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Evidence of gastric ulcer healing activity of *Maytenus robusta* Reissek: In vitro and in vivo studies

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ABSTRACT

Ethnopharmacological relevance: *Maytenus robusta* Reissek (Celastraceae) is traditionally used in Brazilian folk medicine to treat gastric ulcer, as a substitute for *M. ilicifolia*, which is almost extinct. The gastroprotective properties of *M. robusta* were demonstrated previously using only preventive approaches, such as acute gastric ulcer models. However, the healing effect of *M. robusta* in gastric ulcers remains unclear.

Aim of the study: The current study was carried out to investigate the healing effectiveness of *M. robusta* hydroalcoholic extract (HEMR) from aerial parts in the acetic acid-induced chronic ulcer model and to determine its effect on cell proliferation, scavenging free radicals, and inflammatory and oxidative damage.

Material and methods: To evaluate the healing properties of HEMR in vivo, chronic gastric ulcer was induced in rats by 80% acid acetic. Next, different groups of animals ($n=6$) were treated orally with vehicle (water plus 1% tween, 1 ml/kg), omeprazole (20 mg/kg), or HEMR (1–10 mg/kg), twice daily for 7 days. At the end of the treatment, the total ulcer area (mm^2) was measured and a sample of gastric tissue was taken for histological and histochemical analysis. Evaluation of GSH and LOOH levels, GST, SOD, CAT and MPO activity was also performed at the site of the lesion. In parallel, radical scavenging activity, cytoprotective effect, and cell proliferation activity in fibroblasts (L929 cells) were determined by in vitro trials. The antisecretory properties were evaluated using the pylorus ligature model in rats, and the anti-*Helicobacter pylori* activity was determined in vitro. Acute toxicity was evaluated by relative organ weight and biochemical parameters in serum. The prokinetic properties were also evaluated in mice.

Results: Oral administration of HEMR (10 mg/kg) reduced the gastric ulcer area by 53%, compared to the vehicle group (120.0 ± 8.3 mm^2), the regeneration of gastric mucosa was evidenced in histological analysis. Moreover, HEMR treatment increased gastric mucin content and reduced oxidative stress and inflammatory parameters at the site of the ulcer. In vitro, HEMR (1–1000 μg/ml) was able to scavenge free radical DPPH and promote cytoprotection against H_2O_2 in fibroblasts at 0.1–100 μg/ml. Moreover, HEMR healing properties also were confirmed by enhancement of proliferation and coverage of scratched wounds in fibroblast monolayer. However, HEMR (10 mg/kg) by the intraduodenal route did not promote changes in volume, pH, total acidity or pepsin activity in the pylorus ligature model, and HEMR up to 2000 μg/ml also did not present considerable activity against *H. pylori*. In relation to gastrointestinal motility, HEMR (10 mg/kg, p.o) did not provoke alterations. It is also important to mention that oral administration of HEMR did not produce any sign of acute toxicity in animals.

Abbreviations: ALFAC, 85% alcohol, 10% formaldehyde and 5% acetic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHT, butylated hydroxytoluene; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's modified eagle medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DMSO, N,N-dimetyl sulfoxide; DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid); ECL, enterochromaffin-like cells; EDTA, ethylenediaminetetraacetic; FBS, fetal bovine serum; FOX2, ferrous oxidation-xylenol orange; GE, gastric emptying; GSH, glutathione reduced; GST, glutathione-S-transferase; HEMR, hydroalcoholic extract of *Maytenus robusta*; HTAB, hexadecyltrimethylammonium bromide; IT, intestinal transit; LOOH, lipoperoxide; MIC, minimum inhibitory concentration; MPO, myeloperoxidase; MTT, 3,4,5-dimethylthiazolyl-2-2, 5-diphenyltetrazolium bromide; Ome, omeprazole; PAS, periodic acid of Schiff; PBS, phosphate buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TMB, 3,3′,5,5′-tetramethylbenzidine; Veh, vehicle

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Conclusions: The data here obtained show that *M. robusta* has evident ulcer healing potential, mainly through the strengthening of protective factors of gastric mucosa, such as mucus layer, antioxidant defenses and cell proliferation. Taking into account the advantages of cultivation and harvesting of *M. robusta* compared to *M. ilicifolia*, and the evidence presented here, it is plausible to conclude that hydroalcoholic extract obtained from aerial parts of *M. robusta* is an interesting source for the development of a phytotherapeutic formulation to treat gastric ulcer.

1. Introduction

Maytenus robusta Reissek (*cafezinho do mato* or *coração de bugre)* is used in traditional folk medicine to gastric ulcer (Balbach, 1980; Cunha, 2003, Niero et al., 2011). Similarly to other *Maytenus* sp, the traditional use of *M. robusta* consist on the intake of its decoction or infusion, prepared with dry and pulverized leaves, up to four times per day (Teske and Trentini, 2001). Based on this, our research group has attempted to find a scientific basis for the development of an alternative phytotherapeutic formulation prepared from *M. robusta*, given that this species is very well adapted to the South of Brazil (Niero et al., 2001). In addition to its traditional use, harvesting of *M. robusta* is favored because this species is a medium-sized tree with a dense crown, and its leaves have no thorns, unlike those of *M. ilicifolia*. It is therefore suitable for sustainable cultivation in order to obtain standardized phytotherapic preparations. Previously, the gastroprotective properties of *M. robusta* were demonstrated by Andrade et al. (2007, 2008) using only preventive approaches, such as acute gastric ulcer models. However, the healing effect of *M. robusta* in gastric ulcer remains unclear, and it is important to emphasize that confirmation of gastroprotective activity is not necessarily an indication of healing activity on existing gastric ulcers (Vasconcelos et al., 2008; Périco et al., 2015).

Gastric ulcer is a lesion characterized by necrosis, neutrophil infiltration, reduction in blood flow, increased oxidative stress, and inflammation (da Silva et al., 2013). It occurs due to an imbalance between aggressive injurious factors (pepsin, HCl) and defensive mucosa-protective factors (prostaglandins, mucus and bicarbonate barrier and adequate blood flow) (Tygat, 2011). Besides, stress (Levenstein et al., 2014), smoking, nutritional deficiencies (Duggan and Duggan, 2006), prolonged ingestion of nonsteroidal-anti-inflammatory drugs (NSAIDs) (Belaiche et al., 2002), and *Helicobacter pylori* infection (Beltrán-Anaya et al., 2014) are all relevant etiological factors for the development of gastric ulcer.

Currently the treatment of gastric ulcer is based on the inhibition of gastric acid secretion by H2-antagonists, such as ranitidine; or proton-pump inhibitors, such as omeprazole (De Vault and Talley, 2009). However, the main problem is that despite a healing rate, ulcers treated with H2-antagonists and proton pump inhibitors can present recurrence within 1 year after the end of treatment (Kangwan et al., 2014). This is mainly due to neutrophil accumulation and ROS production, resulting in an incomplete healing process (Tarnawski et al., 1990). Furthermore, side effects such as osteoporosis (Panday et al., 2014), hypergastrinemia and hyperplasia of enterochromaffin-like cells (ECL) (Sheen and Triadafilopoulos, 2011) are common in the prolonged therapy with antisecretory drugs.

In view of the above, the search for new antiulcer treatments is essential, focusing mainly on the search for agents that promote effective healing of gastric ulcer. The present study was carried out using the acetic acid-induced chronic gastric ulcer as an experimental model. This model is highly similar to human ulcers in terms of pathological aspects and healing process. Consequently, this model is an appropriate tool to evaluate the gastric ulcer healing process of different substances in the gastric tissue (Okabe and Amagase, 2005). The aim of the study was to investigate the effectiveness of healing activity of *Maytenus robusta* hydroalcoholic extract from aerial parts in the acetic acid-induced chronic ulcer model, and to determine its effect on cell proliferation, scavenging free radicals, and inflammatory and oxidative damage regulation.

2. Materials and methods

2.1. Drugs and reagents

The following substances were used: Bovine serum albumin, 2,2-diphenyl-1-picrylhydrazyl, 5,5′-dithiobis (2-nitrobenzoic acid), glutathione, MTT, omeprazole, pyrogallol, xylenol orange (all from Sigma, St. Louis, USA), absolute ethanol, acetic acid, ascorbic acid, hydrochloric acid, diethyl ether, formaldehyde, hydrogen peroxide, magnesium chloride, methanol, sodium acetate, sodium carbonate, sucrose, trichloroacetic acid (Vetec, Rio de Janeiro, RJ, Brazil), dimetilsufoxide and N,N-dimethylformamide (DMSO, Synth, Diadema, SP, Brazil), Dulbecco's Modified Eagle Medium (DMEM, Vitrocell, Campinas, SP, Brazil), and fetal bovine serum (FBS, Gibco).

2.2. Plant material and preparation of the extract

Maytenus robusta was collected at the Morro do Baú Ecological Park, Ilhota, Santa Catarina, and identified by Dr. Ademir Reis (Department of Botany, Universidade Federal de Santa Catarina). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí-SC), and identified as V.C. Filho 016.

Briefly, air-dried and ground leaves (500 g) were macerated in 70% aqueous ethanol (v/v) at room temperature for 7 days, as described previously (Andrade et al., 2007). The macerated leaves were then filtered, and the solvent was eliminated under reduced pressure. The dried material yielded 38.0 g (7.6%) of the crude hydroalcoholic extract of *M. robusta* (HEMR). The phytochemical profile of the extract used in this work was studied previously, and the main compounds present were identified as pentacyclic triterpenes (Niero et al., 2001, 2006; Andrade et al., 2008).

2.3. Animals

The ulcer experiments were conducted with female Wistar rats (2–3 months, 180–200 g). Swiss female mice (8 weeks, 20–25 g) were used in the gastric emptying and intestinal experiments. The animals were provided by the Universidade do Vale do Itajaí colony and maintained under standard laboratory conditions (12 h light/dark cycle, temperature of 22 ± 2 °C) with free access to standard pellet food (Biobase, Águas Frias/SC, Brazil) and water. The animals were deprived of food (12 h) prior to the experiments. All the experiments were performed after approval by the Institutional Animal Ethics Committee of Universidade do Vale do

Itajaí (CEUA/UNIVALI; approval number 033/14p, and were conducted in accordance with the "Principles of Laboratory Animal Care" (NIH Publication 85-23, revised 1985).

2.4. Evaluation of healing properties

2.4.1. Acetic acid-induced gastric ulcer

Chronic gastric ulcers were induced in rats by the method of Okabe et al. (1971), with slight modifications. Rats were randomized into five groups, with six rats per group. First, the animals were anaesthetized with xylazine/ketamine (7.5 mg/kg and 60 mg/kg, i.p) and a laparotomy was performed to expose the stomach. Thereafter, 500 μl 80% acetic acid (v/v) was instilled via a plastic tube of 6 mm diameter, which was applied to the serosal surface of the stomach for 1 min. The acetic acid was aspirated and the area was washed with sterile saline. The treatment period was started on the second day after ulcer induction. Animals were treated orally with vehicle (water plus 1% tween, 1 mL/kg), omeprazole (20 mg/kg, an inhibitor of gastric H^+, K^+-ATPase and positive control), or HEMR (1, 3 and 10 mg/kg), twice daily for 7 days. Omeprazole and HE were dissolved immediately before oral administration in water plus 1% tween. Animals were sacrificed on the day following the last treatments; their stomachs were removed and opened along the great curvature. The length (mm) × width (mm) of ulcer was measured using a ruler to assess the total ulcer area (mm^2).

2.4.2. Histological and histochemical analysis

The gastric ulcers induced by acetic acid in rats submitted to different treatments were located, sectioned, and fixed in ALFAC solution (85% alcohol 80°GL, 10% formaldehyde at 40% and 5% glacial acetic acid). The samples were then dehydrated with alcohol and xylene and embedded in paraffin wax, and sectioned at 5 μm for histological evaluation after hematoxylin/eosin and Periodic Acid–Schiff (PAS) staining as described by Maria-Ferreira et al. (2014). Histological analysis of the gastric sections was carried out using a stereoscopic microscope with magnification of 10 ×. Periodic acid–Schiff (PAS)-stained mucin-like glycoproteins positive pixels were quantified using ImageJ® software in a magnification of 400 ×.

2.4.3. Cell culture

The murine fibrosarcoma L929 (NCTC clone 929) cell line was purchase from the Rio de Janeiro cell bank. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 5% CO_2, with humidified atmosphere, at 37 °C. The experiments were performed when the cells had reached about 90% of confluence.

2.4.4. Cell viability assay (MTT)

L929 cells (1×10^5) were cultured in 96-well plates (in triplicates) in the presence of a vehicle (culture medium with 0.1% of DMSO), 10% DMSO (control) or *M. robusta* extract (0.1, 1 or 10 μg/mL) at 37 °C for 24 h. MTT (0.5 mg/mL) was added to cells, the cultures were incubated for 3 h at 37 °C, and the absorbance was analyzed at 570 nm after solubilization of reduced formazan crystals with pure DMSO (Chen et al., 2003). The percentage of cell viability was calculated as the ratio (Abs. sample − Abs. blank/Abs. vehicle × 100).

2.4.5. Cell proliferation activity

The proliferation ability of fibroblasts exposed to HEMR was assessed using the scratch wound assay as previously described by Balekar et al. (2012) with few modifications, which measures the expansion of a cell population on surfaces. Monolayers of L929 cells were allowed to form in a 24-well plate containing an enriched medium of DMEM/10% FBS. When nearly confluent, the plate was taken out and artificial wounds were created in the monolayers by making a linear scratch in the center of each well using the tip of a sterile 200 μL plastic pipette tip. Any cellular debris created from the scratch was removed by gently washing the wells with phosphate buffered saline (PBS).

Once ready, the scratched wounds were divided into groups. The cells were treated with a lower (0.1 μg/ml) or a high (1 μg/ml) concentration of HEMR, DMEM/5% FBS was used as negative control. The plates were then incubated at 37 °C in a humidified incubator with 5% CO_2 atmosphere. The plates were evaluated after 30 h of incubation to assess the closure of the scratched wounds. Micrographs were used to record the wound closure activity, which was captured under an inverted microscope (Olympus, CK40) with magnification of 100 ×. All the experiments were performed in triplicate.

2.5. Preparation of subcellular fractions of stomachs

The stomachs were homogenized with 200 mM potassium phosphate buffer (pH 6.5). The homogenate was used to determine the reduced glutathione (GSH) and then centrifuged at 9000*g* for 20 min. The supernatant was used to determine the activity of the enzymes: glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT); the precipitate was used to determine myeloperoxidase (MPO) activity.

2.6. Protein assay

Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (0.125–1.0 mg/mL) as standard, according to the manufacturer's instructions.

2.7. Evaluation of antioxidant properties

2.7.1. Determination of GSH levels

GSH levels in gastric mucosa were determined by the method of Sedlak and Lindsay (1968). Aliquots of tissue homogenate were mixed with 12.5% trichloroacetic acid, for 10 min, and centrifuged for 15 min at 900*g*. Thereafter, the supernatants were mixed with TRIS buffer (0.4 M, pH 8.9) and 5,5,9-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 M) and the absorbance was measured by spectrophotometry at 415 nm. The procedures were performed at 4 °C, and the individual values were interpolated into a standard curve of GSH (1.25–10 μg/mL) and expressed as mg/g of tissue.

2.7.2. Determination of GST activity

The total GST activity was determined as described by Habig et al. (1974). Briefly, reactions were carried out in the presence of supernatant aliquots, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM GSH and 100 mM potassium phosphate buffer (pH 6.5) at room temperature. The reaction of CDNB with GSH was monitored at 340 nm for 90 s. Specific activity was calculated using an extinction coefficient of 9.6/mM/cm for GSH, and the results were expressed as mmol/min/mg of protein.

2.7.3. Determination of SOD activity

SOD activity was measured based on the ability of the enzyme to inhibit the process of pyrogallol autoxidation, according to Marklund and Marklund (1974) and Gao et al. (1998). Supernatant aliquots were added to buffer solution (200 mM Tris HCl–EDTA, pH 8.5) and pyrogallol (1 mM), and then mixed for 1 min. The reaction was incubated for 20 min at room temperature, stopped with the addition of 1 N HCl and centrifuged for 4 min at 18,700*g*. The absorbance of the resulting supernatant was measured at

405 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The SOD activity was expressed as U/mg of protein.

2.7.4. Determination of CAT activity

CAT activity was defined as the amount of enzyme required to decompose 1 μmol of H_2O_2 per minute at 25 °C and pH 7.0 and measured by adding supernatant aliquots to buffer solution (200 mM Tris HCl–EDTA, pH 8.5) containing 20 mM H_2O_2, as previously described by Aebi (1984). The enzymatic activity was immediately determined spectrophotometrically at 240 nm and the results were expressed as μmol/min/mg protein.

2.7.5. Determination of lipoperoxide content (LOOH)

The lipid peroxidation content was determined using the method of Ferrous Oxidation-Xylenol Orange (FOX2), as described by Jiang et al. (1992). Briefly, 30 μl of 90% methanol was added to 100 μl of homogenate, mixed and centrifuged at 9000*g* for 20 min at 4 °C. Next, the supernatant was added to FOX2 reagent [4 mM butylated hydroxytoluene (BHT), 250 mM $FeSO_4$, 25 mM H_2SO_4 and xylenol orange at 100 mM] and incubated for 30 min at room temperature. The absorbance was determined at 560 nm and the concentration of LOOH was equalized to 1 mg of tissue. The results were expressed as mmol/mg of tissue using the extinction coefficient of 43.6/M/cm for H_2O_2, cumene hydroperoxide or butyl hydroperoxide.

2.7.6. In vitro study of radical scavenging activity-2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The free radical scavenging ability of the HEMR was evaluated by the decrease of DPPH absorbance, following Blois (1958) and Chen et al. (2004), with a few modifications. Samples of HEMR (1, 10, 100 and 1000 μg/ml) were mixed with DPPH methanolic solution (10 μg/ml). Ascorbic acid (50 μg/ml) was used as positive control, and distilled water was used as negative control. The solutions were mixed and incubated for 5 min at room temperature and the absorbance was read at 517 nm. The individual values were interpolated to a standard curve of DPPH (0–60 μM) and expressed as μM de DPPH. All the experiments were performed in triplicate.

2.7.7. Cytoprotective activity assay

In order to investigate of the protective effects of *M. robusta* extract against H_2O_2 cytotoxicity, a co-treatment protocol was used. Before treatment, the cells were seeded in DMEM containing 10% FBS in 96-well culture plates (5000 cells/well) and incubated for 24 h at 37 °C. The cells where then simultaneously incubated with different concentrations of HEMR extract (0.1, 1, 10 or 100 μg/mL) and 200 mM H_2O_2 for 2 h. Next, the medium was replaced with fresh DMEM containing 10% FBS and the cell viability was quantified by MTT (Chen et al., 2003). All the experiments were performed in triplicate.

2.8. Evaluation of gastric inflammatory parameters

2.8.1. Determination of in vivo MPO activity

Neutrophil infiltration in the ulcerated gastric mucosa was accessed by determining gastric MPO activity according to the method described by Bradley et al. (1982) and modified by De Young et al. (1989). Briefly, the precipitate obtained from the ulcer homogenate was mixed in 80 mM potassium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB), and centrifuged at 11,000*g* for 20 min at 4 °C. MPO activity in the presence of H_2O_2 and 3, 3′, 5, 5′-tetramethylbenzidine (TMB) in the supernatant was determined at 620 nm and expressed as units of milli optical density (mO.D.)/mg of protein.

2.8.2. Determination of in vitro MPO activity

The in vitro MPO activity was determined using precipitate from homogenated sample of ulcerated rats treated with vehicle, which is a sample with high MPO activity, using the protocol described above. The supernatants obtained were then incubated with HEMR (1, 10 and 100 μg/ml) at room temperature for 15 min, and the MPO activity was determined and expressed as previously described. All the experiments were performed in triplicate.

2.8.3. In vivo leukocyte migration: air pouch model

Animals were anesthetized and 10 mL of sterile air was injected subcutaneously into the dorsal region as described by Marinho et al. (2011) with few modifications. After six days, the pouch was refilled with 10 mL air. On the tenth day following the first air injection, the animals were divided into groups and received one of the following treatments by oral gavage: (1) Sham; (2) vehicle; (3) indomethacin (30 mg/kg; positive control) or (4) HEMR (10 mg/kg). After 1 h, carrageenan (1% PBS) was injected directly into the pouches. Four hours after carrageenan injection, the animals were anesthetized and sacrificed. The pouches were washed with 2 mL of ice-cold PBS, and the total leukocyte number was determined using a Neubauer chamber. Differential cell counts were performed on smears stained with May Grunwald-Giemsa.

2.9. Evaluation of antisecretory properties

2.9.1. Induction of hypersecretion by pylorus ligature in rats

Pylorus ligature was carefully performed in fasted rats under anesthesia (Shay, 1945). Briefly, to maintain the gastric content in the stomach, the pylorus was located and ligated with sutures. The animals were intraduodenally treated with vehicle (water plus 1% tween, 1 ml/kg) or HEMR (10 mg/kg) immediately after pylorus ligature. The positive control of the test was omeprazole (20 mg/kg) administered by oral route 1 h before pylorus ligature. After 4 h of pyloric ligation, the animals were euthanized, the stomachs opened, and the gastric secretion collected. Measurements of volume, pH and total gastric acidity were performed immediately after collection, as described previously (Berté et al., 2014).

2.9.2. Peptic activity

The quantification of pepsin activity was performed as described by Anson (1938) in gastric content from rats submitted to pylorus ligature and treated with vehicle (water plus 1% tween, 1 ml/kg), omeprazole (20 mg/kg) or HEMR (10 mg/kg) as described above. Briefly, 100 μl of gastric acid secretion was collected and transferred to polypropylene tubes. The samples were added to 500 μl of bovine albumin solution (5 mg/ml in 0.06 M HCl) and incubated at 37 °C for 10 min. Afterwards, the reaction was interrupted by addition of 500 μL of 10% trichloroacetic acid, the samples were centrifuged at 1500*g* for 20 min, and the supernatant was separated and alkalized with 5 ml of 0.55 M sodium carbonate. Thereafter, 500 μL of 1 N Folin reagent was added to the tubes and incubated for 30 min at room temperature. Next, 300 μl from each tube was transferred to microplates and the absorbance was determined at 660 nm. Individual values were interpolated on a tyrosine standard curve (30–1000 mmol/mL) and the results expressed as tyrosine μmol/ml/4 h.

2.10. Evaluation of prokinetic properties

Fasted female swiss mice were orally treated with vehicle (Veh: water plus 1% tween, 1 ml/kg), atropine (Atro: 3 mg/kg, s.c.) or HEMR (10 mg/kg, p.o) 30 min (s.c route) or 1 h (p.o route) prior to administration of semisolid marker solution (0.05% phenol red plus 1.5% carboxymethylcellulose), with the same volume in each animal (0.5 mL). After 20 min, the mice were euthanized, and the

stomachs and small intestine were immediately removed to measure gastric emptying and intestinal transit, respectively.

2.10.1. Evaluation of gastric emptying (GE)

GE was measured as the amount of marker that remained in the stomach at the end of the experiment. The stomachs were individually homogenized with 7 mL of distilled water and centrifuged at 1500*g* for 20 min. Next, 150 μL of supernatant was added to 150 μL of 0.01 M NaOH, and the absorbance was measured at a 560 nm. GE was expressed as a percentage and calculated as follows: $GE\% = 100 - (X \times 100/Y)$, where X is the absorbance of marker solution recovered from the stomach of mice euthanized 20 min after the administration of marker, and Y is the mean ($n=6$) of the absorbance of marker recovered from the stomachs of zero control mice (an animal euthanized at 0 min following administration of the marker).

2.10.2. Evaluation of intestinal transit (IT)

The small intestine was dissected from the pylorus to the ileocaecal junction to measure IT. The total length of the small intestine and the distance covered by phenol red solution were then measured. IT was expressed as a percentage, calculated as follows: $IT = X/Y \times 100$, where X is the distance traveled by phenol red and Y is the total length of the small intestine.

2.11. Evaluation of toxicity

During the treatment period of the acetic acid-induced gastric ulcer experiment, the possible toxic effects of HEMR were evaluated, using parameters such as mortality and changes in body mass. At the end of this period, the animals were sacrificed and the adrenal gland, heart, kidney, liver, lung, spleen, ovaries and uterus were removed and weighed. Thereafter, relative organ weight [(organ weight/body weight) × 100] was reported. In addition, biochemical parameters (alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and urea levels) were evaluated in serum samples using a commercial kit (Bioclin/Quibasa, Belo Horizonte, MG, Brazil).

2.12. Assessment of anti-helicobacter pylori activity

The Minimum Inhibitory Concentration (MIC) was determined by the agar dilution solid method, according to the recommendations of the Clinical Laboratory Standards Institute (CLSI-Clinical and Laboratory Standards Institute, 2006) and previously described by Mégraud and Lehours (2007). From stock extract, solutions of 40 mg/mL were serially diluted. In individual glasses, 50 μL of each dilution was added to 950 μL of *Brucella* agar supplemented with 10% sheeps' blood, with 45–50 °C of fluid, reaching concentrations of 2000; 1000; 500; 250; 125; 62.5; 31, 25 and 15.625 μg/ml. The bacterial inoculums were prepared based on a scale of 0.5 MacFarland turbidity. After the medium solidification, 1 μL of bacterial suspension was seeded in each glass with the diluted extract agar. It was incubated in humidity and microaerophilic optimal conditions, at 35 °C, for 48–72 h. The MIC was defined as the lowest the concentration of fractions capable of completely inhibiting bacterial growth. All the experiments were performed in triplicate.

2.13. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) with 6 animals per group. Statistical significance was determined using one- or two-way analysis of variance (ANOVA), when applicable, followed by Bonferroni's test using Graph-Pad 5.0 software (GraphPad software, San Diego, CA, USA). Differences were considered to be significant when $p < 0.05$.

3. Results and discussion

Acetic acid-induced chronic gastric ulcer was used as an experimental model to determine the gastric healing effect of the hydroalcoholic extract obtained from aerial parts of *M. robusta* (HEMR). This model was used because it closely resembles chronic ulcers in humans, particularly in the healing process (Okabe and Pfeiffer, 1972). In this assay, oral administration of HEMR (10 mg/kg) or omeprazole (20 mg/kg), twice daily for seven days, promoted reductions in the gastric ulcer area of 53% and 45%, respectively, when compared to the ulcerated vehicle group (120.0 ± 8.3 mm^2) (Fig 1A). Accordingly, in the tissue observation it was possible verify that the instillation of acetic acid induced extensive and deep damage in the gastric mucosa (Fig. 2A), characterized by the single-layered regenerating epithelium originating from the marginal epithelium of the ulcer and extended over the granulation tissue in the prolonged ulcer base (Fig. 2D). On the other hand, the regeneration of gastric mucosa promoted by omeprazole (20 mg/kg) or HEMR (10 mg/kg) was evidenced by contraction of the ulcer base and by regenerating glands appeared in the ulcer margin (Fig. 2E and F, respectively).

Studies of healing properties of substances or extracts are needed, even though the gastroprotective effect has been proven (Vasconcelos et al., 2008). For example, ascorbic acid prevents gastric lesions induced by ethanol (Potrich et al., 2010) or indomethacin (Koc et al., 2008) but does not promote the healing of gastric ulcers induced by acetic acid (da Silva et al., 2013; Potrich et al., 2010) therefore it is not clinically used for the treatment of peptic ulcers. Andrade et al. (2007 and 2008) demonstrated the gastroprotective activity of *M. robusta*, but healing activity was not evaluated. In this context, the confirmation of HEMR healing activity using the chronic ulcer model induced by acetic acid was important.

PAS histochemical staining is a technique used to detect the presence of macromolecules typically found in the mucus, such as glycoproteins, proteoglycans and glycogen, which protect the gastric mucosa. As observed in Fig. 3A, the PAS staining in the stomach from animals treated with HEMR (10 mg/kg) was increased by 37.6% when compared to ulcerated vehicle group (Veh: $3.7 \pm 0.5 \times 10^{12}$ pixels/field). Microscopic observations of the PAS staining in gastric mucosa from rats treated with vehicle, omeprazole (20 mg/kg) or HEMR (10 mg/kg) are represented in Fig. 3B, C and D, respectively. This finding implies that the healing effect

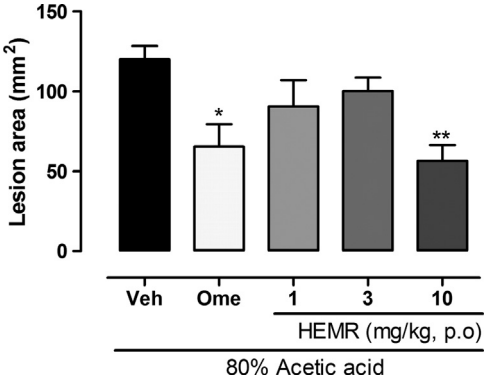

Fig. 1. Effects of HEMR on the chronic gastric ulcer induced by 80% acetic acid in rats. The animals were orally treated with vehicle (Veh: water plus 1% tween, 1 ml/kg), omeprazole (Ome: 20 mg/kg) or HEMR (1, 3 and 10 mg/kg) during 7 days. The results are expressed as mean ± S.E.M. ($n=8$) and statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni's test. $^{*}P < 0.05$ or $^{**}P < 0.01$ when compared to vehicle group (Veh).

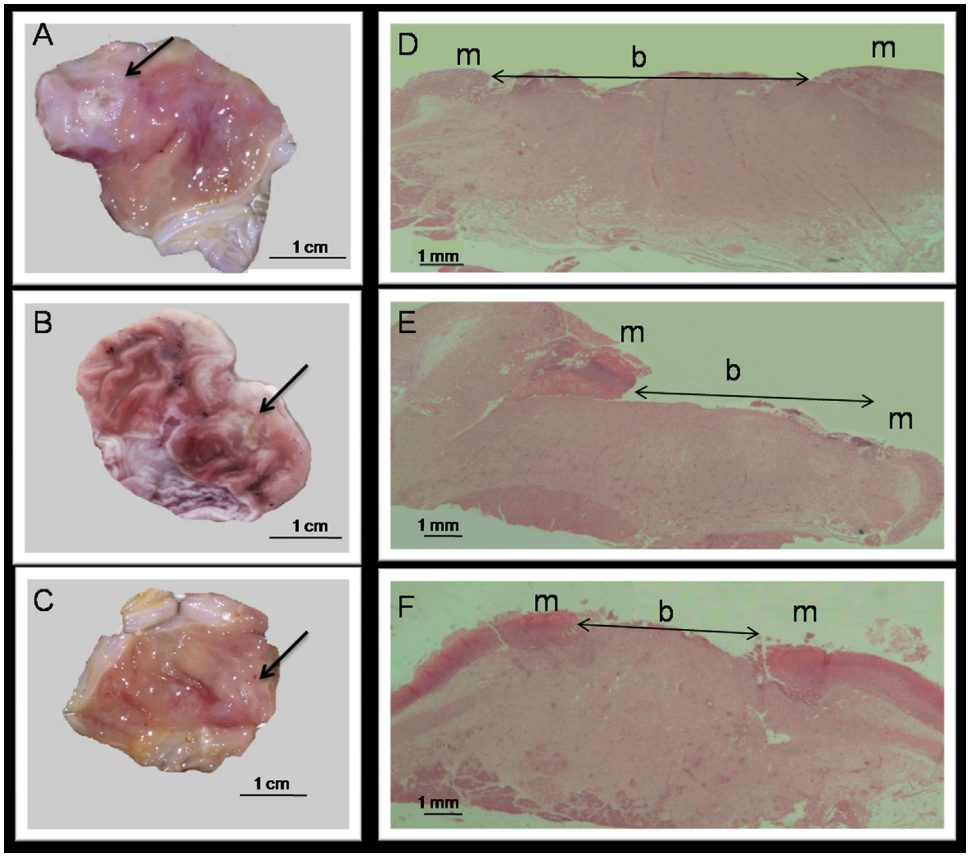

Fig. 2. Representative macroscopic photograph and histological hematoxylin/eosin appearance of site of 80% acetic acid induced-gastric ulcer. The rats were treated orally daily for 7 days with vehicle (water plus 1% tween, 1 ml/kg – panels A and D), omeprazole (20 mg/kg – panels B and E) or HEMR (10 mg/kg – panels C and F). Histological section increased by 16×, *m* indicate margin of ulcer, *b* indicate base of ulcer and arrows indicate site of the ulcer.

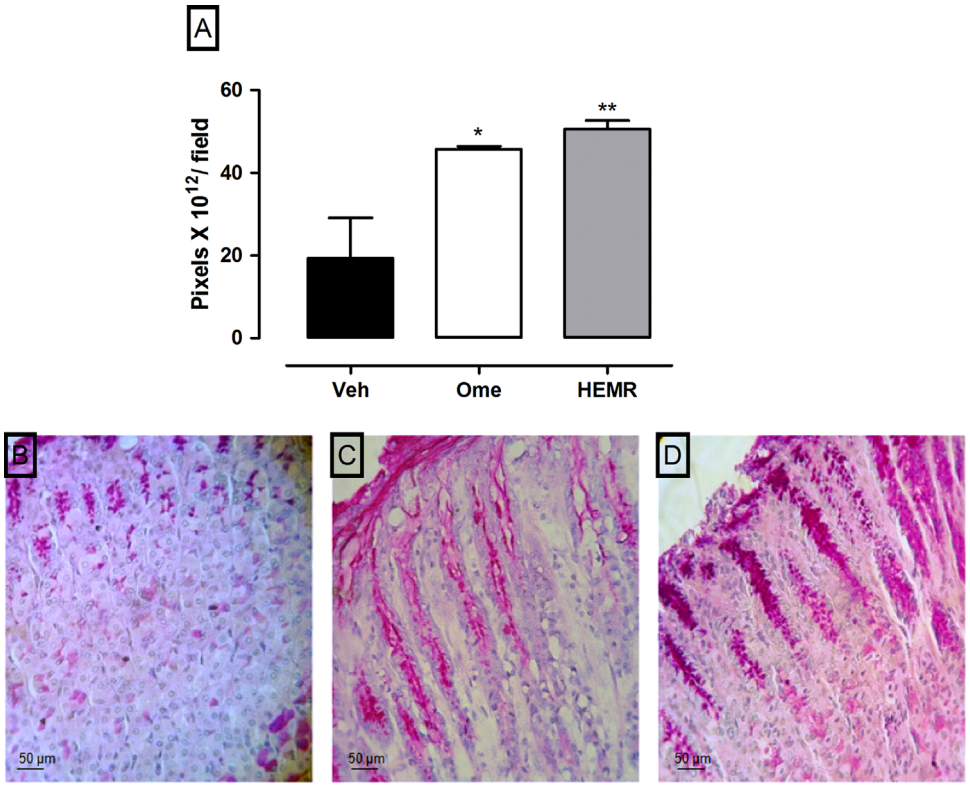

Fig. 3. Effect of HEMR on staining for mucin-like glycoproteins (PAS) in 80% acetic acid induced-gastric ulcer in rats (Panel A). Representative images of groups orally treated with vehicle (water plus 1% tween, 1 ml/kg; Panels B), omeprazole (20 mg/kg; Panels B) or HEMR (10 mg/kg; Panels C) for 7 days after. Panels B–D: magnification = 400×.

promoted by *M. robusta* is mediated by an increase in mucus layer, an important protective factor in the gastric tissue, which helps regenerate gastric mucosa, mainly through its lubricant action. Moreover, the mucus layer reduces physical abrasion in gastric mucosa and provides an effective protection against gastric acid, bacteria, luminal toxins, and other xenobiotics (Laine et al., 2008).

Gastric ulcer healing is a complex and dynamic genetically programmed process that includes cell proliferation, re-epithelialization, granulation tissue formation, angiogenesis, cell interaction, extracellular matrix deposition and tissue remodeling (Tarnawski, 2005). It is well-established that myofibroblasts are an important component of wound healing (Chai et al., 2007), responsible for extracellular matrix production, morphogenesis and the inflammatory process related to tissue repair (Hinz, 2010; Kemény et al., 2013; Mutoh et al., 2010). In this context, data obtained from previous studies showed that fibroblasts have an important role in the gastric and esophageal ulcer healing in mice and rats (Chai et al., 2007; Konturek et al., 1993; Nishida et al., 2006). In this way, murine fibroblast L929 (NCTC clone 929) cells were used as an alternative in vitro model to confirm the cell proliferation effect of HEMR. Furthermore, taking into account that L929 fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests (ISO, 1999), the cytotoxicity of HEMR was also evaluated. Based on the results obtained from the MTT assay, no cytotoxicity signal has been found after 24 h of incubation of L929 cells with HEMR (1–100 μg/ml) (Fig. 4). Furthermore, as observed in Fig. 4C, HEMR was able to cover scratched wound areas made on the L929 cell monolayer. The enhancement of proliferation and coverage of scratched wounds by HEMR at 0.1 μg/mL and 1 μg/mL was found to be 70% and 72%, respectively, compared with the negative control (Fig. 5). Fibroblast cell cultures have been proposed as a suitable method for testing wound healing activity in vitro (Abe et al., 2000), and the data obtained here reinforce the healing potential of *M. robusta*.

The generation of reactive oxygen species (ROS) and oxidative damage is crucial step in the pathogenesis of gastric ulcer (Naito et al., 2014). For this reason, we determined the ability of HEMR to act in favor of antioxidant defenses such as GSH, antioxidant enzymes (SOD and CAT), and as a ROS scavenger. Similar to what is described by Maria-Ferreira et al. (2014), we observed that acetic acid instillation promotes a decrease in GSH levels and in SOD activity by 42% and 71%, respectively, when compared with normal non-ulcerated stomach (474.5 ± 42.1 μg of GSH/g of tissue and 1.7 ± 0.3 U SOD/mg of protein) (Table 1). As expected, the acetic acid-induced ulcer markedly reduced the CAT activity and increased lipid peroxidation (an important biomarker of oxidative damage) in gastric tissues by 92% and 74%, when compared with normal values (3.1 ± 0.01 mmol de H_2O_2/min/mg of tissue and 7.3 ± 0.5 mmol LOOH/mg of tissue) (Table 1). On the other hand, treatment with HEMR (10 mg/kg) was able to restore the GSH level, SOD and CAT activity and LOOH content to similar levels to the not ulcerated group (naive group). Animals treated with omeprazole (20 mg/kg) showed an increase in CAT activity and a decrease in LOOH content to 2.0 ± 0.7 mmol/min/mg of tissue and 7.4 ± 0.4 mmol LOOH/mg of tissue, respectively, when compared with the ulcerated vehicle group (Table 1). The in vitro DPPH radical scavenging profile and cytoprotective effect against H_2O_2 (200 mM) presented by HEMR was also evaluated, and the results showed the scavenging effect of the extract at 10 and 100 μg/ml by 36% and 85%, respectively, compared to the vehicle group (53.9 ± 0.4 μM of DPPH, Fig. 6A) and highlighted the cytoprotective activity of HEMR at 0.1, 1, 10 and 100 μg/ml in L929 cells exposed to H_2O_2 (Fig. 6B). These findings clearly reinforce that the reduction in oxidative damage is correlated with the antioxidant properties of *M. robusta*, including scavenger activity. These effects can contribute to the gastric healing presented by *M. robusta*. In addition, this is the first scientific evidence of the intracellular redox status during the healing process promoved by an extract from a *Maytenus* ssp.

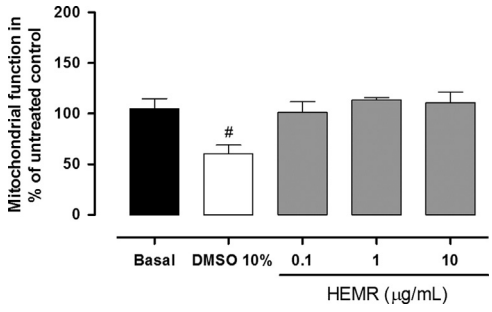

Fig. 4. Effects of HEMR on the cell viability of L929 fibroblasts as measured by MTT assay. L929 cells were treated with different concentrations of HEMR. After incubation for 24 h MTT assay was performed. The results represent mean ± SEM in triplicated experiments. Statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni's test. $^{\#}P < 0.05$ compared to basal group.

Leukocyte migration to the site of the lesion is an important event in the healing process, and neutrophils are the first cells to arrive, these cells being crucial for the onset of the gastric inflammatory process (Santin et al., 2013). However, the persistence of neutrophils in the regenerated epithelium after an incomplete healing process is a key event for ulcer recurrence (Kangwan et al., 2014). Moreover, there is plenty of evidence linking the catalytic activity of MPO, a cationic enzyme present in the azurophilic granules of neutrophils, and the neutrophil role in the oxidative stress by ROS production (Martin-Ventura et al., 2012; Pattison et al., 2012; Yoshida et al., 2014). For this reason, the MPO activity measurement is commonly used as an indirect indicator of neutrophil migration to tissue. As shown in Fig. 7A, the MPO activity was increased by 51% in damaged stomachs when compared to non-ulcerated group (Naive: 7.3 ± 1.3 mO.D./mg of protein), this result confirms the hypothesis of increased neutrophil migration in the occurrence of ulcer. However, oral administration of omeprazole (20 mg/kg) or HEMR (10 mg/kg) was able to reverse the increased myeloperoxidase activity in the stomach of acetic acid-ulcerated rats to the basal level. In view of this result, the next step was to evaluate whether this effect is mediated by neutrophil influx blockage or by direct inhibition of enzyme activity. To answer this question, a sample of homogenate obtained from ulcerated tissue (vehicle-treated animal) was incubated with vehicle or HEMR (1, 10 or 100 μg/mL) and MPO activity was measured again. Interestingly, HEMR at 10 and 100 μg/mL inhibited the MPO activity in vitro by 51% and 50% (Fig. 7B), and proved that the extract can act directly in the enzymatic reaction and therefore reduce ROS generation mediated by neutrophils. In addition, to study the effects HEMR on neutrophil trafficking, we used carrageenan-induced inflammation in the air pouch model. The results showed that HEMR treatment (10 mg/kg) does not inhibit the migration of neutrophils to the air pouch, compared to vehicle group (Veh: 89.6 ± 7.6 × 10^6 cells/mL) (Table 2). Thus, the reduction in MPO activity described above is probably related to enzyme inhibition, and not to a reduction in neutrophil migration.

Continuing with the evaluation of the healing mechanisms, we carried out hypersecretion induced by pylorus ligature. The data obtained show that HEMR (10 mg/kg, i.d.) does not promote changes in volume, pH, total acidity or pepsin activity when compared to the vehicle group (Table 3). As expected, omeprazole increased the pH to 6.7 ± 0.20 and reduced gastric volume, total acidity and peptic activity by 63%, 89% and 41%, respectively, when compared to the vehicle group (Veh: 1.5 ± 0.07 pH value;

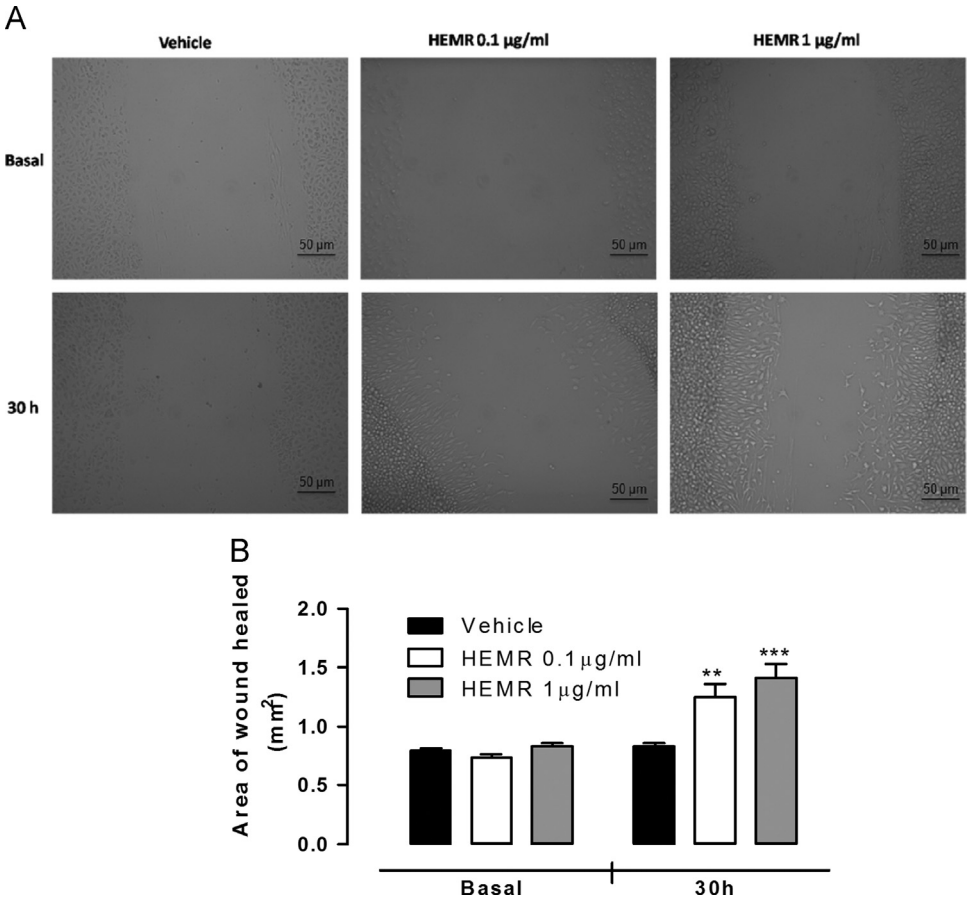

Fig. 5. Effects of HEMR on cell migration in the in vitro scratch assay. A fibroblast L929 layer subjected to scratch and treated with HEMR 0.1 µg/ml, HEMR 1 µg/ml and with vehicle. In Panel A: Images captured at 100× magnification at hour 0 and 30 after incubation. In Panel B: Measurement the rate of migration by quantifying the total distance that the cells moved from the edge of the scratch toward the center. The results represent mean ± SEM in triplicated experiments. Statistical comparison was performed using analysis of variance (two-way ANOVA) followed by Bonferroni's test. ***$P < 0.001$ and **$P < 0.01$ compared to the matched-time vehicle group.

Table 1
Effects of HEMR in GSH and LOOH levels and SOD and CAT activity.

Group	SOD (U/mg of protein)	CAT (mmol/min/mg protein)	GSH (µg/g of tissue)	LOOH (mmol/g of tissue)
Naive	1.7 ± 0.3	3.1 ± 0.01	475.0 ± 35.5	20.6 ± 1.3
Vehicle (0.1 ml/100 g, p.o.)	0.5 ± 0.1#	0.2 ± 0.09#	307.8 ± 37.8###	35.8 ± 2.9#
Omeprazole (20 mg/kg, p.o.)	0.48 ± 0.1#	2. 0 ± 0.7*	282.6 ± 24.8###	21.7 ± 1.2*
HEMR (10 mg/kg, p.o.)	1.5 ± 0.3*	1.0 ± 0.4*	452.2 ± 32.7*	24.3 ± 2.4*

Values are mean ± S.E.M. ($n=6$).
# $P < 0.01$.
$P < 0.001$ when compared with the naive group.
* $P < 0.01$ when compared with the vehicle group.

7.9 ± 0.47 mL; 72.1 ± 2.20 mEq[H^+] and 1.1 ± 0.07 µM of tyrosine/4 h) (Table 3). Thus, based on our data, we can infer that gastric acid inhibition is not related to the mechanism of gastric ulcer healing presented by HEMR at the dose evaluated. On the other hand, previous studies conducted by Andrade et al. (2007) showed that HEMR at doses of 50, 250 or 500 mg/kg present gastric acid antisecretory effect. The present study therefore shows that HEMR at a dose of 10 mg/kg displays an interesting healing effect, without interfering in the gastric secretion parameters. Considering the side effects linked to antisecretory therapy, is interesting that the extract keep its antiulcer potential in the absence of antisecretory effect. In addition, an herbal formulation that promotes gastroprotection and gastric ulcer healing and not alter gastric acid secretion can be used in parallel to classical therapies. Also, in relation to anti- *H. Pylori* activity, HEMR incubation at all the tested concentrations did not present considerable activity against this bacterium (data not shown).

Concerning the gastrointestinal motility experiments, GE and IT of semisolid phenol red after 15 min in control mice was 70.6 ± 2.2% and 64.6 ± 3.1%, respectively; oral treatment with HEMR (10 mg/kg) did not alter GE or IT. Atropine, the positive control for the test, reduced GE and IT by 24% and 68%, respectively, when compared to the vehicle group (Table 4). Thus, it was found that HEMR (10 mg/kg) did not alter gastrointestinal motility. The GE rate and IT are related to a neurohumoral mechanism, and the endogenous neurotransmitter acetylcholine (Ach) is described as the major mediator of gastrointestinal motility (Sagar et al., 2005). We can therefore infer that the change in the cholinergic pathway is not involved in the gastric healing of the extract.

Phytochemical analysis performed with extract from *M. robusta* demonstrated mainly the presence of pentacyclic triterpenes, i.e. friedelin, *β*-friedelinol, 3-oxo-21*β*-*H*-hop-22(29)-ene, 3,4-*seco*-

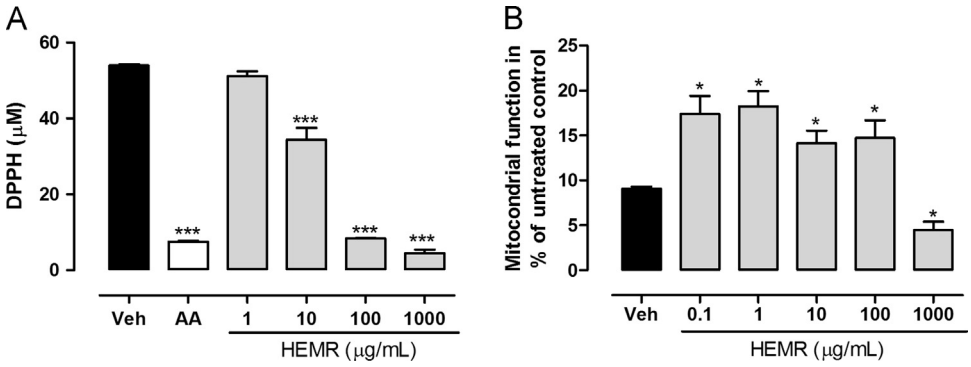

Fig. 6. Effects of HEMR on the ability to scavenge the free-radical DPPH and in to protect L929 cells against hydrogen peroxide-induced cytotoxicity. Panel A shows the scavenging of DPPH radical by HEMR (1–1000 μg/ml) or ascorbic acid (AA, 50 μg/ml) in vitro and Panel B shows the protective effect of HEMR against H_2O_2-induced cytotoxicity in L929 cells. Cells were co-treated with HEMR and 200 μM H_2O_2 for 2 h. The results are expressed as mean ± S.E.M, in triplicated experiments. Statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni's test. $^{*}P < 0.05$ or $^{***}P < 0.001$ when compared to vehicle group (Veh).

friedelan-3,11β-olide, 3β-hydroxy-21β-*H*-hop-22(29)-ene, 3,4-*seco*-21β-*H*-hop-22(29)-en-3-oic acid, 3,4-*seco*-friedelan-3-oic acid (Sousa et al., 2012), 3,15-dioxo-21alpha-hydroxy friedelane (Andrade et al., 2008; Niero et al., 2006). Pentacyclic triterpenes are secondary metabolites widely distributed in nature, and the gastroprotective and antiulcer activities are one of the most reported biological effects of these compounds. These effects are attributed to its ability to strengthen the defense factors in gastric mucosa, e.g. by stimulating mucus synthesis or maintaining the prostaglandin contents of gastric mucosa at high levels (Klein et al., 2012; Lewis and Hanson, 1991). Moreover, the antiulcer effect of 3,15-dioxo-21alpha-hydroxy friedelane isolated from hydroalcoholic *M. robusta* extract was reported by De Andrade et al. (2008). In contrast, Queiroga et al. (2000) showed that triterpenes friedelin and friedelanol was not displayed gastroprotective effect. In addition, is important to emphasize that the presence of other compounds may contribute to antiulcer effect of *M. robusta,* including steroids and flavonoids; compounds previously identified by thin layer chromatography in samples from *M. robusta* extract by Niero et al. (2001).

It is also important to mention that the oral administration of HEMR not produced any sign of acute toxicity in the animals. Over the 7 days following the oral administration HEMR (10 mg/kg), none of the animals died and no significant changes in organ weight, hepatic enzymes (AST and ALT) or renal functions (creatinine and urea) were observed at the end of this period (data not shown). Previous studies by our group demonstrated that methanol extract of *M. robusta* presents genotoxicity but not clastogenicity at 250 or 500 mg/kg, when administered orally

Table 2
Effects of HEMR in leukocyte migration.

Group	Total leukocytes (10^6/mL)	Neutrophils (10^6/mL)
Naive	0.9 ± 0.87	0.7 ± 0.5
Vehicle (1 ml/kg)	89.6 ± 7.6###	83.4 ± 4.9###
Indomethacin (30 mg/kg, p.o)	12.9 ± 5.4***	11.9 ± 1.2***
HEMR (10 mg/kg, p.o)	84.6 ± 9.2	83.4 ± 6.6

Values are mean ± S.E.M. (n=6).
$P < 0.001$ when compared with the naive group.
*** $P < 0.001$ when compared with the vehicle group.

Table 3
Effects of HEMR on gastric acid secretion.

Group	pH	Volume (mL)	Acidity (mEq [H^+])	Pepsin activity (μM of tirosin/4 h)
Vehicle (1 mL/kg, i.d.)	1.5 ± 0.07	7.9 ± 0.47	72.1 ± 2.20	1.1 ± 0.07
Omeprazole (20 mg/kg, p.o.)	6.7 ± 0.20*	2.9 ± 0.26*	7.9 ± 3.00*	0.6 ± 0.06*
HEMR (10 mg/kg, i.d.)	2.2 ± 0.34	6.6 ± 0.73	54.3 ± 9.50	0.9 ± 0.10

Values are mean ± S.E.M. (n=6).
* $P < 0.05$ when compared with the vehicle group.

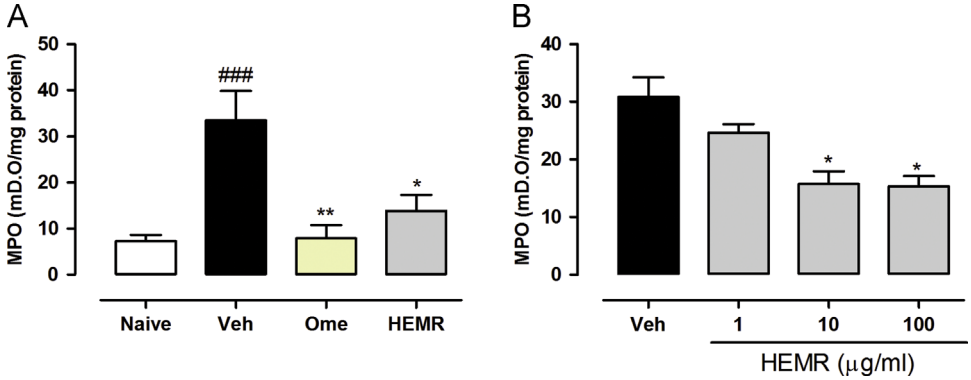

Fig. 7. Effects of HEMR on the myeloperoxidase (MPO) activity. Panel A shows the MPO activity in normal non-ulcerated rats (Naive), vehicle- (Veh, 1 ml/kg), omeprazole- (Ome, 20 mg/kg) or HEMR- (10 mg/kg) treated- rats and Panel B shows the HEMR effects in MPO activity in vitro by its incubation at 1, 10 and 100 μg/ml in a sample with high level of MPO activity. The results are expressed as mean ± S.E.M, (n=8). Statistical comparison was performed using analysis of variance (one-way ANOVA) followed by Bonferroni's test. $^{###}P < 0.001$, when compared to non-ulcerated groups (N) and $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared to vehicle group (Veh).

Table 4
Effects of HEMR on gastric emptying and intestinal transit.

Group	Gastric emptying (%)	Intestinal transit (%)
Vehicle (1 mL/kg, i.d.)	64.6 ± 3.1	70.6 ± 2.2
Atropine (3 mg/kg, s.c.)	21.0 ± 5.2*	53.7 ± 5.9***
HEMR (10 mg/kg, i.d.)	54.8 ± 5.3	69.6 ± 1.2

Values are mean ± S.E.M. ($n=6$).

* $P<0.05$ when compared with the vehicle group.
*** $P<0.001$ when compared with the vehicle group.

(Raymundo et al., 2012). However, in the same study, no genotoxic or clastogenic effects were observed at the dose of 50 mg/kg.

In conclusion, the data obtained here showed that *M. robusta* has evident ulcer healing, mainly through the strengthening of protective factors of the gastric mucosa, such as mucus layer, antioxidant defenses and cell proliferation. Taking into account the advantages in cultivation and harvesting of *M. robusta* it is plausible to conclude that hydroalcoholic extract obtained from aerial parts of *M. robusta* is an interesting source for the development of a new phytotherapeutic formulation to treat gastric ulcer.

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