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Byrsonima intermedia A. Juss.: Gastric and duodenal anti-ulcer, antimicrobial and antidiarrheal effects in experimental rodent models

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ABSTRACT

Ethnopharmacological relevance: An ethnopharmacological survey indicated that the leaves of *Byrsonima intermedia* A. Juss. (Malpighiaceae), a medicinal species commonly found in the Brazilian Cerrado, can be used against gastroduodenal disorders, such as gastric ulcers and diarrhea.
Aim of the study: The objective of this study was to evaluate the effects of a methanolic extract of *Byrsonima intermedia* (MBI) leaves on gastric and duodenal ulcers and to assess the antimicrobial and antidiarrheal effects of this extract.
Material and methods: The anti-ulcerogenic effect of MBI was investigated with different ulcerogenic agents in rodents (mice and rats), including non-steroidal anti-inflammatory drug (NSAID), HCl/ethanol, pyloric ligature, absolute ethanol, cysteamine and ischemia–reperfusion. The gastroprotective effect of MBI was assessed by analysing the volume of gastric juice, pH, total acidity, mucus, NO, sulfhydryl compound, vanilloid receptor, glutathione (GSH) levels, and myeloperoxidase (MPO) activity in the gastric and duodenal mucosa. The gastric and duodenal healing effects of MBI were also evaluated during 7 or 14 days of treatment. The antidiarrheal action (measured by intestinal motility and diarrhea induced by castor oil) and anti-bacterial action of MBI against *Staphylococcus aureus*, *Escherichia coli* and *Helicobacter pylori* were also evaluated by microdilution methods.
Results: The phytochemical profile from MBI indicated the presence of phenolic acids, flavan-3-ols, oligomeric proanthocyanidins, and flavonoids. MBI (500 mg/kg, p.o.) significantly inhibited totally gastric and duodenal lesions (69%) and healed gastric (49% on 14 days) and duodenal lesions (45% on 7 and 14 days). The MBI exert gastroprotective action by participation of endogenous sulfhydryl compounds, vanilloid receptors and increase in GSH level to effective gastric and duodenal protection. MBI also displayed curative (42%) and preventive (49%) antidiarrheal effects by involvement of opiate receptors and also antimicrobial effects *in vitro*.
Conclusions: Byrsonima intermedia leaves present gastroprotective, healing and antidiarrheal activities, supporting previous claims that its traditional use can treat gastrointestinal disorders.

1. Introduction

Byrsonima intermedia A. Juss. (Malpighiaceae) is a keystone species that plays a critical role in the maintenance of the Cerrado Biome structure (Vilas Boas, 2009). This species is popularly called "murici-pequeno" and is commonly found in the Cerrado of the city of Botucatu, São Paulo, Brazil. This medicinal species is used in popular medicine in cases of fever, skin infection, stomach ache, diarrhea and dysentery and as a diuretic and anti-asthmatic (Sannomiya et al., 2007; Rinaldo et al., 2010). Rodrigues & Carvalho (2001) have described the preparation of this medicinal plant in an infusible form as astringent against diarrhea or dysentery (3–4 cups/day). Rinaldo et al. (2010) have evaluated the difference between the methanolic extract and the infusible form from the leaves of *Byrsonima intermedia* and have shown that the extract presents higher amounts of flavan-3-ols than the infusible form per gram of leaves. Previous

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investigations regarding the chemical composition of this species have resulted in the isolation of quercetin-3-*O*-β-D-galactopyranoside, (+)-catechin, (−)-epicatechin, gallic acid, methyl gallate, quercetin-3-*O*-α-L-arabinopiranoside and amentoflavone (Sannomiya et al., 2007). Although some phytochemical studies of this species exist, no pharmacological studies have been performed to assess the anti-ulcer or antidiarrheal action of this species as a folk medicine. Therefore, the aim of the present study was to evaluate the protective and healing effects of methanolic extract from the leaves of *Byrsonima intermedia* against gastric and duodenal ulcers and to evaluate the antidiarrheal effect of this medicinal plant in a rodent experimental model.

2. Materials and methods

2.1. Drugs and chemicals

Analytical grades of the following chemicals were from Sigma Chemical Co. (St. Louis, USA) and used in this study: absolute ethanol, hydrochloric acid, (Sinth, Brazil), lansoprazole and piroxicam (Hexal, Brazil), *N*-nitro-L-arginine methyl ester (L-NAME), carbenoxolone, cimetidine, *N*-ethylmaleimide (NEM), Alcian Blue, cysteamine, castor oil, atropine, loperamide, morphine, methanol, and dichloromethane. Gallic acid, (+)-catechin, (−)-epicatechin, methyl gallate and trifluoroacetic acid (TFA) were also obtained from Sigma Chemical Co. (St. Louis, USA). 3,4-Di-*O*-galloylquinic acid, 1,3,5-tri-*O*-galloylquinic acid, 1,3,4,5-tetra-*O*-galloylquinic acid, quercetin-3-*O*-β-galactopyranoside, quercetin-3-(2″-*O*-galloyl)-*O*-β-galactopyranoside, quercetin-3-*O*-α-arabinopyranoside and quercetin-3-*O*-(2″-*O*-galloyl)-α-arabinopyranoside were isolated by the authors, and their structures were fully characterized by UV, MS, ^{1}H-NMR and ^{13}C-NMR data. The purity of each compound was determined to be higher than 98%. HPLC-grade methanol was obtained from Tedia (Phillipsburg, USA). HPLC-grade water (18 MΩ cm) was obtained using a Direct Q5 Milli-Q purification system (Millipore Co., Bedford, USA). Sep-Pak RP18 cartridges (500 mg/6 mL) for solid phase extraction (SPE) were obtained from Waters Co. (Waters, Milford, USA). In the SPE step, the MBI (10 mg) was dissolved in 1 mL of methanol and applied to a Sep-Pak RP18 cartridge that had been preconditioned with methanol (2 × 6 mL). The cartridge was eluted with methanol (6 mL) to remove chlorophyll, and the effluent was collected and evaporated under a nitrogen stream. The solid obtained was re-dissolved in methanol/water (1:1, v/v), filtered through a 0.45 μm nylon filter membrane (Sigma–Aldrich, St. Louis, USA), and aliquots (20 μl) were submitted to HPLC analysis. All samples and reagents were prepared immediately before use.

2.2. Plant material and extraction

Leaves of *Byrsonima intermedia* A Juss. were collected in July 2007 from Pratânia city, São Paulo, Brazil. This species was identified by Prof. J. Tamashiro from the Institute of Biology, UNICAMP, Campinas, SP, Brazil, and a voucher specimen (Ref. No. 24164) was deposited at the IBB herbarium at the UNESP-Botucatu, SP, Brazil. After collection, the plant material was shade-dried, pulverized by a mechanical grinder, passed through a 40 mesh sieve and stored at room temperature until extraction. The dried powdered plant material (0.59 kg) was extracted exhaustively with successions of methanol at room temperature (48 h). The extract was filtered and concentrated under reduced pressure at 60 °C with a rotary evaporator to generate the methanolic extract from *Byrsonima intermedia*, which was named MBI (85.2 g). Upon low-pressure evaporation of the solvent from the extract, residues were obtained (14.5% (w/w) with respect to dry powder material).

2.3. Chromatographic analyses of MBI

The MBI TLC analyses, which were performed according to the method of Wagner et al. (1984), showed positivity for flavonoids and tannins. The crude extract (10.0 g) was then suspended in H_2O and partitioned with EtOAc. The concentrated EtOAc fraction (4.0 g) was chromatographed on a gel permeation column (GPC, Sephadex LH-20, Pharmacia) (57 cm × 3 cm H × i.d.), eluted with MeOH, and yielded 195 fractions (15 mL). The fractions were analyzed by Thin Layer Chromatography (TLC) on silica gel plates eluted with a mixture of $CHCl_3$/MeOH/H_2O (80:18:2, v/v/v) and revealed either with NP/PEG reagent or with anisaldehyde/sulfuric acid solution (Wagner et al., 1984). Fractions showing similar TLC data were grouped, furnishing 17 major fractions. Fractions 48–52 (319.0 mg) were separated over a successive silica gel column (Merck), and its compounds were eluted with $CHCl_3$/MeOH (75:25, v/v) furnishing quercetin-3-*O*-β-D-galactopyranoside **1** (7.0 mg), a mixture of (+)-catechin **2** and (−)-epicatechin **3** (11.0 mg). Fractions 39–42 (74.0 mg) were processed by semi-preparative HPLC using a Phenomenex Luna RP18 (2) column (250 mm × 10 mm; H × i.d., 10 μm, flow rate: 2.0 mL/min). The mobile phase used was MeOH/H_2O (7:3) and furnished the following pure compounds: gallic acid **4** (6.0 mg), methyl gallate **5** (7.2 mg) and quercetin-3-*O*-α-L-arabinopiranoside **6** (7.0 mg). The biflavonoid amentoflavone **7** (9.0 mg) was obtained from fractions 77–94 (222 mg) by successive chromatographic columns on silica gel (Merck) eluted with $CHCl_3$/MeOH (90:10, v/v). The elucidation of the structure of the isolated compounds was determined using spectral data, such as ^{1}H, ^{13}C, HSQC, HMQC, COSY and NOESY-NMR spectra elucidation and EI-MS spectra evaluation. The NMR spectra were obtained on a Varian Inova 500 MHz.

2.4. Chromatographic profiling and identification of MBI

The chemical composition of MBI was investigated by High Performance Liquid Chromatography coupled to a Photodiode Array Detector (HPLC-PAD), using a Jasco (Tokyo, Japan) HPLC equipped with a PU-2089 quaternary solvent pump, a MD-2010 PAD and an AS-2055 autosampler. The analytical column maintained at room temperature (25 °C), was a Phenomenex Synergi Hydro RP18 (250 mm × 4.6 mm H × i.d.; 4 μm) with a Phenomenex security guard column (4.0 mm × 2.0 mm H × i.d.). 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 operation and data processing. The identification of the compounds was performed by retention time comparison, UV spectral analyses and by spiking with commercial or isolated standards under the same conditions.

2.5. Animals

Male Swiss albino mice (25–35 g) and male Wistar albino rats (150–250 g) from the Central Animal House of the UNESP were used. The animals were fed 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 fasted prior to all assays because standard drugs and MBI were always administered orally (gavage) using a saline solution (0.9% NaCl, 10 mL/kg) as the vehicle. The animals were housed in cages with raised floors of wide mesh to prevent coprophagy. All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993). The UNESP Institutional Animal Care and Use Committee approved all of the employed protocols (CEUA no. 18/05).

2.6. Evaluation of gastroprotective activity

Ethanol-induced ulcer – This experiment was performed as described by Morimoto et al. (1991). Male rats were distributed into 5 groups ($n = 8$) and fasted for 24 h prior to receiving an oral dose of the vehicle (10 mL/kg), lansoprazole (30 mg/kg) or MBI (250, 500 or 1000 mg/kg body weight). After 60 min, all groups were treated orally with 1 mL of absolute ethanol to induce gastric ulcers. After another 1 h, the animals were sacrificed, and the extent of the lesions was measured by AvSoft® Bioview Spectra, Brazil; the lesion measurements are expressed as mm^2.

NSAID-induced gastric ulcers in mice – In this model (Puscas et al., 1996), gastric lesions were induced with piroxicam (30 mg/kg, s.c.) administered to male Swiss mice ($n = 8$) after a 24 h fast. MBI (250, 500 or 1000 mg/kg body weight), cimetidine (100 mg/kg) or vehicle was orally administered 30 min before the induction of the gastric lesions. The animals were sacrificed 4 h after treatment with the ulcerogenic agent. The stomachs were removed, and gastric lesions were measured as described above.

HCl/ethanol-induced ulcer – The experiments were performed as described by Mizui and Doteuchi (1983), with modifications. Male Swiss mice ($n = 5$) were distributed into groups of 5 animals that were fasted for 24 h prior to receiving an oral dose of the vehicle (10 mL/kg), lansoprazole (30 mg/kg) or MBI (250, 500 or 1000 mg/kg body weight). After 50 min, all groups were orally treated with 0.2 mL of a 0.3 M HCl/60% ethanol solution (HCl/ethanol) for gastric ulcer induction. The animals were sacrificed 1 h after the administration of HCl/ethanol, and the gastric damage was measured as described above.

Shay ulcer – All male mice ($n = 8$–9) were randomly divided into three groups and fasted for 18 h with free access to water. Thirty minutes after oral administration of MBI (500 mg/kg, which is the lowest dose that presents significant results in all acute experiments), cimetidine (100 mg/kg) as a positive control or vehicle (10 mL/kg), pylorus ligature was performed as described by Shay (1945). Four hours later, the animals were sacrificed, the abdomen was opened, and another ligature was placed around the oesophagus close to the diaphragm. The stomach was removed and lesion was measured (mm^2), and the gastric juice volume (mL), hydrogen ion concentration (mEq/mL/4 h) and pH (unit) were recorded.

Determination of gastric secretion – The assay was performed according to the method of Shay (1945) with a few modifications. All groups of male mice ($n = 9$–10) were fasted for 18 h with free access to water. Immediately after pylorus ligature, MBI (500 mg/kg), cimetidine (100 mg/kg) as positive control or vehicle (10 mL/kg) was administered by an intraduodenal route. The animals were sacrificed 4 h later, the abdomen was opened, and another ligature was placed around the oesophagus close to the diaphragm. The stomachs were removed, the gastric lesions were measured (mm^2) and the gastric content was collected to determine the total amount of gastric juice acid (mL) and pH values (unit). Distilled water was added, and the resultant solution was centrifuged at $3000 \times g$ for 10 min. Total acid in the gastric secretion volume was determined in the supernatant by titration to pH 7.0 with 0.01 N NaOH (mEq/mL/4 h).

Assessment of mucus adhered to the gastric wall – The procedure was performed according to the methods of Rafatullah et al. (1990). After fasting for 18 h, a longitudinal incision was made below the xiphoid apophysis for the pylorus ligature in anaesthetized rats ($n = 6$–7). Vehicle (10 mL/kg), carbenoxolone (200 mg/kg) or MBI (500 mg/kg) was administered (p.o.) for 1 h before the ligature. Four hours later, the animals were sacrificed, and the glandular portion of the stomach was separated, weighed and immersed in a solution of Alcian Blue to quantify the mucus. For each sample, the absorbance at 598 nm was measured in a spectrophotometer, and the results are expressed as μg of Alcian blue/g of tissue.

Ethanol-induced gastric lesions in L-NAME-, NEM-, and Ruthenium Red-pretreated rats – The procedure was performed according to the methods of Pongpiriyadacha et al. (2003), Matsuda et al. (1999) and Morimoto et al. (1991).

Gastric mucosal lesions were induced in male Wistar rats. To investigate the involvement of SHs in the protective effect of MBI (500 mg/kg), NEM (an SH-blocker, 10 mg/kg) or vehicle was injected 30 min before the administration of MBI. To investigate the possible involvement of endogenous NO in the protective effect of MBI, L-NAME (an inhibitor of NOS, 70 mg/kg) or vehicle was administered 30 min before the administration of MBI. To investigate the possible involvement of the vanilloid receptor in the protective effect of MBI, Ruthenium Red (RR, 6 mg/kg), which has been reported to be an antagonist against capsaicin, or vehicle was injected 30 min before the administration of MBI. After 60 min, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. The animals were sacrificed 1 h after ethanol administration, the stomachs were excised, and the gastric lesions were measured as described above.

Determination of total glutathione (GSH) in gastric mucosa in NEM-pretreated rats and RR-pretreated rats – The GSH present in the samples was quantified by the method described by Anderson (1985). Stomachs obtained from the investigation of the role of sulfhydryl compounds and the involvement of the vanilloid receptor in the gastric protection experiment were cut into slices and placed in an Eppendorf containing 5% TCA at a 1:20 (w/v) proportion. The samples were homogenized in 5% TCA using a Potter cell macerator (Marconi®, Brazil). After two centrifugation steps ($2000 \times g$ and $9000 \times g$ for 5 and 10 min, respectively) at 4 °C, the supernatants were removed to quantify the glutathione through reaction with DTNB (5,5′-dithiobis(2-nitrobenzoic acid). The results were obtained as nmol/g of wet tissue.

Acute gastric mucosal lesions induced by ischemia–reperfusion in rats – Ischemia–reperfusion (I/R) erosions were produced in three groups of rats ($n = 8$–10) by the method originally proposed by Ueda et al. (1989). The rats were deprived of food but allowed access to tap water *ad libitum* for 18 h before the experiments. Briefly, under anaesthesia (0.8 mg/kg of ketamine hydrochloride and 0.4 mg/kg xilazine hydrochloride), the celiac artery was clamped with a small clamp for 30 min. Then the clamp was removed, and reperfusion occurred for 60 min. Vehicle (10 mL/kg), lansoprazole (30 mg/kg) or MBI (500 mg/kg) was administered orally 60 min prior to the experiments. The untreated group comprised animals that were submitted to the abdominal incision, but not to I/R. After 60 min of reperfusion, the animals were sacrificed, and their stomachs were excised. The gastric mucosal injury was measured by AvSoft® Bioview Spectra, Brazil and expressed as mm^2. Then the gastric mucosa was stored at −80 °C for biochemical measurements of GSH levels and MPO activity.

Biochemical determinations from gastric mucosa after gastric ischemia–reperfusion (I/R) injury – The MPO level was measured according to the technique described by Krawisz et al. (1984). Samples were suspended in 1 mL of 50 mM phosphate buffer with 0.5% hexadecyltrimethylammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was

subsequently diluted to a final 1:20 (w/v) ratio and homogenized for 1 min with an automatic Heidolph homogenizer, sonicated for 10 s and subjected to three freeze–thaw cycles. The homogenates were then centrifuged at 7000 × *g* at 4 °C for 10 min, and the supernatants were assayed for MPO activity. The results are expressed as MPO units per gram (U/g) of wet tissue. The measurement of GSH content has already been described.

2.7. Evaluation of healing of gastric ulcers induced by acetic acid in rats

The experiments were performed according to the method described by Okabe et al. (1971) modified by Konturek et al. (1988). Six groups of male Wistar rats that were fasted for 18 h were used in this experiment ($n = 8$). Under anaesthesia, a laparotomy was performed on all animals through a midline epigastric incision. After exposing the stomach, 0.05 mL (v/v) of 80% acetic acid solution was injected into the subserosal layer in the glandular part of the anterior wall. The stomach was bathed with saline (20 °C) to avoid adherence to the external surface of the ulcerated region. The abdomen was then closed, and all of the animals were fed normally. All treatments, including MBI (500 mg/kg body weight), lansoprazole (30 mg/kg) or vehicle (10 mL/kg), were administered orally once a day for 7 or 14 consecutive days beginning one day after surgery. On the day after the last drug administration, the rats were sacrificed and their stomachs were removed. The gastric lesions were evaluated by examining the inner gastric surface with a dissecting magnifying glass. The macroscopic ulcer area was recorded using AvSoft® Bioview Spectra, Brazil and expressed as mm^2.

2.8. Evaluation of the preventive effect of MBI on duodenal ulcers in rats

Cysteamine duodenal ulcer in rats and determination of GSH and MPO activities – Male Wistar albino rats, weighing 150–250 g were used, and food and water were available *ad libitum* throughout the assay. Duodenal ulcers were induced by two oral administrations of cysteamine hydrochloride (400 mg/kg) in saline solution at an interval of 4 h. MBI (500 mg/kg), lansoprazole (30 mg/kg), or vehicle (10 mL/kg) were administered 30 min before the first dose of cysteamine. One untreated group was used to evaluate the normal parameters of the duodenum mucosa. All of the animals were sacrificed 48 h after the first dose of cysteamine. Each duodenum was cut open along the antimeseteric side and rinsed with saline. The duodenal score was determined using the following 4-point scale: 0 = no lesion; 1 = superficial mucosal erosion; 2 = deep ulcer or transmural ulcer; and 3 = perforated or penetrated ulcer (Szabo, 1978).

2.9. Evaluation of the healing of duodenal ulcers induced by acetic acid in rats

The experiments were performed according to the method described by Okabe et al. (1971) modified by Konturek et al. (1988). Six groups of male Wistar rats (200–250 g) were deprived of food for 18 h, but had free access to water. An incision was made in the abdomen of animals under anaesthesia, and a plastic mould (4.2 mm in diameter) was placed tightly on the serosal surface of the duodenal wall, approximately 5 mm distal to the pylorus. Acetic acid (80%, 70 μl) was poured into the mould and allowed to remain for 10 s. After removal of all acetic acid, the abdomen was closed, and the animals were fed normally. All treatments were administered orally once a day for 7 or 14 consecutive days beginning one day after surgery. To assess the duodenal healing effects, the selected dose of MBI (500 mg/kg body weight), lansoprazole (30 mg/kg) or vehicle (10 mL/kg) was administered once a day to 3 groups for 7 days and to another 3 groups for 14 days. On the day after the last drug administration, the rats were sacrificed, and their duodena were removed. The duodenal lesions were evaluated by examining the inner duodenal surface with a dissecting magnifying glass. The macroscopic duodenal ulcer area was recorded and expressed as mm^2.

2.10. Antidiarrheal activity

Gastrointestinal motility (charcoal meal) in mice – Male Swiss mice ($n = 7$) were weighed and deprived of food for 6 h, but had free access to water. At time zero, the animals received vehicle (saline) as the negative control, 5 mg/kg of atropine as the positive control, or MBI at doses of 250, 500 or 1000 mg/kg by oral gavage. Thirty min after the treatments, all groups received 10 activated charcoal ((01, mL/10 g), orally) and were sacrificed 30 min later. The results were expressed as percentage of the total length of the small intestine. The distance travelled by the charcoal relative to the total length of the small intestine was calculated for each mouse to express the percentage of distance travelled and converted by arcosine (Stickney and Northup, 1959, with modifications).

Intestinal fluid accumulation – Following the method of Robert et al. (1976), male Swiss mice were divided into six groups of seven or eight animals per group and half of them were pretreated with saline and another one with naloxone – opioid antagonist (30 mg/kg, i.p.). Fifteen minutes later, groups were treated with vehicle (10 mL/kg, p.o.), morphine (10 mg/kg, i.p.), or MBI (500 mg/kg, p.o.) and one hour later, the mice received castor oil (2 mL/animal) orally. The animals were sacrificed 1 h later, and the small intestines were removed after ligation at the pyloric end and ileocaecal junction and weighed. The contents of the intestine were then expelled into a graduated tube, and the volume was measured. The intestines were re-weighed, and the differences between the full and empty intestines were calculated.

Diarrhea induced by castor oil: evaluation of preventive effect of MBI – Three groups of male Swiss mice ($n = 7$) were orally treated with vehicle (10 mL/kg), MBI (500 mg/kg body weight) or loperamide (5 mg/kg) 30 min before castor oil (0.2 mL/animal) administration. Immediately after ingesting castor oil, each animal was kept in an individual cage, whose floor was lined with blotting paper, and observed for 5 h. The following parameters were monitored: time to initial evacuation (min), evacuation classification – 1 (normal stool), 2 (semi-solid stool), and 3 (watery stool) and evacuation index (EI), expressed according to the formula: EI = 1 × (no stool 1) + 2 × (no stool 2) + 3 × (no stool 3) (Awounters, 1978, modified by Mukherjee et al., 1998).

Diarrhea induced by castor oil: evaluation of curative effect of MBI – Three groups of male Swiss mice ($n = 8$–9) were orally treated with 0.2 mL/animal with castor oil (p.o.). Then 15 min after administration of the cathartic agent, the groups were treated with vehicle (10 mL/kg), MBI (500 mg/kg body weight) or loperamide (5 mg/kg). Immediately after ingesting the different treatments, each animal was kept in an individual cage whose floor was lined with blotting paper and observed for 4 h. The same parameters as described above for the preventive evaluation (Awounters, 1978; Mukherjee et al., 1998) were monitored.

2.11. Antibacterial susceptibility test

The antibacterial properties of MBI were also tested. The bacterial strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Helicobacter pylori* ATCC 43504) were tested using a microdilution methods followed by CLSI (2006a, b), and minimal inhibitory concentration (MIC) values were determined. *E. coli* and *S. aureus* strains were inoculated on Mueller–Hinton agar

plates and incubated at 37 °C for 24 h, and *H. pylori* was inoculated on Mueller–Hinton agar plates containing 5% sheep blood and incubated at 36 °C for 3 days in a 10% CO_2 atmosphere. Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [10^8 colony-forming units (CFU)/mL] for *E. coli* and *S. aureus* and 2.0 McFarland turbidity standard for *H. pylori*. The working suspension for each microorganism was diluted 1:10, and a 100 μl volume was added to each well of a microplate. A 100 μl volume of Mueller–Hinton broth or the same volume of Mueller–Hinton broth supplemented with 10% foetal bovine serum was added to each well of the microplates of *E. coli* and *S. aureus*, and *H. pylori*, respectively. The concentrations for each extract, prepared in 2% DMSO, ranging from 0.5 to 1.000 μg/L were obtained when a 100 μl volume of the extract was transferred to the first well of each row, and serial 2-fold dilutions were performed. Amoxicillin, metronidazole, and tetracycline were used as reference antimicrobial compounds. The MICs were recorded after incubation of the microplates at 37 °C for 24 h (*E. coli* and *S. aureus*) or 36 °C for 3 days in a 10% CO_2 atmosphere (*H. pylori*), and the growth was visually examined. The absorbance was determined using an automatic microplate reader adjusted to 600 nm. The lowest concentration of the test extract that ultimately caused an inhibition of bacterial growth of more than 90% was taken as the MIC.

2.12. Statistical analysis

The results are expressed as the mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's test, or no parametric results was determined by Kruskal–Wallis followed by Dunn's test; levels of $P < 0.05$ were considered to be statistically significant.

3. Results and discussion

The HPLC-PAD phytochemical characterization showed that MBI presented several peaks that eluted in the retention time (R_t) range of 5–65 min (Fig. 1). The metabolite classes were identified with PAD scanning from 200 to 600 nm. In the chromatogram in Fig. 1, the constituents of MBI can be divided into the following three main classes: (**I**) phenolic acids and catechin derivatives, (**II**) oligomeric proanthocyanidins and (**III**) flavonoids. Based on this information and after co-injection experiments, eleven peaks were unambiguously identified as being associated with gallic acid (**1**, R_t = 15.1 min), 3,4-di-*O*-galloylquinic acid (**2**, R_t = 23.9 min), methyl gallate (**3**, R_t = 28.6 min), (+)-catechin (**4**, R_t = 29.9 min), (−)-epicatechin (**5**, R_t = 38.1 min), 1,3,5-tri-*O*-galloylquinic acid (**6**, R_t = 39.5), 1,3,4,5-tetra-*O*-galloylquinic acid (**7**, R_t = 41.7 min), quercetin-3-*O*-β-D-galactopyranoside (**8**, R_t = 56.8 min), quercetin-3-(2″-*O*-galloyl)-*O*-β-D-galactopyranoside (**9**, R_t = 57.9 min), quercetin-3-*O*-α-L-arabinopyranoside (**10**, R_t = 59.4 min) and quercetin-3-*O*-(2″-*O*-galloyl)-α-L-arabinopyranoside (**11**, R_t = 61.2 min). In addition to the monomeric flavan-3-ols found in MBI, oligomeric proanthocyanidins were suggested as the broad unresolved peak that eluted in the R_t range of 35–60 min. This result is in accordance with those obtained by Rinaldo et al. (2010), who compared the methanolic extract and infusible form from the leaves of *Byrsonima intermedia*. The extraction with methanol instead of water did not show any selectivity in the extraction process; however, the concentration of (+)-catechin and (−)-epicathechin was lower in the infusible form.

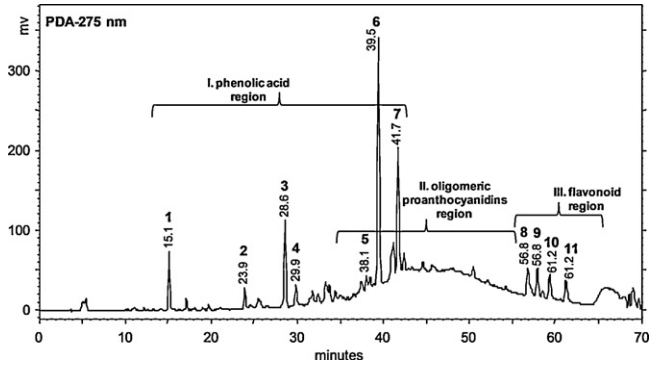

Fig. 1. Analytical HPLC-PAD chromatogram recorded at 275 nm of the compounds identified on the **MBI**. Peak identification: (**1**) gallic acid, (**2**) 3,4-di-*O*-galloylquinic acid, (**3**) methyl gallate, (**4**) catechin, (**5**) epicatechin, (**6**) 1,3,5-tri-*O*-galloylquinic acid, (**7**) 1,3,4,5-tetra-*O*-galloylquinic acid, (**8**) quercetin-3-*O*-β-galactopyranoside, (**9**) quercetin-3-(2″-*O*-galloyl)-*O*-β-galactopyranoside, (**10**) quercetin-3-*O*-α-arabinopyranoside and (**11**) quercetin-3-*O*-(2″-*O*-galloyl)-α-arabinopyranoside. For chromatographic conditions see the Materials and Methods section.

As a part of the pharmacological study of the gastroprotective effects of MBI, the extract was evaluated in experimental models of gastric ulcers induced by multiple damaging agents (Table 1). Gastric mucosal injury frequently occurs when noxious factors, such as NSAID, increased acid secretion, the presence of the bacteria *Helicobacter pylori*, ingestion of alcohol or mucosal ischemia overwhelm the mucosal defence factors (Laine et al., 2008). Among the three different doses of MBI examined in this study, the gastroprotective effect was found to be greatest at the dose of 500 mg/kg in all experimental models. The gastric damage in the group treated

Table 1
Effects of different doses from methanolic extract of *Byrsonima intermedia* (MBI) on models of gastric lesions induced in rodents.

Methods (animal)	Treatments (p.o.)	Dose (mg/kg)	*N*	Gastric lesion (mm^2)	Inhibition (%)
Absolute ethanol (rats)	Vehicle	–	8	150.5 ± 17.1	–
	Lansoprazole	30	8	65.6 ± 16.7**	56
	MBI	250	8	68.4 ± 13.9**	55
		500	8	4.2 ± 1.8**	97
		1000	8	0**	100
NSAID (mice)	Vehicle	–	8	27.2 ± 4.3	–
	Cimetidine	100	8	11.8 ± 2.6**	57
	MBI	250	8	21.3 ± 3.6	–
		500	8	12.4 ± 1.5**	54
		1000	8	9.8 ± 2.6**	64
HCl/ethanol (mice)	Vehicle	–	5	91.4 ± 7.7	–
	Lansoprazole	30	5	34.8 ± 6.6**	62
	MBI	250	5	61.4 ± 3.2**	33
		500	5	45.4 ± 6.6**	50
		1000	5	20.6 ± 3.4**	78

Results are mean ± S.E.M. ANOVA followed by Dunnett's test: gastric lesion. ** $P < 0.01$.

Table 2
Effects of cimetidine and methanolic extract of *Byrsonima intermedia* (MBI) administered orally or intraduodenally on gastric juice parameters in pylorus ligature-induced gastric lesions in mice.

Treatments	Route	Dose (mg/kg)	*N*	Gastric lesion (mm^2)	Inhibition (%)	pH (unit)	Volume (mL)	$[H^+]$ (mEq/mL/4 h)
Vehicle	p.o.	–	8	16.7 ± 2.5	–	3.5 ± 0.3	0.9 ± 0.1	10.3 ± 1.0
Cimetidine		100	8	12.0 ± 1.7*	28	5.3 ± 0.5**	1.0 ± 0.1	6.2 ± 1.0*
MBI		500	9	7.5 ± 1.1**	55	3.3 ± 0.3	1.5 ± 0.2*	7.3 ± 1.2
Vehicle	i.d.	–	10	19.1 ± 2.8	–	3.0 ± 0.3	1.1 ± 0.1	10.2 ± 0.7
Cimetidine		100	10	7.6 ± 1.3**	60	4.9 ± 0.5*	0.8 ± 0.1	6.1 ± 1.3*
MBI		500	9	6.6 ± 1.3**	66	3.7 ± 0.5	1.5 ± 0.1*	8.8 ± 1.0

Results are mean ± S.E.M. ANOVA followed by Dunnett's test: *$P < 0.05$, **$P < 0.01$

with MBI was significantly reduced by 50% (HCl/ethanol model), 54% (NSAID model) and 97% (absolute ethanol model) relative to the control groups treated with vehicle. The lower dose of MBI (250 mg/kg) did not exert a complete gastroprotective action against NSAIDs, and a statistically significant difference existed between the dose of 250 and 500 mg/kg ($P < 0.05$).

The next step of this study was the evaluation the effect of MBI on gastric juice parameters to evaluate the possible anti-secretory action of the extract. The administration of this extract showed (Table 2) that oral treatment with MBI decreased the gastric lesion (55%) and increased the gastric volume (67%) without changing the pH or H^+ concentration relative to the control group treated with vehicle. The systemic effect of gastroprotection by MBI was also observed by administration of this extract through an intraduodenal route that induced the same alterations in the gastric mucosa and gastric juice. Both results in Table 2 excluded a possible anti-secretory action of MBI, like cimetidine, and showed that MBI did not present a coating effect on gastric mucosa. Therefore, the gastroprotective activity of MBI could be related to a strengthening of the defence mechanisms of gastric mucosa, such as mucus (Laine et al., 2008). The copious quantities of mucus from the gastric mucosa are the first line of mucosal defence against noxious agents in the lumen (Abdel-Salam et al., 2001). The effect of MBI on increasing the gastric mucus production was evaluated in pylorus ligature rats. In the group treated only with carbenoxolone (positive control), the amount of mucus adhered to the gastric mucosa (12.4 ± 1.2 μg/g of tissue) in rats increased significantly ($P < 0.05$) in relation to control group. The group treated with MBI maintained the gastric mucus amount at the same level as the control group treated with vehicle (8.6 ± 1.2 and 8.5 ± 0.7 μg/g of tissue, respectively). This result also discards the effect of MBI in increasing the amount of mucus as a gastroprotective defence mechanism. In addition to mucus, other defence mechanisms were also investigated, such as endogenous NO (nitric oxide), NP-sulfhydryl compounds and the TRPV1 receptor (transient receptor potential vanilloid type 1), which are important factors that maintain mucosal integrity (Szabo and Nagy, 1992; Holzer, 2006).

Mucosal blood flow is essential for the delivery of oxygen and nutrients, removal of toxic substances and normally blood flow in the gastric mucosa is increases in response to acid secretion (Abdel-Salam et al., 2001). Gastric blood flow is mediated in part by NO, however sensory nerves are also involved in the regulation of gastric blood flow (Pawlik et al., 1998; Mártin et al., 2001; Jaworek et al., 2002). The gastric mucosa is densely innervated by capsaicin-sensitive afferent neurons containing vasodilator peptides, such as the related peptide calcitonin gene (CGRP) (Sternini et al., 1987). Capsaicin affects these nerves by two different ways; a low-doses stimulation of sensory nerves, accompanied by the release of CGRP, while high doses of capsaicin lead to ablation or functional inactivation of sensory nerves (Warzecha et al., 2000). The release of CGRP by capsaicin-sensitive nerves have also been involved in the maintenance of mucosal integrity because the ablation of functional neurons results a decrease in reactivity to CGRP in the mucosa and leads to a worsening of gastric lesions induced by various ulcerogenic compounds (Brzozowski et al., 1996; Kwiecień et al., 2002). For example, ethanol provides not only harmful effect by decreasing the barrier function of mucus, but also activates TRPV1 receptors in sensory neurons of the gastric mucosa by stimulating the release of substance P in the stomach, which in turn activates NK1 receptors of gastric epithelial cells promoting the increase of ROS (oxidative stress), that cause hemorrhagic lesions in the stomach wall due to lipid peroxidation (Gazzieri et al., 2007).

Our results showed that pretreatment of animals with L-NAME (a NO-synthase inhibitor) did not change the gastroprotective effect of MBI ($P > 0.05$) compared to the saline-pretreatment MBI group (inhibition 98% *vs.* 99%). Therefore, our results also exclude an involvement of endogenous NO in the gastroprotective effect of MBI (data not shown). However, the group of animals that was pretreated with Ruthenium Red (which blocks the TRPV receptor) and treated with extract displayed a significant increase in gastric lesions by approximately 10-fold (37.7 ± 12.5 mm^2) compared to the group treated with MBI (3.1 ± 2.9 mm^2). The protective effect of MBI was also eliminated when the group was treated with a sulfhydryl inhibitor (NEM) ($P < 0.001$). Both of these results, which are shown in Table 3, suggest that the activity of MBI may involve the TRPV receptor and the presence of SH compounds in the gastric mucosa. This gastroprotective effect of MBI demonstrated in Table 3 was also evidenced by a significant increase in the levels of total glutathione (GSH). GSH acts as an important antioxidant in the gastric mucosa, and its presence is important for the maintenance of mucosal integrity (Szabo and Nagy, 1992). The levels of GSH in the gastric mucosa tend to decrease after administration of absolute ethanol, but our results showed that pretreatment with MBI was able to inhibit this decrease in GSH ($P < 0.05$).

Ischemia also has deleterious effects on gastric mucosa and is one of the stress-inducing gastric mucosal injuries (Laine et al., 2008). The restoration of blood flow (reperfusion) after a period of ischemia initiates a cascade of changes, including the release of local of reactive oxygen species (ROS) and an increase in the adhesion of neutrophils to endothelial cells, which causes damage to the integrity of the mucosal lining; this phenomenon is known as ischemia–reperfusion (I/R) (Abdallah et al., 2009).

The gastric ulcerogenic response to I/R has been reported to be significantly mitigated by antioxidants systems (Harada et al., 2001). Our results (Fig. 2) show that gastric injury significantly increased after 60 min of reperfusion following 30 min of ischemia by clamping the celiac artery of rats. Our results show that MBI markedly protected against gastric ulceration induced by I/R (88%). This effect of MBI was not mediated by GSH because the level of GSH decreased relative to the untreated group. Kobata et al. (2007) have described a marked increase in mucosal MPO activity in the stomach after I/R, confirming the infiltration/activation of neutrophils in the gastric mucosa during I/R. However, our results show a significant decrease in MPO ($P < 0.01$), which breaks the vicious cycle that exists between the formation of ROS and the infiltration of inflammatory cells during I/R. Our results are consistent with those obtained by Orlandi et al. (2011) that also characterized the

Table 3
Effect of methanolic extract of *Byrsonima intermedia* (MBI) on gastric lesions and total glutathione level (GSH) in rats ($n = 7–8$) pretreated with NEM (SH blocker) or Ruthenium Red (RR – TRPV blocker) and treated with absolute ethanol.

Pretreatment (i.p.)	Treatment (p.o.)	Dose (mg/kg)	Ulcerative lesion (mm^2)	Gastric lesion Inhibition (%)	GSH (nmol/g)
Untreated					1070.2 ± 92.3
Saline+	Vehicle	–	212.8 ± 49.8	–	607.7 ± 46.7$^{\&}$
Saline+	Carbenoxolone	100	0.1 ± 0.1**	100	895.2 ± 48.0**
Saline+	MBI	500	4.35 ± 4.4**	98	768.1 ± 27.8*
NEM+	Vehicle	–	360.3 ± 91.9$^{\#}$	–	411.2 ± 40.1$^{\&}$
NEM+	Carbenoxolone	100	240.2 ± 101.4$^{*,\#}$	33	661.6 ± 73.2$^{\&}$
NEM+	MBI	500	147.3 ± 42.2$^{**,\#\#}$	59	486.7 ± 51.1$^{\&}$
Saline+	Vehicle	–	131.6 ± 42.4	–	711.4 ± 43.2$^{\&}$
Saline+	Capsaicin	4	1.6 ± 0.9**	99	1077.4 ± 52.2**
Saline+	MBI	500	3.0 ± 2.9**	68	991.8 ± 58.1*
RR+	Vehicle	–	151.5 ± 12.4	–	647.8 ± 61.8$^{\&}$
RR+	Capsaicin	4	103.8 ± 44.7$^{\#\#}$	31	967.3 ± 98.3*
RR+	MBI	500	37.7 ± 12.5$^{**,\#}$	75	793.3 ± 34.7$^{\&}$

Data are presented as mean ± S.E.M. ANOVA followed by Dunnett's test. Ulcerative lesion: $^{*}P < 0.05$, $^{**}P < 0.01$ represents difference in relation to control group treated with vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ represents difference in relation to pre-treatments saline + treatment and RR + treatment or saline + treatment and RR + treatment. Total Glutathione $^{\&}$ represents difference in relation to untreated group and $^{*}P < 0.05$, $^{**}P < 0.01$ represents statistical difference in relation to control group pre-treated with saline + vehicle, NEM + vehicle or RR + vehicle.

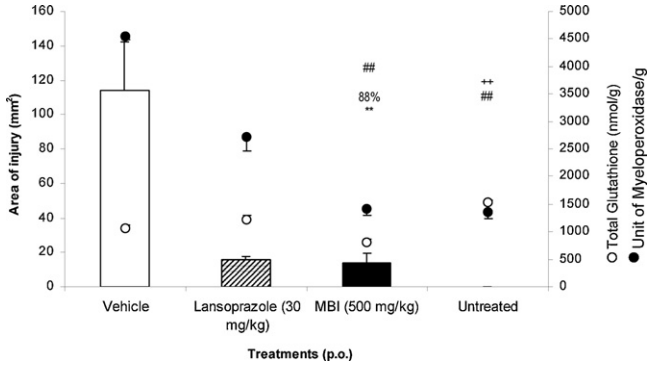

Fig. 2. Gastroprotective activity of methanolic extract of Byrsonima intermedia (MBI) on level of total glutathione and mieloperoxidase on model of gastric injury induced by ischemia/ reperfusion in rats (n = 8–10). The bars represent mean ± S.E.M. The numbers indicate the percentage protection in relation to the control group treated with vehicle. $^{++}P < 0.01$ for total gluthatione; $F_{(3,21)}$=9,70 $^{\#\#}P < 0.01$ for myeloperoxidase, ANOVA followed by Dunnett's test; $^{**}P < 0.01$.

anti-inflammatory effect of this species. The phytochemical composition of MBI (mainly flavonoids) is probably involved in this result because a study by Rao and Vijayakumar (2008) has already described the effect of (+)-catechin, the main flavan-3-ol present in MBI, as an important gastroprotective compound against gastric mucosal injury induced by ischemia–reperfusion in rats.

In addition to the protective effect of MBI in gastric experimental models *in vivo*, we also determined the protective effects of MBI against duodenal lesions induced by cysteamine in rats. Cysteamine hydrochloride inhibits alkaline mucus secretion from the Brunner's gland in the proximal duodenum and stimulates the rate of gastric acid secretion (Schwedes et al., 1977). According to Khomenko et al. (2009) animals treated with cysteamine also display a significant increase in duodenal lesions due to the generation of free radicals. Fig. 3 shows that duodenal injuries caused by cysteamine significantly decreased upon treatment with MBI relative to the group treated with vehicle. The duodenal protective effect of MBI (69%) was also accompanied by a significant decrease in MPO, a marker of neutrophil activity. Neutrophils are the major inflammatory cell type that infiltrates the injured gastroduodenal mucosa. Our results indicate that MBI counteracts this infiltration to protect the duodenal mucosa against ulcers.

Based on all of the results regarding the gastroprotective effect of MBI, the protective effect of this extract against gastric and duodenal injuries induced by different ulcerogenic agents was confirmed. However, for any new anti-ulcer drug, it is desirable for this preventive effect to also be accompanied by ulcer-healing effects in both of these tissues.

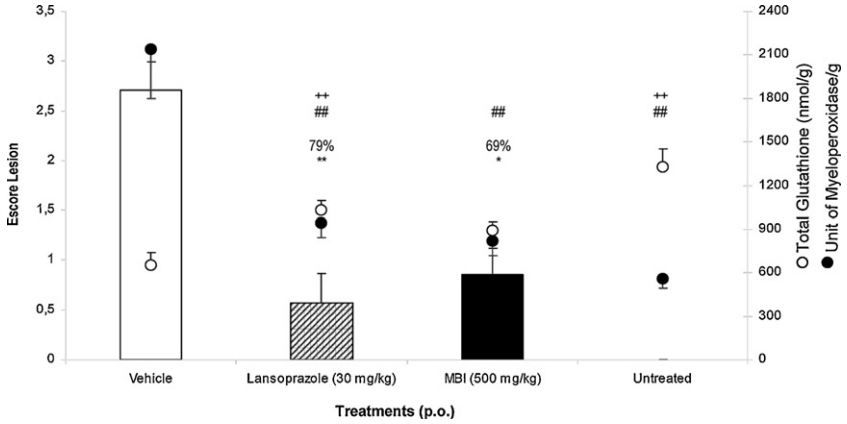

Fig. 3. Effect of methanolic extract of *Byrsonima intermedia* (MBI) on level of total glutathione and mieloperoxidase on model of duodenal injury induced by cysteamine in rats (n = 8–10). The bars represent mean ± S.E.M. The numbers indicate the percentage protection in relation to the control group treated with vehicle. ANOVA followed by Krusal/Wallis's test; $^{*}P < 0.05$, $^{**}P < 0.01$. $^{++}P < 0.01$ for total glutathione; $F_{(3,21)}$ = 9,70 $^{\#\#}p < 0.01$ for myeloperoxidase, ANOVA followed by Dunnett's test; $^{**}P < 0.01$.

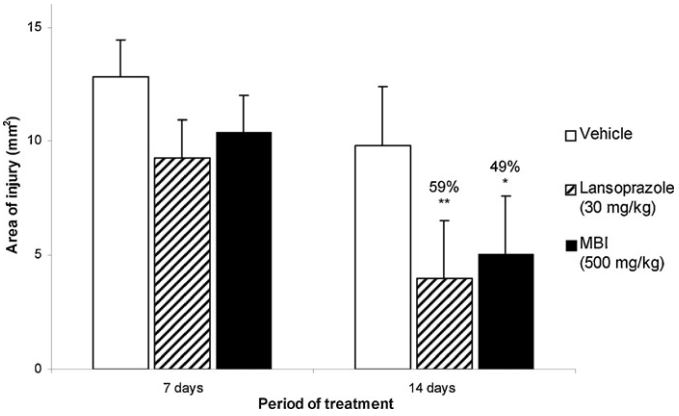

Fig. 4. Gastric healing effect of methanolic extract of *Byrsonima intermedia* (MBI) by treatment during 7 or 14 days after injury induced by acetic acid in rats (n = 8). The bars represent mean ± S.E.M.. The numbers indicate the percentage protection in relation to the control group treated with vehicle. ANOVA followed by Dunnett's; *P < 0.05, **P < 0.01.

Okabe and Amagase (2005) have elegantly described the benefit of the appearance of acetic acid ulcer models for pathophysiological and pharmacological studies of peptic ulcers. New drugs that prevent ulcer relapse and enhance ulcer healing could potentially be developed using this established method. Thus this study also determined the effect of MBI during 7 or 14 consecutive days on the healing of gastric (Fig. 4) and duodenal ulcers (Fig. 5) induced by acetic acid in rats.

Due to severity of the lesions induced by acetic acid in the gastric mucosa of rats, neither lansoprazole nor MBI were able to heal gastric lesions within 7 consecutive days (Fig. 4). However, the results in Fig. 4 also show that treatment with MBI (500 mg/kg) or with lansoprazole for 14 consecutive days were able to heal gastric ulcers by decreasing the lesion area (49% and 59%, respectively) in relation to the control group treated with vehicle. Fig. 5 shows that MBI was more effective in healing duodenal ulcers than gastric ulcers in rats. MBI was able to heal duodenal ulcers within 7 (44%) and 14 consecutive days of treatment (45%). Taken together, these results reinforce the marked effect of MBI in inhibiting gastric and duodenal lesions and in promoting the healing of a chronic type of gastric and duodenal ulcer in rats. Other important data obtained in this assay indicated that during the consecutive treatments of animals with MBI (7 or 14 days), no animals died or showed signs of toxicity from this extract, such as weight loss. However, more specific studies regarding potential *in vivo* toxicity of MBI must be performed, based on data obtained from Sannomiya et al. (2007), which describes the absence of *in vivo* mutagenic action of this extract but the presence of a mutagenic effect *in vitro*.

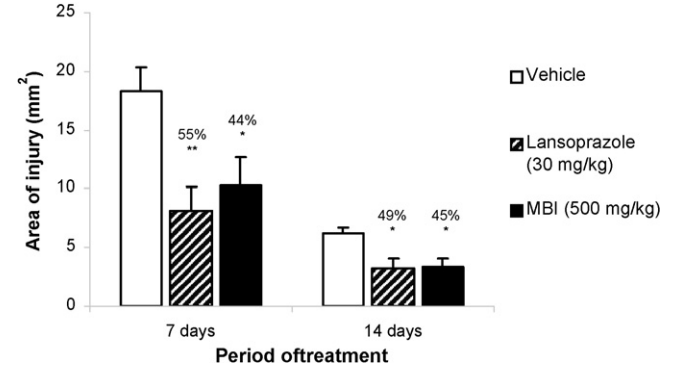

Fig. 5. Duodenal healing effect of methanolic extract of *Byrsonima intermedia* (MBI) by treatment during 7 or 14 days after injury induced by acetic acid in rats (n = 8). The bars represent mean ± S.E.M. The numbers indicate the percentage protection in relation to the control group treated with vehicle. ANOVA followed by Dunnett's; *P < 0.05, **P < 0.01.

Table 4
Effect of methanolic extract of *Byrsonima intermedia* (MBI) on intestinal propulsion induced by activated charcoal in mice.

Treatment (p.o.)	Dose (mg/kg)	*N*	Distance moved by charcoal (arcosine)
Vehicle	–	7	56.1 ± 2.6
Atropine	5	7	35.8 ± 3.8**
MBI	250	7	62.6 ± 5.0
	500	7	51.4 ± 1.9
	1000	7	52.4 ± 2.0

Data are presented as mean ± S.E.M. ANOVA followed by Dunnett's test.
** $P < 0.01$.

Additionally to proving the anti-ulcer activity of MBI, this study also aimed to evaluate the claimed antidiarrheal effect of the traditional indication of this species. The relevance of this therapeutic effect is demonstrated by intestinal infection being the most common cause of diarrhea in the world and being responsible for the deaths of approximately 4 million individuals each year (WHO, 1996).

We started to evaluate this potential effect of MBI on normal intestinal propulsion of mice with different doses of the extract (Table 4). In the MBI-treated group of mice, all doses of extract did not change the normal intestinal propulsive movement relative to the atropine group; atropine produced a greater anti-motility effect ($P < 0.05$). To evaluate the antidiarrheal effect of MBI, we challenged this extract against the cathartic agent, castor oil. Oral administration of castor oil produced a marked and significant increase in the intestinal fluid volume of castor oil-treated mice (Table 5). The standard drug (morphine) and MBI (500 mg/kg) produced a significantly inhibitory effect on the castor oil-induced fluid accumulation. These finding suggest that this extract produces diarrhea relief through its anti-enteropooling effect.

We evaluated the antidiarrheal activity of this extract by pretreament with naloxone (competitive opioid antagonist) to determine the involvement of opioid receptors at the MBI effects on management of diarrhea. Our results shown that pretreament with naloxone was able to reversed the antidiarrheal effect of MBI confirming the action MBI may occur by opioid receptors. These results are also in concordance with obtained by Orlandi et al. (2011) that found expressive antinociceptive activity of *Byrsonima intermedia* extract. In this study, the authors suggest that at least part of the anti-hyperalgesic effect was due to involvement of opioid system.

Table 5
Effects of methanolic extract of *Byrsonima intermedia* (MBI) on castor oil-induced enteropooling (fluid accumulation) in mice.

Pretreatment (i.p.)	Treatment (p.o.)	Dose (mg/kg)	N	Intestinal fluid (g)	Inhibition (%)
Saline +	Vehicle	-	7	1.5 ± 0.1	-
Saline +	Morphine	10	7	0.8 ± 0.1 **	48
Saline +	MBI	500	7	0.9 ± 0.1 **	36
Naloxone +	Vehicle	-	8	1.4 ± 0.1	-
Naloxone +	Morphine	10	8	1.4 ± 0.1 ##	0
Naloxone +	MBI	500	8	1.6 ± 0.1 ##	-14

Data are presented as mean ± S.E.M. ANOVA followed by Dunnett's. * $P < 0.05$, ** $P < 0.01$ represents difference in relation to control group treated with vehicle. ## $P < 0.01$ represents difference in relation to pretreated group with saline and naloxone.

Table 6
Preventive and curative effects of methanolic extract of *Byrsonima intermedia* (MBI) on antidiarrheal induced by castor oil in mice.

Effects	Treatment (p.o.)	Dose (mg/kg)	*N*	Time to initial evacuation (min)	Evacuation classification			Evacuation index (EI)	Inhibiton (%)[a]
					Normal	Semi-solid	Liquid		
Preventive effect	Vehicle	–	7	74.1 ± 16.3	1.7 ± 0.3	1.3 ± 0.4	3.9 ± 0.5	15.9 ± 0.9	–
	Loperamide	5	7	220.8 ± 19.2**	0.0 ± 0.0**	0.0 ± 0.0	0.5 ± 0.5**	1.5 ± 1.3**	91
	MBI	500	7	167.0 ± 30.8*	2.4 ± 0.2	1.6 ± 0.4	0.9 ± 0.6*	8.1 ± 1.9*	49
Curative effect	Vehicle	–	8	71.1 ± 5.7	1.5 ± 0.3	0.9 ± 0.3	5.0 ± 0.4	18.3 ± 1.2	–
	Loperamide	5	9	187.0 ± 28.7**	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.2***	3.8 ± 0.9***	79
	MBI	500	9	103.4 ± 18.8	1.8 ± 0.4	1.1 ± 0.2	2.2 ± 0.4*	10.7 ± 0.7*	42

Data are presented as mean ± S.E.M. ANOVA followed by Dunnett's test; *$P<0.05$, **$P<0.01$. For classification of evacuations and calculation of IE, Kruskal-Wallis followed by Dunn *$P<0.05$, **$P<0.01$. [#]Inhibition in relation to evacuation index.

In addition to the preventive effect of MBI on diarrhea induced by castor oil, we also evaluated the curative effect of this extract to reverse existing diarrhea, simulating a more realistic situation in the therapeutic use of the medicinal plant. The diarrhea inducing by castor oil is known to be due to ricinoleic acid that presents a laxative action, cause local irritation, affects intestinal motility, increases gastrointestinal mucosal permeability, induced damage of intestinal mucosa and also has pro-inflammatory action by increased prostaglandins levels and activated platelet activating factors (Izzo et al., 1998; Vieira et al., 2001; Saito et al., 2002). All rats in the control group (treated with vehicle) after castor oil administration produced copious diarrhea (Table 6). However, pre-treatment with MBI (500 mg/kg) induced a significant delay in the onset of the copious diarrhea (49%). The decrease in the severity of the diarrhea (evacuation index) was almost the same with pre-treatment with MBI (preventive effect) or post-treatment with this extract (curative effect). Treatment with MBI after or before the cathartic agent (castor oil) was able to produce a significant decrease in liquid evacuation, similar to loperamide, but only MBI showed preventive effects by increasing the time to initial evacuation ($P<0.05$).

Together, all of these results show that MBI was able to both prevent and reverse diarrhea induced by the cathartic agent by decreasing the liquid faeces and intestinal fluid without changing the intestinal motility. Our phytochemical study demonstrated the presence of oligomeric proanthocyanidins in this extract and these compounds have been demonstrated antidiarrheal activity (Fischer et al., 2004). But the presence of compounds such as flavonoids in MBI with demonstrated anti-inflammatory effects also contributes to antidiarrheal action against the cathartic agent.

The antidiarrheal effects of MBI were disabled because the most common intestinal infections that induce acute watery diarrhea in approximately 80% of people are caused by *Escherichia coli* and *Staphylococcus aureus* (Hunter et al., 2010). In this study, we also evaluated the *in vitro* antibacterial activity of MBI. MBI was found to present antibacterial activity against *Helicobacter pylori* (MIC = 0.125 mg/mL). But we also evaluated the effect of MBI which the most important pathogenic agents, such as *Staphylococcus aureus* (MIC = 0.250 mg/mL) and *Escherichia coli* (MIC = 0.500 mg/mL).

4. Conclusions

The present study clearly demonstrates the gastroprotective, ulcer-healing, antibacterial and antidiarrheal action of the methanolic extract from *Byrsonima intermedia* leaves. The protective action of the extract against various ulcerogenic agents is related to the strengthening of the gastric protective barrier through the action of endogenous sulfhydryl compounds, increases in the glutathione level and activation of vanilloid receptors. The protective effect of the extract is also mediated by a reduction of the myeloperoxidase levels in gastric and duodenal mucosa. The ulcer-healing action against gastric (14 days) but mostly duodenal ulcers (7 and 14 days) demonstrated the powerful healing effect of this extract. This work also confirmed that the anti-diarrheal action of this extract is executed by decreasing diarrheal faeces and intestinal fluid formation and suggest the involvement of opiate receptors. These effects of the extract are accompanied by an antimicrobial action against the most common bacteria that cause ulcers.

Conflicts of interest

There is not conflict of interest.

Acknowledgments

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