Byrsonima intermedia A. Juss partitions promote gastroprotection against peptic ulcers and improve healing through antioxidant and anti-inflammatory activities

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ABSTRACT

Byrsonima intermedia is a species of bush popularly used to treat gastrointestinal disorders, such as gastric ulcers, gastritis, and diarrhea. Previous studies have revealed that the methanolic crude extract of *B. intermedia* leaves has gastroprotective and healing properties. In this new study, we specifically investigated two purified partitions, ethyl acetate (EtOAc) and water (AcoAq), obtained from the crude extract to characterize the antiulcer effects of these two partitions and the mechanisms of action of this medicinal plant. The healing effects of these partitions on the gastric and duodenal mucosa were assessed after ischemia-reperfusion (I/R) or acetic acid-induced injury. The involvement of tumor necrosis factor-alpha (TNF-alpha), interleukin 1β (IL-1β), interleukin 10 (IL-10), and myeloperoxidase (MPO) activity and glutathione (GSH) levels were determined. The antibacterial activity against *Helicobacter pylori* was evaluated using microdilution methods. The phytochemical analysis of AcoAq revealed a predominance of oligomeric proanthocyanidins and galloyl quinic esters, whereas EtOAc was found to contain concentrated flavonoids. Both partitions led to a significant reduction in gastric lesions, but AcoAq was more effective than EtOAc with regard to anti-*Helicobacter pylori* activity in addition to protecting the gastric mucosa against ethanol, non-steroidal anti-inflammatory drugs (NSAIDs) and duodenal mucosal damage induced by cysteamine.

Additionally, both partitions were associated with a significant increase in gastric and duodenal healing and increased gastric mucosal GSH content after damage induced by acetic acid. On the other hand, after 6 days of treatment, EtOAc was more effective than AcoAq in ameliorating gastric damage upon initiation of the gastric I/R, which was accompanied by a significant reduction in the activity of gastric mucosal MPO, IL 1-β and TNF-alpha, as well as an elevation in IL-10 and GSH content. These results demonstrate that the oligomeric proanthocyanidins and galloyl quinic esters present in AcoAq were more effective in the prevention of gastric and duodenal ulcers due to the antioxidant effects of these compounds, whereas the flavonoids present in EtOAc were more effective due to their anti-inflammatory activity on the gastric and duodenal tissue. All these results confirm that the rich phytochemical diversity of *B. intermedia* contributes to the pharmacological actions of this medicinal plant on the gastrointestinal tract in addition to its activity against *H. pylori*.

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1. Introduction

Peptic ulcer disease (PUD) is an important cause of morbidity, especially in older age groups, representing an important global health problem. The medical costs of PUD have been estimated at more than $6 billion per year, and the annual per person attributable direct medical cost of PUD was $1.183, which resulted from longer durations of medication regimens and a higher proportion of hospitalization [1].

The complex and multifactorial pathogenesis of PUD has been studied over several decades. This disease results from gastrointestinal mucosal damage caused from an imbalance of aggressive gastric luminal agents (acid and pepsin) and defensive mucosal factors [2]. Several environmental factors contribute to ulcer formation by increasing gastric acid secretion or weakening the mucosal barrier; additionally, lifestyle habits, such as smoking, excessive alcohol consumption, use of NSAIDs and emotional stress are also important contributors to ulcer pathogenesis [3]. Furthermore, *Helicobacter pylori* infection has been identified as a major risk factor for PUD in both clinical and epidemiological studies. After the introduction of proton pump inhibitors (PPIs) and antibiotics to combat *H. pylori,* the number of PUD cases decreased; the current challenge is to limit the unnecessary use of PPIs and to treat the unexpected side effects that are being revealed secondary to the excessive use of these medications [4].

According to Singh *et al.*, [5] peptic ulcer disease can be treated with curative agents used to heal the ulcer and may be prevented through gastroprotective or cytoprotective mechanisms. Herbs have exhibited prophylactic and/or curative antiulcer activities *via* multitarget actions, creating a research opportunity for drug discovery for the treatment of ulcers.

Byrsonima intermedia A. Juss is a species of the Malpighiaceae family. Popularly known as "murici-pequeno", this plant is native throughout the Brazilian Cerrado Ecosystem. Ethnopharmacological studies have demonstrated the traditional use of different parts of *B. intermedia* for the treatment of dysentery and diarrhea, stomachache, asthma, skin infections, tuberculosis, fever and as a diuretic [6–9].

In the region of Alto Rio Grande, Minas Gerais state (Brazil), the bark and leaves of *B. intermedia* are commonly used in an infusible form to treat gastrointestinal disorders, such as gastritis, dysentery, and diarrhea [6].

The phytochemical profile of this species indicates the presence of oligomeric proanthocyanidins and flavonoids [8,10,11]. Previous pharmacological studies of *B. intermedia* have already characterized this plant's anti-inflammatory effects, antioxidant activity, anti-*Helicobacter pylori* properties and strong antimutagenic activity [11–15]. Specian *et al.* [16] encouraged new studies with *B. intermedia* to investigate its therapeutic potential and mechanisms of action, based not only on ethnopharmacological knowledge but also on the absence of any mutagenic activity and the low cytotoxicity of this species in two cell lines (HepG2 and primary gastric cells). Our group has previously studied the effect of the methanolic extract of *B. intermedia* on peptic ulcers and clearly demonstrated its gastroprotective and healing effects in *in vivo* experimental models [17]. Therefore, the aim of this study was to characterize the effects of the ethyl acetate (EtOAc) and water (AcoAq) partitions obtained from the crude extract of the leaves of *Byrsonima intermedia*.

2. Materials and methods

2.1. Reagents and chemicals

According to the experimental protocols, the reagents used were: acetic acid, absolute ethanol (Labimpex, Brazil), 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), glutathione reductase from baker's yeast (*Saccharomyces cerevisiae*), indomethacin, N-ethylmaleimide (NEM), carbenoxolone, capsaicin, ruthenium red (RR), o-dianisidine dihydrochloride, cysteamine, trichloroacetic acid (TCA), n-nitro-L-arginine methyl ester (L-NAME), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), hexadecyltrimethylammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, USA), lansoprazole (Cruz Vermelha, Brazil), and TNF-alpha, IL-1β and IL-10 assay kits (kit R&D System, Minneapolis, USA). To dilute both partitions, a saline solution (0.9% NaCl, 10 mL/kg) was used as a vehicle and as a negative control.

2.2. Plant material and extraction

The *Byrsonima intermedia* A. Juss. used in this study was collected in Pratânia city, São Paulo in July 2007. The plant material was identified by Professor Jorge Tamashiro from the Institute of Biology, University of Campinas (UNICAMP), SP, Brazil, and the voucher (no. 24164) was deposited in the Herbarium of the Institute of Biosciences, UNESP, in Botucatu-SP. The leaves of *B. intermedia* were dried in an oven at 40°C and powdered on a knife mill. The powder (2000 g) was macerated with methanol (4 L, P.A.) for seven days. The mixture was filtered, and the solvent was evaporated at 60°C under reduced pressure, yielding the methanolic extract of *B. intermedia* (318.4 g, 16% yield), which was stored at 5°C. A portion of the methanolic extract (100 g) was partially solubilized in 30 mL of methanol: water (7:3, v:v). The suspension was transferred to a separation funnel containing 500 mL of water and 500 mL of ethyl acetate, and the mixture was gently shaken. After equilibration and separation of the phases, the ethyl acetate portion (EtOAc) was removed, and a new aliquot of 500 mL of this solvent was added to the funnel. This procedure was repeated three times, and the EtOAc portions were combined. The solvents were evaporated (at 60°C) under reduced pressure, yielding 26.76 g (29.3%) of the EtOAc fraction and 64.5 g (70.7%) of the aqueous portion (AcoAq).

2.3. Chromatographic analyses and chemical identification

The chemical compositions of the methanolic extract and the AcoAq and EtOAc partitions were determined after high-performance liquid chromatography analyses coupled to a photodiode array detector (HPLC-PAD). The experimental and instrumental conditions applied were the same as reported previously by Santos *et al.* [10]. Complete separation of the chemical composition was achieved, and the main classes of secondary metabolites were attributed to: phenolic acids, flavonoids, catechins and proanthocyanidins, which were putatively confirmed after the comparison of retention time and ultraviolet (UV) spectral analyses. Confirmation of these metabolites was established by performing experiments of spiking with commercial or isolated standards under the same conditions.

The analytical column was a Phenomenex Synergi Hydro RP18 (250 mm × 4.6 mm L × i.d.; 4 m) with a Phenomenex security guard column (4.0 mm × 2.0 mm L × i.d.) maintained at room temperature (25°C). Separation of phenolic acids, flavonoids, flavan-3-ols and proanthocyanidins was established using the mobile phase of water (eluent A) and methanol (eluent B), both containing 0.1% trifluoroacetic acid (TFA), with the following gradient program: 0–20% B (20 min), 20% B isocratic (2 min), 20–50% B (38 min), 50–100% B (5 min), 100% B isocratic (5 min), return to 0% B (2 min), and the column was re-equilibrated with the initial conditions for 18 min before the next injection. The flow rate was 1.0 mL/min, and the total run time was 90 min. EZChrom Elite Data System software (Chromatec, Idstein, Germany) was used for both detector operations and data processing.

2.4. Animals

The *in vivo* experimental procedures were conducted using male Wistar albino rats (160–260 g, 6–8 weeks) obtained from the Central Biotherium of UNESP (Botucatu, São Paulo, Brazil) and from Anilab Laboratory Animal Creation and Trade Ltd. (Paulínia, São Paulo, Brazil). A total of 335 animals were used in this study. The rats were

divided into groups of 4–10 individuals and maintained in metabolic cages under controlled conditions (12:12 h light: dark cycle and room temperature of 22 ± 2°C). The rats' diet was composed of Presence® feed and water provided *ad libitum*. The animals were subjected to fasting before the test because the standard drugs and both partitions were always administered orally (gavage) using a saline solution (0.9% NaCl, 10 mL/kg) as vehicle. The experimental procedures were performed in accordance with the experimental protocols approved by the Ethics Committee on Animal Experimentation of the Institute of Biosciences, UNESP – Botucatu, identified under protocol number 18/05.

2.5. Evaluation of gastric and duodenal protective activities of partitions

2.5.1. Absolute ethanol-induced gastric ulcers

The rats were separated into groups and underwent fasting for 16 h prior to receiving the vehicle (saline 0.9% NaCl, 10 mL/kg, p.o., n = 7), carbenoxolone (100 mg/kg, p.o., n = 7) and EtOAc (50, 100 and 200 mg/kg, p.o., n = 5–6) or AcoAq (50, 100 and 200 mg/kg, p.o., n = 5–6). Next, all groups were treated with absolute ethanol (1 mL) by oral route after 60 min [18]. The animals were euthanized 1 h later, and the extent of the lesions (mm^2) was measured using AvSoft® Bioview Spectra (Brazil).

2.5.2. Role of nitric oxide (NO), sulfhydryl groups (SH) and vanilloid receptors in the cytoprotection

The possible involvement of NO in the protective activity partitions was investigated using rats (n = 5–7 per group) treated with the NO-synthase inhibitor L-NAME (70 mg/kg, i.p.) or vehicle (10 mL/kg, i.p.) 30 min before the administration of EtOAc or AcoAq (100 mg/kg, lower dose, p.o.). The possible involvement of SH in the gastroprotective effects of the partitions was evaluated in the presence of the SH blocker NEM (10 mg/kg, i.p.) or vehicle (10 mL/kg, i.p). The animals were injected with NEM 30 min before the oral administration of EtOAc (100 mg/kg) or AcoAq (100 mg/kg). The participation of vanilloid receptors by transient receptor potential vanilloid type 1 (TRPV1) was investigated with the administration of an antagonist against capsaicin, ruthenium red (RR, 6 mg/kg, s.c.), 30 min before the administration of the partitions (EtOAc and AcoAq, 100 mg/kg, p.o). The animals received an oral administration of 1 mL of absolute ethanol per animal after one hour. One hour after administration of the harmful agent, all animals were euthanized, and their stomachs were placed on glass plates and scanned for evaluation of the lesion area, as previously described. Strips from the stomachs were removed and weighed for later biochemical analysis [19–21].

2.5.3. Non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcer

Male Wistar rats were separated into groups (n = 5–7 per group) after 16 h of fasting. The rats were then submitted to the different treatments: saline (vehicle, 10 mL/kg, p.o., n=7), cimetidine (100 mg/kg, p.o., n=7) and EtOAc (100 mg/kg, p.o., n=6) or AcoAq (100 mg/kg, p.o., n=7). Indomethacin 50 mg/kg (solubilized in sodium carbonate 0.5%) was administered by oral route to all groups 30 min after the pretreatment. Six hours after NSAID administration, all animals were euthanized and the stomachs removed. The gastric lesions were measured as described above, and stomach strips were removed and weighed for later biochemical procedures [22,23].

2.5.4. Cysteamine-induced duodenal ulcer

Duodenal ulcers were induced in rats by administration of cysteamine (300 mg/kg, p.o.) at intervals of 4 h in 2 administrations. After 2 h of fasting, the rats were pretreated by oral route with vehicle (10 mL/kg, n=10), lansoprazole (30 mg/kg, n=8) and EtOAc (100 mg/kg, n=9) or AcoAq (100 mg/kg, n=7) thirty min before the first administration of cysteamine. The animals were euthanized twenty-four hours after the start of fasting, and the duodenums were completely removed

for measurement of the lesions. The lesion counts were scored according to the scale from Szabo [24].

2.5.5. Gastric acute lesion induced by ischemia-reperfusion (I/R)

The rats were separated into different groups: vehicle (10 mL/kg, p.o., n = 10), positive control (lansoprazole 30 mg/kg, p.o., n = 10) and EtOAc (100 mg/kg, p.o., n = 9). and AcoAq treatments (100 mg/kg, p.o., n = 8). Thirty minutes after the oral treatments described earlier, the animals were anesthetized with xylazine (8 mg/kg) and ketamine (80 mg/kg) administered intramuscularly. The abdomens were then opened to expose and isolate the celiac artery, and a microvascular clamp was placed in the artery to disrupt gastric blood flow for thirty minutes (period of ischemia). Next, the clamp was completely removed and remained open for 60 min (period of reperfusion). After the period of reperfusion, the animals were euthanized and the stomachs were removed for characterization of the gastric lesions, as previously described. The tissue strips were stored for later biochemical analysis [25].

2.6. Evaluation of gastric and duodenal healing activity of partitions

2.6.1. Gastric and duodenal ulcers induced by acetic acid

Male Wistar rats (n = 7–8 per group) were anesthetized as previously described, and the abdomen was opened to expose the stomach or duodenum. A plastic tube of 4.2 mm internal diameter (for the standardization of the area) was applied to the serosal surface of the stomach wall or duodenum. A volume of 70 μL of 80% acetic acid was applied for 20 s to the gastric surface or 10 s to the serosal surface of the duodenum. The acetic acid was then completely removed, and the surface of the tissue was washed with saline solution. The animal was sutured and kept under observation for 24 h after surgery. After the determination of the postsurgical condition of the animal, the animal was treated orally with vehicle (saline 0.9% NaCl, 10 mL/kg), lansoprazole (30 mg/kg), or EtOAc (100 mg/kg) or AcoAq (100 mg/kg) for 7 or 14 days (once a day) for both procedures (gastric or duodenal lesions). All animals were euthanized at the end of the treatment period, and the stomach or duodenum was removed for microscopic analysis of tissue injury. Strips were weighed and stored for biochemical determinations [19,20]. The organs (heart, liver, kidneys, lungs, spleen and testicles) were removed for weighing, and blood was collected for biochemical analysis of parameters such as glucose, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Gamma glutamyl transpeptidase (Gamma-GT).

2.6.2. Gastric subacute lesions induced by I/R

The rats (n = 5–8 per group) were anesthetized with ketamine and xylazine (administered intramuscularly at doses of 0.80 and 0.8 mg/kg, respectively), and the abdomen was opened to expose the celiac artery. This artery was isolated and subjected to a cleaning process to eliminate adhesions and possible adipose tissue. A microvascular clamp was placed on this artery, interrupting gastric blood flow for thirty minutes. Following the period of ischemia, the clamp was completely removed, and the incision was sutured. Twenty-four hours after the surgery, the animals were separated randomly into different groups: negative control (vehicle, saline 0.9% NaCl, 10 mL/kg, p.o), positive control (lansoprazole, 30 mg/kg, p.o.) and treatments with EtOAc (100 mg/kg, p.o.) or AcoAq (100 mg/kg, p.o.). The treatments were performed in a single administration (24 h after surgery) or for a period of 6 consecutive days after surgery (once a day). At the end, the animals were euthanized, the stomachs were removed for characterization of the gastric lesion area as previously described, and the tissue strips were weighed and stored for biochemical determinations [25,26].

2.7. Biochemical analysis

2.7.1. Determination of glutathione (GSH) levels

The method described by Anderson [27] was performed to determine the content of glutathione. Gastric or duodenal tissue samples taken from the experimental procedures previously described were weighed and stored in 1 mL solution of TCA 5%. The GSH content of the stomach was determined using DTNB. The enzymatic reaction consisted of a 200 μL sample containing 2 mg/mL of protein, 0.2 M pH 8.0 phosphate buffer, and 0.5 mM DTNB (10 mg in 2 mL of 1% sodium citrate) in a final volume of 2 mL. The absorbance was determined using a spectrophotometer at 412 nm, and total glutathione concentration was expressed using the extinction coefficient of 13.6 mM.

2.7.2. Determination of myeloperoxidase (MPO) activity

Tissue samples (stomach and duodenum) were weighed and stored at -80°C until the time of measurement. The reaction with HTAB buffer (0.5% in 50 mM sodium phosphate buffer, pH 6.0) acts to break the detergent granules of neutrophils that contain the enzyme, which is then released. The enzymatic activity was determined following the enzyme reaction kinetics with hydrogen peroxide in the reaction buffer, where 1 unit of MPO is determined capable of degrading 1 nmol/min of hydrogen peroxide at 25°C. The MPO activity is proportional to the number of infiltrating neutrophils in the mucosa. The absorbance was determined at 450 nm using a spectrophotometer [28].

2.7.3. Tumor necrosis factor alpha (TNF-alpha)

To quantify TNF-alpha in the stomach, samples were placed into tubes with PBS buffer, pH 7.4 (1/20 v/v). The tubes were centrifuged at 13,680 × g for 10 min. The supernatants were frozen at -80°C until analysis. TNF-alpha (R&D Systems RTA00) was quantified by immunoenzymatic assay (ELISA) with detectable absorbance at 450 nm following the technical guidelines of the manufacturer [29].

2.7.4. Interleukin-1 beta (IL-1β) or Interleukin-10 (IL-10)

Gastric tissue samples were homogenized in 1 mL of phosphate buffer, followed by centrifugation of samples. The IL-1β or IL-10 concentration was determined using an enzyme immunoassay kit (R&D Systems R1000 and RLB00) and detectable absorbance at 450 nm following the manufacturer's instructions [30].

2.8. Determination of antibacterial activity

2.8.1. Microorganism

Helicobacter pylori (ATCC 43504) was maintained on Mueller Hinton broth (MHB) with glycerol (20%) at −20°C. Bacteria were subcultured for use in 2 mL of MHB and incubated at 37°C for 24 h.

2.8.2. Growth conditions and standardization of H. pylori suspension

H. pylori was cultivated on Mueller-Hinton agar plates with sheep blood (5%). This culture medium was incubated at 36°C for 72 h in 10% CO_2 atmosphere. In liquid culture medium (MHB + fetal bovine serum 50%), the strain was standardized by density adjusted to a 2.0 McFarland turbidity. The concentration of the strain was confirmed by spectrophotometric reading at 620 nm. This suspension was diluted 1/10 (suspension of 10^7 CFU/mL) according to Araújo et al. [31].

2.8.3. Determination of minimal inhibitory concentration (MIC)

The *H. pylori* assay was performed according to CLSI (2006) with modifications. First, 100 μL of MHB supplemented with fetal bovine serum (50%) was added to all wells of the microplates (96 wells), with 100 μL in each of the EtOAc and AcoAq partitions (2000 ug/mL). Then, serial dilutions were carried out from 1000 to 7.81 ug/mL, and 20 μL of the suspensions of previously standardized microorganism was distributed in the microplate and incubated at 37°C for 72 h in 10% CO_2 atmosphere. The tests were performed in triplicate, and amoxicillin was
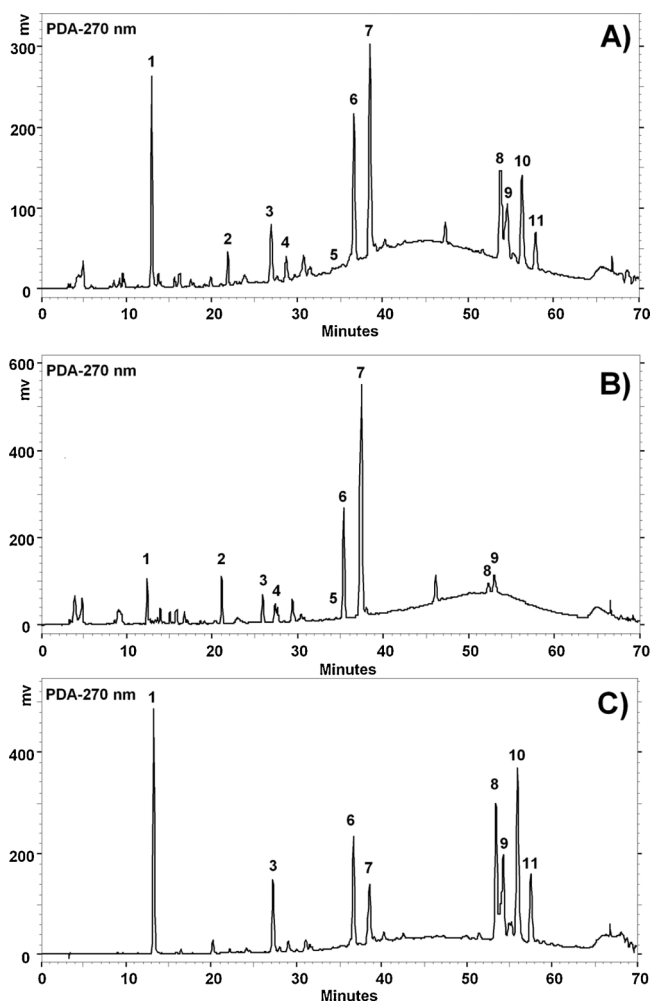

Fig. 1. Analytical HPLC-PAD chromatograms recorded at 270 nm of the secondary metabolites identified in the A) methanolic extract, B) AcoAq fraction and C) EtOAc fraction. Peak identification: (1) gallic acid, (2) 3,4-di-*O*-galloylquinic acid, (3) methyl gallate, (4) catechin, (5) epicatechin, (6) 1,3,5-tri-*O*-galloylquinic acid, (7) 1,3,4,5-tetra-*O*-galloylquinic acid, (8) quercetin-3-*O*-β-galactopyranoside, (9) quercetin-3-(2"-*O*-galloyl)-O-α-galactopyranoside, (10) quercetin-3-*O*-α-arabinopyranoside and (11) quercetin-3-*O*-(2"-*O*-galloyl)-α-arabinopyranoside.

used as a positive control (100 to 0.3 ug/mL) [31,32].

2.9. Statistical analysis

Results were expressed as the mean ± standard error of mean (S.E.M.) of all parameters obtained. The average values obtained were subjected to analysis of variance (ANOVA), followed by Kruskal-Wallis and Dunn or Dunnett's tests to compare three or more groups. Student's test (*t*) was used for comparisons between two groups. The statistical analysis considered a minimum significance level of $P < 0.05$.

3. Results

3.1. Chromatographic analyses and chemical identification

The chromatographic HPLC-PAD characterization of AcoAq and EtOAc partitions obtained from crude methanolic extract of B. *intermedia* leaves is shown in Fig. 1. It shows that some compounds are preferably concentrated either in the aqueous AcoAq or in the organic EtOAc phase. Peaks were identified by their UV spectra and by coinjection with authentic standards available in our laboratory. The AcoAq
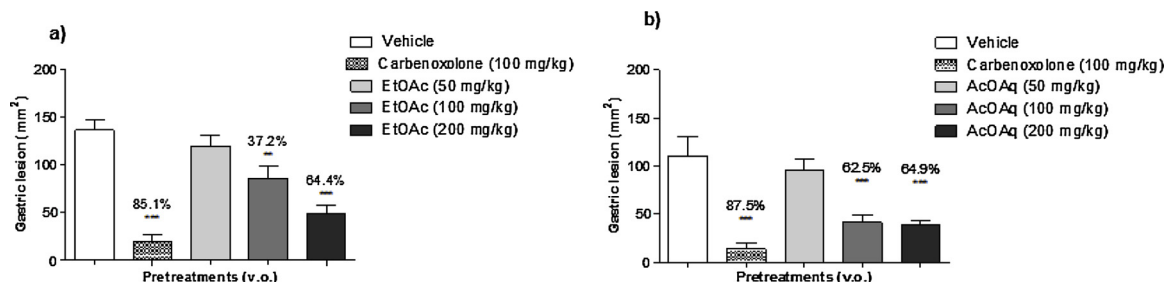
Fig. 2. Effects of the pretreatment with EtOAc (a) and AcOAq (b) obtained from the leaves of *Byrsonima intermedia* (50, 100 and 200 mg/kg) on ethanol-induced gastric ulcers in rats (n = 5–7 per group). Each column represents the mean, and the vertical line indicates the S.E.M. The asterisks denote the significance levels compared with the control group treated with vehicle. ** $P < 0.01$ and *** $P < 0.001$ using one-way ANOVA followed by Dunnett's test.

(Fig. 1b) concentrates mainly catechins (4 and 5), proanthocyanidins (broad peak in the range of 35–65 min) and gallic acid derivatives (1–3, 6 and 7). On the other hand, the EtOAc fraction (Fig. 1c) concentrates flavonoids (8–11) in addition to some gallic acid derivatives (1,3,6 and 7).

3.2. Evaluation of gastric and duodenal protective activities of partitions

3.2.1. Ethanol-induced gastric ulcer

Fig. 2 shows the protective effect on gastric mucosa of EtOAc (Fig. 2a) and AcOAq (Fig. 2b) in the ethanol-induced gastric ulcer model. We observed that the lower dose of 50 mg/kg of each partition did not protect against this ulcerogenic agent ($P > 0.05$). However, oral pretreatment with EtOAc or AcOAq at doses of 100 and 200 mg/kg decreased the gastric injury in 37.2% and 64.4% or 62.5% and 64.9% of animals, respectively, compared to the control group ($P < 0.01$).

3.2.2. Role of nitric oxide (NO), sulfhydryl groups (SH) and vanilloid receptors in the cytoprotection and determination of glutathione (GSH) levels.

After the characterization of the gastroprotective effects of both partitions against absolute ethanol, the next step was the investigation of the mechanisms of action involved in strengthening the gastric mucosa.

Table 1 shows that rats pretreated with L-NAME (NO-synthase inhibitor) then treated with AcOAq maintained gastroprotection ($P > 0.05$). However, the EtOAc group, when pretreated with L-NAME, showed a significant increase in gastric lesions (369.7 ± 81.6 mm^2) compared to the saline-pretreatment group (20.6 ± 17.6 mm^2).

Table 1 also shows that when the rats were pretreated with an SH blocker (NEM), neither partition had any gastroprotective action, while the GSH levels significantly decreased when compared to the groups pretreated with saline (Table 1).

We also evaluated the participation of vanilloid receptors on the gastroprotective action of AcOAq or EtOAc. Pretreatment of animals with RR, an antagonist of the TRPV receptor, did not change the protective action of the AcOAq partition (86% in both treatments). However, rats treated with EtOAc showed an increase in lesions (107.6 ± 26.4 mm^2) compared to the saline-pretreatment EtOAc group (25.7 ± 6.2 mm^2).

3.2.3. Non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcer in rats, GSH levels and MPO activity

NSAIDs increased gastric mucosal lesions (Fig. 3) and MPO activity and decreased the GSH content of the gastric tissue in animals treated with vehicle compared to the untreated group (Table 2). The gastroprotective effect against NSAIDs was found only in the group pretreated with AcOAq (57%) compared to the groups treated with vehicle (Fig. 3, $P < 0.001$). This protective effect of AcOAq on gastric tissue was followed by a significant decrease in MPO activity (54%) (a biochemical marker of neutrophil infiltration in the injured tissue) compared to

vehicle ($P < 0.01$), while EtOAc did not protect against NSAID-related injury and did not change the GSH level or MPO activity ($P > 0.05$).

3.2.4. Cysteamine-induced duodenal ulcer and determination of MPO activity

Our results (Table 2) show an increased duodenal lesion area (Fig. 3) and MPO activity and a decreased GSH content in the duodenal mucosa of animals treated with vehicle compared to the untreated group ($P < 0.001$). Pretreatment with EtOAc did not protect the duodenum against injuries caused by cysteamine ($P > 0.05$). In contrast, pretreatment with AcOAq significantly improved the duodenal injuries, decreasing the lesion area (76%) and MPO level (55%) compared to the saline-treated group (Fig. 3, $P < 0.01$).

3.2.5. Gastric acute lesion induced by ischemia and reperfusion (I/R)

We also evaluated the ability of each partition to protect the gastric mucosa of rats from the oxidative damage induced during an I/R procedure. We observed an increase in the gastric lesion area (Fig. 3), MPO activity and a decrease in GSH content in the gastric mucosa of animals treated with vehicle compared to the untreated group (Table 2). Oral pretreatment with EtOAc or AcOAq significantly decreased the extent of the gastric lesions by 79% and 73%, respectively (Fig. 3), compared to the vehicle-treated group ($P < 0.001$). Pretreatment with partitions also decreased MPO activity ($P < 0.001$), but only pretreatment with EtOAc significantly increased GSH content ($P < 0.01$) compared to the vehicle-treated group.

3.3. Evaluation of gastric and duodenal healing activity of partitions

3.3.1. Healing activity of partitions on gastric and duodenal ulcers induced by acetic acid

Our results show that, due to the pronounced severity of the acetic acid induced injury, neither *B. intermedia* partitions nor lansoprazole were able to heal the gastric lesions within 7 consecutive days of treatment (Fig. 4a). Fig. 4 shows that 14 consecutive days of treatment with lansoprazole healed the gastric ulcers, decreasing the lesion area by 63% ($P < 0.001$). Both partitions were also effective in healing the gastric ulcer compared to the control group treated with saline (decrease of 73.3% and 35.5% by EtOAc and AcOAq, respectively $P < 0.05$). However, only the partitions increased GSH levels after 14 days of treatment (Fig. 4b), similar to the results obtained for the untreated group without gastric lesions ($P < 0.05$). Body weight was recorded daily throughout the experimental period (Appendices, Figure A) and any deaths or significant changes in body weight were associated with treatment with EtOAc and AcOAq. Blood samples were collected for quantification of AST, Gamma-GT, ALT, glucose, urea and creatinine (Appendices, Table A). Data in Table A show a significant decrease in Gamma-GT and ALT levels after treatment with EcoAq (both parameters) and AcOAq (only to ALT level).The decreases in both parameters not constitute hepatotoxicity effect. Additionally, no significant changes in organ weight (heart, liver, kidneys, lungs and

Table 1
Effect of the EtOAc and AcoAq partitions of *Byrsonima intermedia* on gastric lesions and glutathione (GSH) levels in rats pretreated with L-NAME (NO-synthase inhibitor), NEM (SH blocker) or ruthenium red (TRPV antagonist) and treated with absolute ethanol.

Pretreatment (i.p.)	Treatment (p.o.)	Dose (mg/kg)	n	Gastric lesion (mm^2)	Inhibition of gastric lesion (%)	GSH (nmol/g tissue)	Increase in GSH level (%)
Saline +	Vehicle	–	7	204.0 ± 40.6	–	877.4 ± 105.8	–
Saline +	Carbenoxolone	100	6	11.8 ± 3.8	94 ***	1244.0 ± 110.8[+]	42
Saline +	EtOAc	100	5	20.6 ± 17.6	90 ***	1188.0 ± 50.9[+]	35
Saline +	AcoAq	100	5	20.2 ± 10.0	90 ***	1080.0 ± 138.3[+]	23
L-Name +	Vehicle	–	7	456.1 ± 79.2 [#]	–	900.0 ± 71.1	–
L-Name +	Carbenoxolone	100	7	161.8 ± 50.8 [#]	64 *	1079.0 ± 106.5	20
L-Name +	EtOAc	100	6	369.7 ± 81.6 [##]	19	839.1 ± 56.0	–
L-Name +	AcoAq	100	6	141.7 ± 36.8 [#]	69 **	1001.0 ± 68.43	11
Saline +	Vehicle	–	7	195.1 ± 16.1	–	603.0 ± 28.6	–
Saline +	Carbenoxolone	100	7	8.6 ± 1.4	96 ***	953.5 ± 27.0[++]	58
Saline +	EtOAc	100	5	36.3 ± 9.7	84 ***	752.5 ± 23.3[++]	20
Saline +	AcoAq	100	6	31.1 ± 12.6	81 ***	861.3 ± 95.1[++]	43
NEM +	Vehicle	–	7	381.6 ± 93.3 [#]	–	538.3 ± 41.1	–
NEM +	Carbenoxolone	100	6	286.9 ± 90.0 [##]	25	698.2 ± 64.4	30
NEM +	EtOAc	100	6	342.2 ± 56.5 [###]	35	429.9 ± 24.3	–
NEM +	AcoAq	100	5	247.2 ± 18.4 [###]	10	576.2 ± 61.8	7
Saline +	Vehicle	–	7	149.0 ± 33.6	–	756.4 ± 44.3	–
Saline +	Capsaicin	5	7	4.1 ± 1.5	97**	1093.4 ± 49.8[++]	41
Saline +	EtOAc	100	6	25.7 ± 6.2	83**	963.5 ± 61.8[++]	27
Saline +	AcoAq	100	6	20.9 ± 13.1	86**	1056.2 ± 74.4[++]	40
RR +	Vehicle	–	6	171.2 ± 16.3	–	674.8 ± 59.8	–
RR +	Capsaicin	5	6	130.9 ± 42.6 [###]	24*	933.0 ± 87.3[+]	38
RR +	EtOAc	100	5	107.6 ± 26.4 [##]	38	740.4 ± 91.1	10
RR +	AcoAq	100	5	24.9 ± 7.7	86**	1038.1 ± 75.6[++]	54
Untreated			5			1258.0 ± 106.2[++]	

Data are presented as the mean ± S.E.M. ANOVA followed by Dunnett's test. Ulcerative lesion: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ represent the difference compared to the control group treated with vehicle; [#] $P < 0.05$; [##] $P < 0.01$ and [###] $P < 0.001$ represent the difference compared to pretreatments saline + treatment and L-Name + treatment, pretreatments saline + treatment and NEM + treatment or pretreatments saline + treatment and RR + treatment. GSH (glutathione level) [+] $P < 0.05$ and [++] $P < 0.01$ represent the difference compared to the control group treated with vehicle (n = 5–7 per group).

spleen) were noted for EtOAc or AcoAq-treated rats (Table A). The result (Table A) indicated that there was no change between the treatments with both partitions and the control group, indicating that after 14 days, there were no variations that indicated toxicity of the partitions at the administered dose.

EtOAc and AcoAq healed duodenal ulcers within 7 (66% and 65%, respectively; Fig. 5a) and 14 (72% and 49%, respectively; Fig. 5a) consecutive days of treatment. EtOAc also increased the levels of GSH in the duodenal mucosa after 7 and 14 days of treatment (Fig. 5b).

3.3.2. Gastric subacute lesions induced by I/R and biochemical analysis of MPO, TNF-alpha, IL-1β and IL-10

Treatment with the *B. intermedia* partitions one day after I/R-induced injury in rats was unable to heal the deleterious effects on the gastric mucosa ($P > 0.05$). However, treating the rats with EtOAc (100 mg/kg) for 6 consecutive days healed the gastric mucosa (56%) better than lansoprazole (49%) treatment (Fig. 6). The EtOAc partition decreased neutrophil infiltration by reducing the MPO levels (45%) and inhibited pro-inflammatory cytokines such as TNF-alpha and IL-1β in the gastric mucosa (Table 3). Additionally, the healing effect of EtOAc
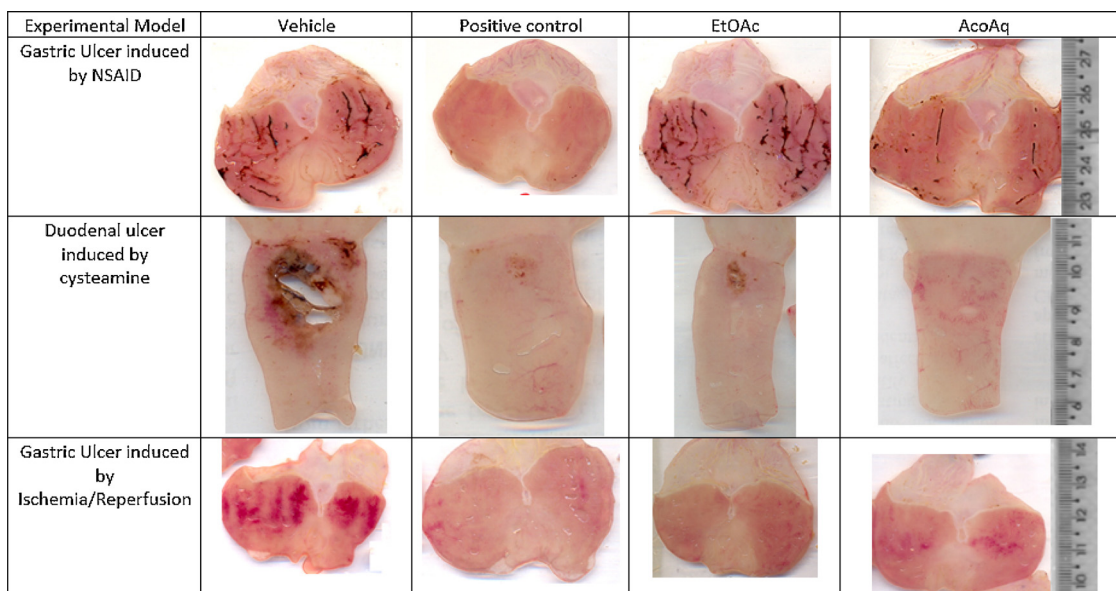

Fig. 3. Macroscopic photograph of the rat stomach or duodenum in different models of lesions.

Table 2

Effect of the treatment with EtOAc and AcoAq partitions from *Byrsonima intermedia* in different models of duodenal and gastric ulcer prevention.

Experimental model	Treatment (p.o.)	Dose (mg/kg)	n	Ulcerative lesion (mm²)	Inhibition (%)	GSH (nmol/g tissue)	Increase (%)	MPO Unit/g tissue	Decrease (%)
Gastric ulcer induced by NSAID	Vehicle	–	7	60.4 ± 3.6	–	1206 ± 81.7	–	7878.1 ± 1190	–
	Cimetidine	100	7	3.5 ± 2.7***	94	1363 ± 103.1	–	4265.9 ± 181.7**	46
	EtOAc	100	6	51.6 ± 12.7	–	1153 ± 86.9	–	5142.3 ± 968.2	–
	AcoAq	100	7	26.2 ± 6.2***	57	1189 ± 96.7	–	3628.0 ± 166.3**	54
	Untreated	–	5	–	–	1502 ± 34.7*	–	2939.2 ± 166.7***	–
Duodenal ulcer induced by cysteamine	Vehicle	–	10	2.9 ± 0.1	–	654.7 ± 73.4	–	10,685.1 ± 1685.9	–
	Lansoprazole	30	8	0.6 ± 0.3**	79	1038.1 ± 54.4	–	4695.0 ± 426.9***	56
	EtOAc	100	9	1.3 ± 0.6	–	872.4 ± 45.8	–	9745.3 ± 1548.7	–
	AcoAq	100	7	0.7 ± 0.3**	76	864.2 ± 95.8	–	4781.2 ± 901.1**	55
	Untreated	–	4	–	–	1390.0 ± 122.8***	–	2910.0 ± 349.5***	–
Gastric ulcer induced by Ischemia/reperfusion	Vehicle	–	10	102.8 ± 20.8	–	962.6 ± 45.2	–	4310.0 ± 569.9	–
	Lansoprazole	30	10	15.1 ± 2.4***	85	1226.2 ± 106.0*	27	2612.7 ± 252.2***	38
	EtOAc	100	9	21.1 ± 6.1***	79	1277.3 ± 60.1**	33	2170.8 ± 93.0***	49
	AcoAq	100	8	27.3 ± 5.7***	73	1146.1 ± 73.5	–	2623.3 ± 234.7***	39
	Untreated	–	5	–	–	1281.2 ± 100.6***	–	1236.4 ± 102.9***	–

Data are presented as the mean ± S.E.M. ANOVA followed by Dunnett's test for gastric ulcer induced by NSAIDs and ischemia/reperfusion. ANOVA followed by the Kruskal-Wallis test for duodenal ulcer induced by cysteamine. Ulcerative lesion: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control group treated with vehicle (n = 4–10 per group).

was followed by a 2-fold increase in GSH levels and an increase in IL-10 levels in the gastric mucosa compared to the group of animals not subjected to I/R (7409.6 ± 568.6 pg/mL *vs.* 6571.0 ± 33.6 pg/mL, respectively).

3.4. Determination of anti-Helicobacter pylori activity of partitions

Our results show that both partitions were minimally active (increased MIC) against *Helicobacter pylori* (EtOAc > 0.1000 mg/mL and AcoAq = 0.500 mg/mL).

4. Discussion

As demonstrated previously, the chromatographic HPLC-PAD profile of the crude methanolic extract of *B. intermedia* presents a broad range of compounds classified as catechin derivatives, flavonoids, phenolic acids and oligomeric proanthocyanidins [10]. Similar chromatographic characterization was applied to the partitions obtained from the liquid-liquid extraction, demonstrating that some compounds are preferably concentrated in the aqueous AcoAq (mainly catechins, proanthocyanidins and gallic acid derivatives) and in the EtOAc fraction (flavonoids and gallic acid derivatives).

Due to the distinct chemical profiles of the two partitions, we chose to perform a series of *in vivo* tests to investigate their protective and healing activities on the gastric and duodenal mucosa and the factors involved in the protection and maintenance of the gastric mucosa. Based on the previous results with the crude extract (lower effective

dose of 500 mg/kg), we started this study with a dose 10 times lower (50 mg/kg), and to determine the lowest effective dose of each fraction, we evaluated three doses of each partition.

First, we evaluated the ability of EtOAc and AcoAq to protect the gastric mucosa of rats from injuries caused by the oral administration of ethanol. Ethanol induces gastric mucosal damage with hemorrhagic erosions associated with microcirculatory disorders, increased vascular permeability and infiltration of inflammatory cells, forming edema in the mucosa, subepithelial hemorrhage, and cellular exfoliation, culminating in necrosis [33–35].

Our results show the gastroprotective effect of EtOAc and AcoAq in the model of ethanol-induced gastric ulcer at different doses (50, 100 and 200 mg/kg). The oral pretreatment with EtOAc or AcoAq at doses of 100 and 200 mg/kg decreased gastric injury. For comparison between the two partitions, the next experiments were performed using only the dose of 100 mg/kg by oral route.

The next step was the investigation of the mechanisms of action involved in the gastroprotection, which included SHs, the TRPV1 receptor and NO, which are involved in gastric mucosal strengthening. Cho [36] and Ancha *et al.* [37] described the involvement of NO with the preservation of the gastric mucosa in an experimental ulcer model because NO induces vasodilatation, decreases lipid peroxidation and plays a role in anti-inflammatory factors in the tissue [38].

Although our previous study excluded the involvement of endogenous NO on the gastroprotective action of the crude extract of *B. intermedia* [12], the EtOAc and AcoAq partitions behave differently. Independent from NO-synthase inhibitor (L-NAME) action, AcoAq
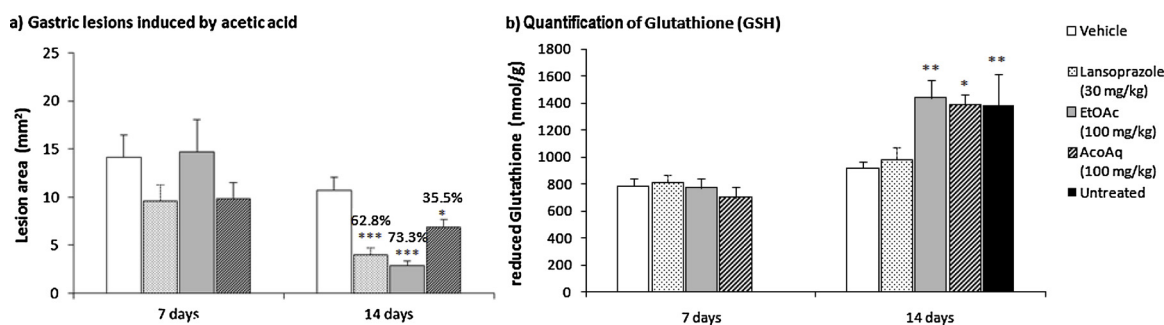

Fig. 4. Healing effect of the EtOAc and AcoAq partitions of *Byrsonima intermedia* administered for 7 or 14 days after gastric injury induced by acetic acid in rats (n = 7–8 per group). The bars represent mean ± S.E.M. a) gastric lesions induced by acetic acid. b) glutathione quantification. ANOVA followed by Dunnett's test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared to vehicle. The numbers indicate the percentage of healing in relation to the control group treated with vehicle.
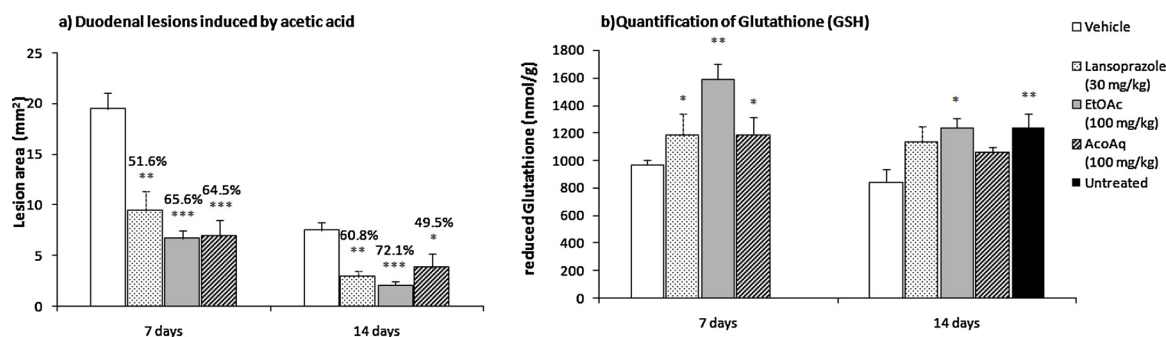
Fig. 5. Healing effect of the EtOAc and AcoAq partitions of *Byrsonima intermedia* administered for 7 or 14 days after duodenal injury induced by acetic acid in rats (*n* = 7–8 per group). The bars represent mean ± S.E.M. a) duodenal lesions induced by acetic acid. b) glutathione quantification. ANOVA followed by Dunnett's test; * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 when compared to vehicle. The numbers indicate the percentage of healing in relation to the control group treated with vehicle.

maintained gastroprotective effects against ethanol, but EtOAc showed a significant increase in gastric lesions, clearly indicating the relevance of NO for the latter and demonstrating that endogenous NO exerts an exclusive action in the gastroprotective effect of EtOAc, probably related to the flavonoids and high phenolic acid content in this fraction, which are known for their antioxidant activity [11].

Another factor that contributes to the integrity of the gastric mucosa is the formation of SH compounds, which strengthen the disulfide bridges and reduce the formation of oxygen-derived free radicals related to cell protection [20,39,40]. Lowering the normal SH levels significantly impacts the mucosa, making it susceptible to the attack of ulcerogenic substances affecting the mucosa and its defensive mechanisms, thereby facilitating the formation of gastric lesions [41,42]. The role of endogenous substances, such as SH, becomes more evident when SH alkylators, such as NEM, counteract any form of gastroprotection [43].

As previously observed for the crude extract of *B. intermedia* [12], the protective effect of both partitions was abolished with a NEM pretreatment. The absence of a protective effect of both partitions were also evidenced by the significant decrease in the GSH levels compared to the groups pretreated with saline and the partitions. The role of GSH has been implicated in the protection against chemically induced lesions and in the protection of the cell from free-radical induced damage [44]. The data obtained suggested that SHs and GSH are involved in gastroprotection of these two partitions, since the administration of NEM led to a loss of gastroprotective activity.

Capsaicin, which is found in a range of tissues, including the gastrointestinal tract, has the potential to modulate metabolism *via* activation of TRPV1 receptors. Capsaicin-mediated activation of TRPV1-expressing neurons in the gastrointestinal tract promotes sympathetically mediated stimulation of brown fat, therefore increasing the metabolic rate [45].

Ruthenium red (RR - an antagonist of the TRPV receptor) is described as a functional antagonist that does not interfere with the receptor binding site but blocks the active ion channel [46–48].

Unlike the crude extract from *B. intermedia*, which exerts its gastroprotective action through the TRPV receptor [12], RR pretreatment

did not change the protective action of the AcoAq partition, excluding the TRPV receptor involvement in its mechanism of action. However, the gastroprotective effect of EtOAc was completely abolished by RR pretreatment. This result was corroborated by a significant decrease in the levels of GSH in the gastric tissue. Like endogenous NO, this result demonstrates that TRPV receptors are involved in the gastroprotective effect of EtOAc.

Low doses of NSAIDs are clinically used for the prevention of cardiovascular and cerebrovascular events in our aging society [49]. Nevertheless, it is well known that NSAIDs have side effects in the gastrointestinal system, caused primarily by a decrease in the levels of prostaglandins (PGs) in the gastrointestinal mucosa [50]. NSAIDs cause significant gastric mucosal damage and oxyradical production and enhance gastric motility, resulting in an increase in mucosal permeability, neutrophil infiltration and MPO activity [50]. The frequent use of these drugs causes mucosal lesions not only in the stomach and duodenum but also in the lower small intestine in humans. Edogawa *et al.* [51] described that small intestinal mucosal injuries have been recognized as common complications associated with NSAID use. Antisecretory drugs, such as the histamine H_2-receptor antagonists, are commonly used for the treatment of peptic ulcers, but these drugs have been thought to be ineffective against NSAID-induced injury [52].

We observed that NSAIDs caused an increase in gastric mucosal lesions, MPO activity and decreased GSH content of the gastric tissue in animals treated with vehicle compared to the untreated group. The EtOAc partition did not protect against NSAID-induced injury. In contrast, the group pretreated with AcoAq demonstrated gastroprotective effects against NSAIDs followed by a significant decrease in MPO activity.

Considering the different phytochemical compositions of the partitions and the presence of oligomeric proanthocyanidins in the AcoAq composition, it is probable that these compounds are directly related to the prevention of NSAID-induced gastric lesions. Kim *et al.* [53] have already shown the gastroprotective effect of proanthocyanidins against NSAID-induced gastric injury in rats. Gil-Cardoso *et al.* [54] also provided evidence that proanthocyanidins ameliorate gastric injury by reducing intestinal mucosal inflammation due to a decrease in the MPO
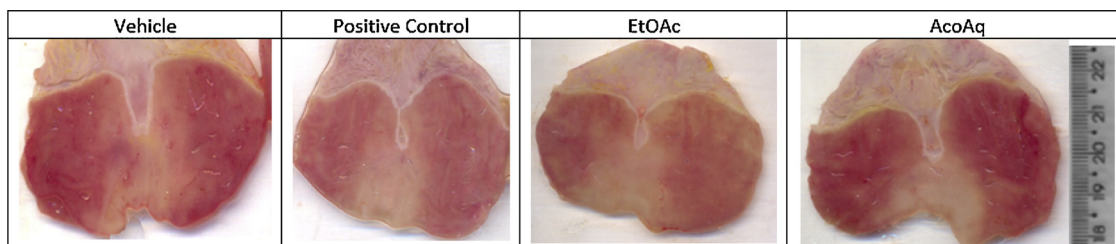

Fig. 6. Macroscopic photograph of rat stomachs treated for 6 days with EtOAc and AcoAq partitions of *Byrsonima intermedia* on gastric lesions induced by I/R.

Table 3
Healing effect of EtOAc and AcoAq partitions of *Byrsonima intermedia* on gastric lesions induced by ischemia and reperfusion followed by treatment for 1 or 6 days.

Days of treatment after I/R	Treatment (p.o.)	n	Dose (mg/kg)	Gastric lesion (mm²)	Inhibition (%)	GSH (nmol/g tissue)	MPO (unit/g tissue)	TNF-a (pg/mL)	IL-1β (pg/mL)	IL-10 (pg/mL)
1	Vehicle	7	–	120.9 ± 22.2	–	964.8 ± 83.4	8457.0 ± 709.2	1269.0 ± 262.9	1307.0 ± 267.5	
	Lansoprazole	7	30	64.9 ± 22.1 *	46.3	1118.0 ± 91.8	6275.0 ± 521.2	826.0 ± 164.5	846.0 ± 141.4	
	EtOAc	7	100	130.1 ± 44.5	–	1267.0 ± 87.7	11352.0 ± 2650.0	842.7 ± 132.7	804.9 ± 82.7	
	AcoAq	8	100	83.1 ± 45.8	–	1090.0 ± 136.3	6546.0 ± 1144.0	847.4 ± 145.5	866.2 ± 139.8	
6	Vehicle	6	–	103.9 ± 9.9	–	651.4 ± 25.2	6138.0 ± 356.8	993.5 ± 94.6	993.5 ± 94.6	3651.0 ± 207.0
	Lansoprazole	6	30	42.7 ± 12.5 *	49.3	903.3 ± 40.2	4499.0 ± 438.1 *	601.6 ± 59.3 **	601.6 ± 59.3 **	3805.1 ± 214.1
	EtOAc	6	100	45.6 ± 10.1 *	56.1	1344.0 ± 154.9 *	3343.1 ± 266.2 **	501.6 ± 126.9 ***	501.6 ± 126.9 ***	7409.6 ± 568.6 ***
	AcoAq	6	100	96.1 ± 20.7	–	1330.1 ± 360.7 *	6421.0 ± 572.1	682.8 ± 48.6	682.8 ± 48.6	5487.2 ± 775.0 *
–	Untreated	5	–	–	–	1507.0 ± 126.5 **	2671.0 ± 336.9 **	513.4 ± 77.4 **	513.4 ± 77.4 **	6571.0 ± 33.6 **

Data are presented as the mean ± S.E.M. ANOVA followed by Dunnett's test (n = 5–7 per group). Ulcerative lesion: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control group treated with vehicle.

activity on high-fat/high-carbohydrate diet-induced intestinal alterations.

In addition to the action of partitions from *B. intermedia* in gastric experimental models *in vivo*, we also determined the protective effects of both partitions against duodenal lesions. Some experimental studies have shown that the administration of high doses cysteamine induces the formation of duodenal ulcers [55]. Cysteamine is able to increase gastric acid secretion and decrease the neutralization of acid in the proximal duodenum [56].

Both partitions have different profiles in the duodenum as well as the stomach. EtOAc pretreatment did not prevent duodenal injuries caused by cysteamine. In contrast, AcoAq pretreatment significantly improved the duodenal injuries, decreasing the lesion area and MPO levels. Thus, the compounds concentrated in AcoAq (mainly proanthocyanidins and gallic acid derivatives) appear to counteract the infiltration induced by neutrophils, as evidenced by the decrease in the MPO levels in both experimental models (NSAID and cysteamine). Zhang *et al.* [57] described the role of proanthocyanidins in the resolution of an acute inflammatory process, observing the effect of proanthocyanidins on MPO activity in intestinal tissues and that the effective anti-inflammatory and antioxidant property of proanthocyanidins contributes to the regulation and termination of acute inflammatory reactions.

Ischemia/reperfusion (I/R) has destructive effects on gastric mucosa because it is caused by interruption of the blood supply to an organ tissue followed by the reintroduction of blood into the affected area [25]. Ischemia is a stress-inducing gastric mucosal injury, which occurs in several clinical settings, including trauma and major surgery [58]. Reperfusion of ischemic tissues aggravates the injury process, resulting in the release of reactive oxygen species (ROS), pro-inflammatory mediators, and chemotaxis of inflammatory cells [58]. The gastric ulcerogenic response to I/R has been reported to be significantly alleviated by antioxidant compounds [59].

The current study shows that both partitions protect the gastric mucosa of rats from the oxidative damage induced during an I/R procedure. Both partitions presented different responses. Oral pretreatment with EtOAc or AcoAq significantly decreased the extent of ulcerative lesions and decreased MPO activity, but only EtOAc increased GSH content in this model. Our results show that pretreatment with EtOAc is more effective than AcoAq, since EtOAc breaks the vicious cycle that exists during I/R between the infiltration of the inflammatory cells and the formation of ROS, which is probably related to the higher antioxidant activity of EtOAc, due to the presence of flavonoids and phenolic acids. Two compounds found in the EtOAc composition have been thoroughly studied for their prevention of I/R deleterious effects. Mojzis *et. al* [60] and Rao Ch and Vijayakumar [61] described the protective effect of quercetin on I/R-induced gastric mucosal injury in rats. Mard *et al.* [62] described the protective effect against ischemia-reperfusion injury on gastric mucosa by gallic acid and found that its action was mainly mediated by reducing the protein expression of inducible nitric oxide synthase (iNOS) and caspase-3.

The anti-secretory effects (gastric juice parameters) of each partition were also evaluated, and similar to the results observed for crude extracts [12], the oral and intraduodenal treatments for each fraction decreased the gastric lesions without changing the H^+ or pH (data not shown), excluding an anti-secretory action of either of the partitions.

Based on all the results regarding the protective effect of the EtOAc and AcoAq partitions, it is possible to conclude that the EtOAc fraction (containing mainly flavonoids and phenolic acids) contains important gastroprotective compounds against gastric mucosal injury induced by ethanol and I/R, since there is a large release of ROS in both methods, which aggravates the injury process in gastric mucosa. The mechanism of action for the gastroprotective effect of EtOAc is related to the endogenous NO pathway, SH content, TRPV receptors and the increased level of GSH. All these factors are known to reduce the formation of oxygen-derived free radicals. The AcoAq partition (containing mainly

proanthocyanidins and gallic acid derivatives) also had a protective effect against gastric and duodenal mucosal injuries induced by ethanol, I/R, NSAIDs and cysteamine. These protective mechanisms involved SH compounds, increased levels of GSH and decreased MPO activity. This set of results clearly demonstrates that pretreatment with AcoAq reduces the inflammatory response induced in the gastric mucosa by NSAIDs and the duodenal injury induced by cysteamine.

In addition to the protective effect of the partitions against ulcerogenic agents, it is also desirable that the preventive effect is accompanied by healing actions in the gastric and duodenal tissues. In fact, sometimes, the protective action of a medicinal plant does not ensure its healing effect [63]. Therefore, models such as chronic ulceration induced by acetic acid are commonly used to elucidate the mechanism of ulcer healing, since it is closer to the human disease in terms of both pathological features and the healing process [64]. According to Kangwan *et al.* [65], ulcer healing is an orchestrated process of filling the mucosal defect with epithelial and connective tissue cells, which includes cell proliferation, migration, differentiation, regeneration, active angiogenesis and extracellular matrix deposition, leading to scar formation. Thus, we evaluated the effect of each fraction on the healing of gastric and duodenal ulcers induced by acetic acid.

Our results show that, due to the severity of the injury induced by acetic acid in the gastric mucosa, within 7 days of treatment, neither the *B. intermedia* partitions nor lansoprazole were able to heal the gastric lesions. However, the *B. intermedia* partitions were effective in healing the gastric ulcer compared to the control group treated with saline after the 14-day treatment, but unlike the lansoprazole effect, the healing effects from both partitions were followed by a significant increase in GSH levels. We highlight the healing effect of EtOAc (73.3%) at a dose of 100 mg/kg when compared to the gastric healing effect of lansoprazole (63%) or the effect of the crude extract (49%) at a dose of 500 mg/kg [12].

Comparisons made between the stomach and duodenum revealed that the healing effect of both partitions were greater in the duodenum. EtOAc and AcoAq were able to heal duodenal ulcers within 7 and 14 consecutive days of treatment. The healing effect of EtOAc in the duodenal tissue in both treatment periods was more pronounced than those of the crude extract (44–45%) with doses 5 times lower [12]. At the same time, the healing effect of EtOAc increased the levels of GSH in the duodenal mucosa in both treatment periods.

In addition to the healing effect of both partitions, other relevant data obtained over 14 consecutive days of treatment showed that no animals died or showed signs of toxicity, such as weight loss, thus confirming the absence of toxicity from these two partitions (Appendices).

Taken together, these results reveal the effects of the EtOAc and AcoAq partitions obtained from the crude methanolic extract of the *B. intermedia* leaves, which inhibited gastric and duodenal lesions and promoted the healing of peptic ulcers in rats. The next studies should evaluate the quality of gastric and duodenal healing promoted by the partitions as well as the angiogenic factors involved in this process.

Based on the results showing that EtOAc and AcoAq improve the inflammatory response, we investigated how the healing effect of these partitions occurred, evaluating the effect of the partitions on gastric lesions induced by I/R followed by treatment for 1 or 6 consecutive days. In addition to the healing effect in gastric lesions caused by I/R, we also evaluated the antioxidant (GSH) and anti-inflammatory (MPO, TNF-alpha, IL-1β and IL-10) parameters in the gastric mucosa.

Exposure of the gastric mucosa to I/R produces numerous gastric lesions due to the increased generation of ROS, microvascular disturbances, increased adhesion of neutrophils to the vascular endothelium, and increased expression and plasma levels of the pro-inflammatory cytokines [66,67]. I/R induces the release of ROS, which not only directly damage the cell structures but also promote the release of IL-1β and TNF-alpha, two important factors in the pathogenesis of gastric ulcer that exacerbate inflammatory reactions [68]. According to

Magierowski *et al.* [66], I/R-induced gastric lesions can progress to deeper chronic ulcerations as the time of reperfusion increases. In this study, it was clear that the I/R process resulted in elevated levels of MPO, IL-1β and TNF-alpha and reduced levels of GSH and IL-10, even after 6 days of gastric mucosal damage. The EtOAc treatment for 6 days after I/R produced very important and promising results leading to significant healing of the gastric mucosa that was better than the reference drug for ulcer treatment (lansoprazole).

Kangwan *et al.* [65] noted that, even after ulcer healing, neutrophils and macrophages persist in and beneath the regenerated epithelium. This persistent chronic inflammation in the gastric mucosa may play a key role in causing future ulcer recurrence in this same regenerated epithelium. In addition to the healing effect of EtOAc, this partition was also able to decrease the neutrophil infiltration, as evidenced by the reduced MPO levels, and inhibit the pro-inflammatory cytokines TNF-alpha and IL-1β in the gastric mucosa, thus demonstrating the anti-inflammatory effect of this partition.

According to Huang *et al.* [69], TNF-alpha plays a major role in the generalized inflammatory process by activating neutrophils, whereas IL-10 may be protective and can limit tissue damage caused by inflammation *via* the inhibition of cytokine synthesis and suppression of TNF-alpha production. Our results showed clearly that the healing effect of EtOAc, unlike lansoprazole, was followed by a 2-fold increase in the level of GSH and a significant increase in IL-10 levels when compared to the group of animals not subjected to I/R.

The findings of this study are significant in three ways. First, the preventive treatment with AcoAq is better than EtOAc, since it can prevent gastric mucosal damage against ethanol, NSAIDs, cysteamine and I/R. Second, EtOAc is more effective than AcoAq in the strengthening of the mucosal defense by the endogenous NO pathway, SH content, TRPV receptor, and the increased level of GSH, *i.e.*, all antioxidant mechanisms. Third, both partitions play a strong role in healing the gastric and duodenal mucosa, with the EtOAc exerting a better effect than AcoAq due to the antioxidant effect inducing a greater production of GSH. EtOAc also promoted anti-inflammatory activity by decreasing the MPO, TNF-alpha and IL-1β levels and increasing IL-10 levels.

Despite the promising results of the partitions compared to the crude methanolic extract from *B. intermedia*, the anti-*Helicobacter pylori* activity was not improved. Santos *et al.* [10] described the antibacterial activity of the crude extract against *Helicobacter pylori*, and our results show that both partitions were less active than the crude extract. These results suggest that the activity of *Byrsonima intermedia* is due to more than one constituent and that a synergistic action of these bioactive compounds occurs.

5. Conclusions

The gastroprotective properties of the *Byrsonima intermedia* species combine the performance of two partitions (AcoAq and EtOAc) that exert their activity through sulfhydryl groups, NO participation, total glutathione action and the activation of neuronal capsaicin sensitive cells. The compounds present in AcoAq and EtOAc effectively contribute differently to the healing of the gastric and duodenal mucosa. The ability of *B. intermedia* to heal gastric and duodenal tissues is due to an increase in the levels of reduced glutathione and IL-10 and reductions in MPO activity, TNF-alpha and IL-1β levels. These results are due to the action of the flavonoids present in the EtOAc partition and the presence of the oligomeric proanthocyanidins in AcoAq partition ensuring the healing activity of the leaves from *Byrsonima intermedia*. Despite the very promising results presented so far, the use of this medicinal plant in humans should be undertaken with cautiously, particularly when the optimal dose and duration of therapy have not yet been established.

Conflicts of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

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