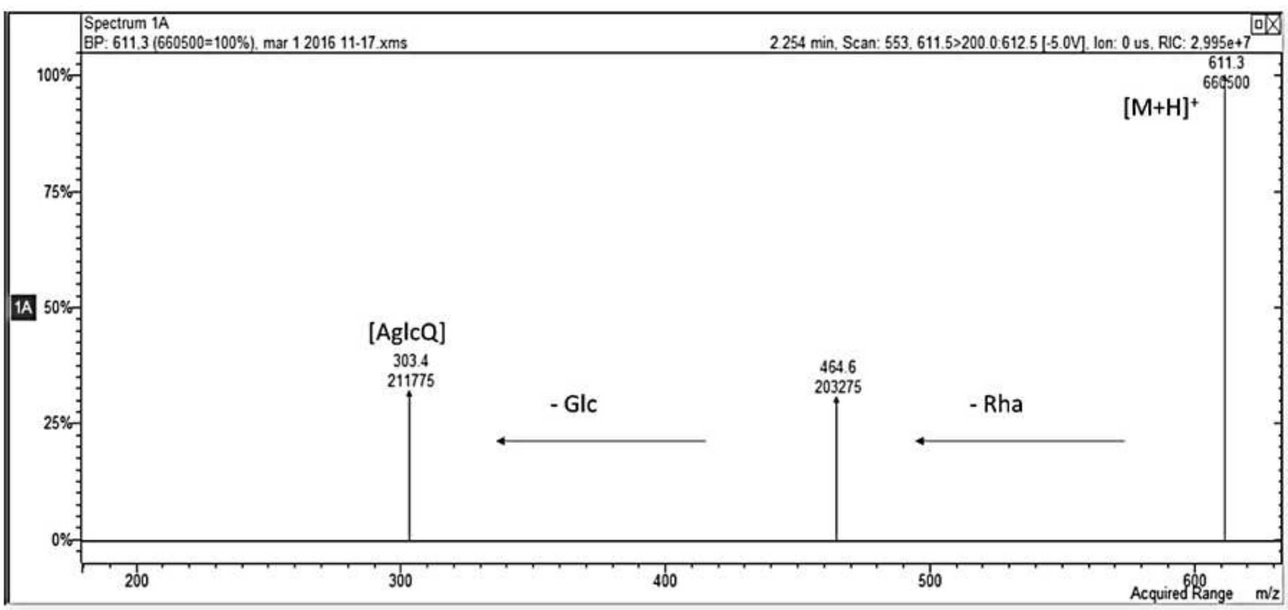

Figure 4. ESI-MS^2 spectra of the compound identified as rutin (ruercetin-3-O-rutinoside) at 611.3 m/z $[M+H]^+$. Fragments at 464.6 and 303.4 m/z correspond to ions arising from the loss of rhamnosyl radical and glucopyranose, respectively, as previously by described by Farias and Mendez (2014) and Ferreres et al. (2012), as the second most abundant phenolic compound identified in *B. forficata*. AglcQ, aglycone quercetin, Rha, rhamnosyl radical, Glc and glucopyranose.

a dose of 12.5 or 6.25 mg kg^{-1}. ButFr administration (6.25 mg kg^{-1}) significantly increased levels of antioxidant enzymes SOD, GPx and GR, while the lipid peroxidation rate and level of MPO (both involved in the gastric ulceration process) were reduced by prior administration of ButFr. The compounds responsible for this pharmacological activity were flavonoids kaempferitrin and rutin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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