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Helicteres sacarolha A. St.- Hil. et al.: gastroprotective and possible mechanism of actions in experimental animals

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ABSTRACT

Ethnopharmacological relevance: *Helicteres sacarolha* A. St.- Hil. et al. popularly known in Brazil as 'semente-de-macaco', is widely employed in the popular medicine in many of parts of Brazil in the alleviation of symptoms of ailments such as peptic ulcer and inflammation. Up to the present, there is no study addressing the gastroprotective activity of the hydroethanolic extract of *H. sacarolha* and its possible mechanism of actions.

Materials and methods: The hydroethanolic (70%) extract of *H. sacarolha* (HEHs) was obtained by maceration. The gastroprotective activity was assessed using gastric ulcer models induced by acidified ethanol, piroxicam, and water restraint stress in mice and rats at doses of 20, 50 and 250 mg/kg p.o. Mechanistic studies involved the antisecretory assay evaluated with pylorus ligation in rats and pre-treatments with appropriate antagonists/inhibitors such as yohimbine, glibenclamide, indomethacin and L-NAME, effect on catalase and myeloperoxidase activities and gastric mucus determination using acidified ethanol- induced ulcer in mice.

Results: HEHs at all doses tested demonstrated potent gastroprotective activities in the acute ulcer models. The gastroprotective activity of HEHs was attenuated by pre-treatments with yohimbine, glibenclamide, indomethacin and L-NAME. HEHs effectively reduced basal gastric juice production without any effect on the free and total acidity. The gastroprotective action of HEHs involved increasing the antioxidant enzyme catalase and mucus secretion and inhibition of neutrophyl infiltration as reflected by the reduction in the myeloperoxidase activity.

Conclusion: The results of this study gave a scientific support for the popular use of the leaves of *H. sacarolha* in the treatment of gastric ulcers and that it has a multi-targeted action.

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1. Introduction

Peptic ulcer is one of the world's major gastrointestinal disorders, embracing both gastric and duodenal ulcers, and affecting 10% of the world population (De Jesus et al., 2012). Gastrointestinal damage is a common adverse effect of nonsteroidal anti-inflammatory drugs (NSAIDs) use, resulting in outcomes ranging from nonspecific abdominal pain to ulceration, haemorrhage, and death (Beck et al., 2000). The goals of treating peptic ulcer disease are to relieve pain, heal the ulcer and prevent ulcer recurrence (Gadekar et al., 2010). In this aspect, medicinal plants are a good source of such therapeutic agents for having pleiotropic effects in many cases (Gadekar et al., 2010). In fact, herbal drugs have been proved in many cases to be very effective in the treatment and prevention of hyperacidity, gastric and duodenal ulcer (Leite et al., 2009).

The use of herbs as medicine is the oldest form of healthcare known to humanity and has been used in all cultures throughout history (Kunle et al., 2012). Moreover, the use of herbal preparations for prevention and treatment of diseases has been on dramatic increase in the Western nations in recent years. In fact with the upsurge in acceptance and public interest in natural therapies in both developing and developed countries, there is availability of these herbal remedies not only in drug stores, but now also in food stores and supermarkets (Ekor, 2014). Although, some of these plants have been scientifically proved in their efficacies, there are still many that

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are yet to be studied for their efficacies and safety (Werner, 2014). It is in the light of this that we accomplished this present study on a medicinal plant from Mato Grosso State, Brazil.

The biological and pharmacological effects of some members of the genus *Helicteres* employed in folk medicine have been confirmed. These include among others, anti-inflammatory, antioxidant, antibacterial, antiviral and antihyperglycemic activities (Pohocha and Grampurohit, 2001; Chakrabarti et al., 2002; Kumar et al., 2006, 2009; Venkatesh et al., 2007; Tambekar et al., 2008; Suthar et al., 2009; De Melo et al., 2012; Huang et al., 2013). In addition, several pharmacologically important secondary metabolites have also been isolated from some of these species (Wang and Liu, 1987; Chen et al., 1990; Venkatesh et al., 2004, 2007; Pan et al., 2008; Tambekar et al., 2008; Bhavsar et al., 2009; Suthar et al., 2009; De Melo et al., 2012; Huang et al., 2012, 2013; Loganayaki et al., 2013; Solanki and Shan, 2013).

Helicteres sacarolha A. St. – Hil. et al. is a native medicinal plant of Brazil and Bolivia (Cristóbal, 2001). It is of the Malvaceae family and belongs to the pantropical genus *Helicteres* that is comprised of approximately 60 species that are native to the tropics of both hemispheres (Goldberg, 2009). In Brazil, the plant is non endemic, and is present in phyto-geographic areas of the Amazon, Cerrado and Atlantic Forest, precisely, in North, Northeast, Midwest and Southeast of Brazil. It is popularly known in Brazil as 'semente-de-macaco', 'saca-rolhas', 'rosquinha' or 'rosquinha-de-gato' (Franceschinelli and Bawa, 2005; Balogun et al., 2014).

The medicinal use of this plant and its description were first documented by the naturalist Saint-Hilaire in 1824 in his book 'Plantes usuelles des brésiliens' (Commonly used plants of Brazil), where he stated that the decoction of the root of the plant is used in the treatment of venereal disorders (Brandão et al., 2012). Preparations from its root or leaves, in the forms of decoction, infusion or maceration are used in the Brazilian popular medicine in the alleviation of gastric ulcer symptoms, hypertension, liver problems, inflammation of the ovaries, itching and diarrhoea due to teeth eruption in children, blood depurative and in the management of women recuperating from child birth (Truiti et al., 2005; Borba and Macedo, 2006; Déborah Luíza and Guarim-Neto, 2009; Silva et al., 2010; Bieski et al., 2012).

We recently reported in our laboratory, the high safety margin of the hydroethanolic extract of *H. sacarolha* in the in vivo acute and subchronic toxicities studies in mice and rats respectively, as well as its not being cytotoxic to Chinese Hamster Ovary (CHO-k1) epithelial cells (Balogun et al., 2014). Phytochemical analysis of the hydroethanolic extract of *H. sacarolha* revealed the presence of ellagic acid, morin and naringin (Balogun et al., 2014).

Until now, there is no available information in the literature addressing the gastroprotective, and possible mechanism of action of *H. sacarolha* despite its widespread use in ethnomedicine. The present study therefore aimed to provide a scientific basis for its use in popular medicine in the treatment of gastric ulcer as well as to elucidate its possible mechanism of action using in vivo ulcer models.

2. Materials and methods

2.1. Plant material

The leaves of *H. sacarolha* used in this study were harvested in the month of March, 2013 from the Bahia de Campo Community, Poconé, Mato Grosso, Brazil. Botanical identification was done by Professor Germano Guarim Neto of Herbário UFMT and a voucher specimen (no. 40722) was deposited at the same Herbarium. The plant name was checked with www.theplantlist.org, on the 14th of August 2014. In order to access the traditional knowledge associated with genetic resources for research purposes from the traditional communities, an ethical clearance (approval no. 247) from the Brazilian Ministry of Environment, under the auspices of the Council for Genetic Heritage Management (CGEN/MMA), was obtained.

2.2. Extract preparation

Fresh leaves of *H. sacarolha* (10 kg) were cleaned and dried in an oven (Model TE-394/4; Tecnal, Brazil) at 40 °C for 72 h, pulverised in an electric miller (Model TE-625; Tecnal, Brazil) with sieved of mesh size 40. The powdered plant material was extracted by maceration in 70% hydroethanolic solution (1:10 w/v) for 7 days, to obtain the hydroethanolic extract. After extraction, the solvent was partially evaporated under reduced pressure (600 mmHg) at 40 °C in a rotary evaporator (Model 801; Fisatom, Brazil). Residual solvent was eliminated in a hot air circulating oven (Model TE-394/4; Tecnal, Brazil) at 40 °C. The extract was then lyophilised in a freeze dryer (Model LL 1500; Heto, Italy) to obtain HEHs, which was bottled in a dark flask and stored in a fridge (Model 350 L; Brastemp, Brazil) at 4 °C. The extract yield per gram of dried powdered leaves was 10%. HEHs hydrosoluble (500 mg/mL) and was dissolved in distilled water at the time of use.

2.3. Animals

Albino mice *Mus musculus*, Swiss-Webster strain (25–30 g) and rats *Rattus norvegicus*, Wistar strain (180–200 g) were used for the studies. Animals were maintained in propylene cages at 26 °C in a 12 h light-dark cycle, with free access to standard laboratory chow and water. Groups of five to six animals were used for each experiment. The experimental protocol followed the International Principles for the Biomedical Research Involving Animal (CIOMS/WHO, 2002) and was approved by the Committee on the Use of Animal for experimentation (CEUA/UFMT) with protocol no. 23108.060195/13-5.

2.4. Evaluation of gastroprotective effect in acute ulcer models

2.4.1. Gastric ulcer induced by acidified ethanol (EtOH/HCl)

The gastroprotective activity of HEHs was studied in EtOH/HCl-induced gastric ulcer. The experiment was done as previously described (Mizui and Doteuchi, 1983). Mice were distributed into groups with 6 animals that had fasted 18 h prior to receiving an oral dose of the vehicle, distilled water (10 mL/kg), carbenoxolone (100 mg/kg) or HEHs (20, 50 and 250 mg/kg body wt.). After 1 h, all groups were treated by gavage (p.o) with 0.3 mL of 60% EtOH and 0.3 M HCl solution for gastric-ulcer induction. Animals were killed by cervical dislocation 1 h after the administration of EtOH/HCl, and the stomachs excised, opened along the greater curvature and were photographed. The lesion area in each animal was measured in mm^2 and was expressed in percentage (%) in relation to the total area of corpus using ImageJ software (Khan, 2004).

2.4.2. Gastric ulcer induced by water immersion restraint stress (WRS)

Rats weighing 150–180 g were used in this study. Rats were distributed into groups of 6 animals that were fasted 20 h prior to receiving an oral dose of the vehicle, distilled water (10 mL/kg), ranitidine (50 mg/kg) or HEHs (20, 50 and 250 mg/kg). After 30 min of the treatments, all animals were subjected to WRS to induce gastric ulcerations as described by Takagi and Susumu (1968). Each animal was immobilised in an individual compartment (restraining tube of 5.5 cm × 5.5 cm × 25.5 cm dimensions), then immersed vertically to the level of the xiphoid region in a water tank containing tap water at temperature of 22 °C. Animals remained in this position for 7 h. After this period, all animals were sacrificed by cervical

dislocation, the stomachs were removed, opened along the greater curvature, gastric contents discarded and the mucosa was gently washed with cold saline. Ulceration was quantified as described in Section 2.4.1.

2.4.2.1. Histopathological evaluation of ulcerated gastric tissues. For histological evaluation, parts of the gastric tissues obtained from each animal were fixed in 10% formalin for 24 h, dehydrated in increasing grades of alcohol, clarified in xylene and embedded in paraffin. Sections (5 μm thick) were taken in the microtome HIRAX M60 (Carl Zeiss, Germany) and stained with haematoxylin and eosin (HE). All tissue sections were analysed with Axioscope A1 microscope (Carl Zeiss, Germany). The images were analysed through the Axiovision Software (Carl Zeiss, Germany) and was used for characterisation of histopathological changes. Samples were evaluated according to the modified criteria and previously reported (Li et al., 2013), by an investigator unaware of the treatments. Briefly, the damage scores were categorised as follows: a segment of 1 cm of each histological section was assessed for loss of epithelial cells (score: 0–4), oedema in the upper mucosa (score: 0–4), haemorrhagic injury (score: 0–4), and the presence of inflammatory cells (score: 0–4).

2.4.3. Gastric ulcer induced by piroxicam

The experiment was carried out as described by Puscas et al. (1997). Gastric ulcers were induced using piroxicam (100 mg/kg p.o.), administered to rats after a 24 h fast. HEHs (20, 50 and 250 mg/kg), ranitidine (50 mg/kg) or vehicle was administered orally, 1 h before the induction of gastric ulcers. The rats were sacrificed by cervical dislocation four hours after ulcer induction and their stomachs processed as described in Section 2.4.1. The length of each lesion was measured using ImageJ software and the lesion index was expressed as the sum of the length of the lesions (Bozkurt et al., 1998).

2.5. Mechanisms of gastroprotective action

2.5.1. Evaluation of anti-secretory activity in pylorus ligation model

Gastric acid secretion (pylorus-ligation ulcer, PL-ulcer) by Shay et al. (1954) method was used for this study. In rats (180–200 g) fasted for 24 h, the effects of HEHs (20, 50 and 250 mg/kg) or omeprazole (20 mg/kg) were evaluated on the basal gastric acid secretion induced by 4 h-pylorus ligation. The test drugs or vehicle (0.5 mL/100 g) were administered intraduodenally immediately after pylorus ligation. Four hours later, the rats were sacrificed by cervical dislocation. The abdomen was opened and another ligature placed around the oesophagus close to the diaphragm. The stomachs was removed, washed in cold saline, the gastric juice was collected in a graduated centrifuge tube, and the juice collected was centrifuged at 1950*g* for 10 min. The gastric juice volume was recorded after centrifuging, by taking into consideration the stomach debris. The pH of the gastric juice was measured with a digital pH meter, while the total acidity was determined by titration with 0.01 M sodium hydroxide and the results were expressed in mEq/L/4 h.

2.5.2. Gastric ulcer induced by acidified ethanol in animals pre-treated with L-NAME, glibenclamide, indomethacin and yohimbine

Separate experiments were conducted in order to examine the roles of α2-receptors, prostaglandins, nitric oxide, and activation of K^+-ATP channel in the gastroprotective effect of HEHs (20 mg/kg). Mice were treated with appropriate antagonists, or inhibitors (Leite et al., 2009).

Briefly, mice were fasted for 18 h with water ad libitum up to 1 h before the experiment was distributed into 11 groups of 6 animals each. Four groups were pre-treated before ulcer induction with L-NAME (10 mg/kg ip.), glibenclamide (5 mg/kg p.o.), yohimbine (2 mg/kg ip.) or indomethacin (10 mg/kg p.o.). Another four groups were pre-treated with these antagonists or inhibitor 30 min before receiving HEHs (20 mg/kg) by gavage. A group that received only HEHs (20 mg/kg) was also included. One hour later, all animals received 0.3 mL of EtOH/HCl to induce ulcer. One hour after ethanol treatment, all animals were sacrificed, and their stomachs were removed, opened along the greater curvature, washed in saline, and photos taken to quantify ulcer as described in Section 2.4.1.

2.5.3. Determination of gastric mucus in the EtOH/HCl ulcer in mice

The assay was performed in EtOH/HCl-induced ulcer animals according to the methodology described by Hariprasath et al. (2012). The stomachs were immersed in 10 mL 0.02% Alcian blue in 0.16 M sucrose/0.05 M sodium acetate, pH 5.8, and incubated for 24 h at 20 °C. After incubation, the stomachs were rinsed in the same solution and then the resulting solution (extract) was centrifuged at 2000 × g for 10 min. The absorbance of the supernatant was measured at 615 nm using a spectrophotometer. The binding mucus in the gastric wall was calculated from the amount of Alcian blue binding (mg/g wet tissue) using standard curve of Alcian blue.

2.5.4. Evaluation of effect of HEHs on antioxidant enzymes in mice with EtOH/HCl ulcer

2.5.4.1. Evaluation of myeloperoxidase (MPO) activity. The stomachs of animals previously subjected to EtOH/HCl-induced ulcer were used to assess the gastroprotective effect of HEHs on the mucosa, through the involvement of MPO according to the method described by De Young et al. (1989). MPO is an enzyme found primarily in the azurophilic granules of the neutrophils and it is commonly used as a marker of tissue polymorphonuclear neutrophil (PMN) content that migrate to the site of the inflammatory stimulus. The homogenate from the ulcerated stomach obtained by adding potassium phosphate buffer (200 mM) of pH 6.5 was centrifuged at 9000 × g for 20 min. The pellet obtained was resuspended with 1 mL of 80 mM potassium phosphate buffer in the presence of 0.5% hexadecyltrimethylammonium and then sonicated for 10 s. After homogenisation, samples were centrifuged at 11,000*g* for 20 min at 4 °C. In 96-well plates 30 μL of the supernatant plus 220 μL of a mix of solutions (containing 100 μL of 80 mM potassium phosphate buffer, 85 μL of 22 mM potassium phosphate buffer and 15 μL of 0.017% H_2O_2) were added. The reaction was initiated with the addition of 20 μL of tetramethylbenzidine, and the sample was incubated for three minutes at 37 °C. The enzyme activity was determined in a microplate reader Multiskan® (Thermo Scientific, USA) at 450 nm.

2.5.4.2. Evaluation of catalase (CAT) activity. Effect of HEHs on CAT activity was evaluated using EtOH/HCl-ulcer model. Animals were distributed into the following groups: sham, vehicle (10 mL/kg, p.o.), HEHs (20, 50 and 250 mg/kg, p.o.) and carbenoxolone (100 mg/kg, p.o.). CAT activity was measured by the decrease of hydrogen peroxide according to a previously described method (Aebi, 1984). The results were expressed in μmol H_2O_2/min/g protein. Total proteins were determined following the method of Lowry et al. (1951).

2.6. Statistical analysis of data

Results of parametric tests were expressed in terms of mean ± standard error of mean (S.E.M). One-way analysis of variance (ANOVA) was used, followed by the Student–Newman–Keuls test when statistical difference was detected among the groups, while for non-parametric test, Kruskal–Wallis followed by Dunn's test were used. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Gastric ulcer induced by EtOH/HCl

Oral administration of acidified ethanol to the vehicle group (distilled water) caused characteristic haemorrhagic lesions in the glandular portion of the gastric mucosa (7.01%). Treatment with HEHs at 20, 50 and 250 mg/kg, p.o. promoted significant reductions in the ulcerogenic response at all doses tested by 63.3% ($p < 0.05$), 78.5% ($p < 0.01$) and 89.1% ($p < 0.01$) respectively. Carbenoxolone (100 mg/kg), the standard drug inhibited the gastric lesions by 79.6% ($p < 0.01$) as shown in Fig. 1.

3.2. Gastric ulcer induced by water immersion and restraint stress (WRS)

Visual inspection showed that WRS for 7 h induced serious gastric bleeding erosions as indicated by the mucosal haemorrhage and the percentage ulcerated area (Fig. 2). The haemorrhage was observed mainly in the gastric body and antrum, but not in the fundus. Pre-treatment with HEHs (20, 50 and 250 mg/kg) and ranitidine (50 mg/kg) effectively attenuated gastric ulcerations due to WRS significantly ($p < 0.001$) inhibiting gastric lesions appearance by 58%, 74%, 75% and 87% respectively, when compared to the vehicle control.

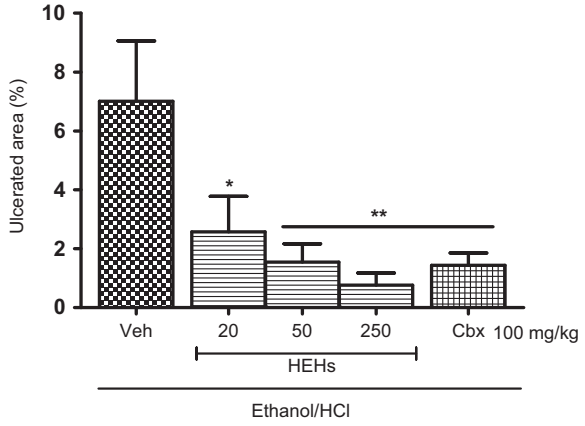

Fig. 1. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), carbenoxolone (Cbx, 100 mg/kg) and vehicle (distilled water) on gastric ulcer induced by 60% ethanol/HCl 0.3 M in mice. Each column represents Mean ± S.E.M of 5–6 animals. One-way ANOVA followed by Student–Newman–Keuls. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. vehicle.

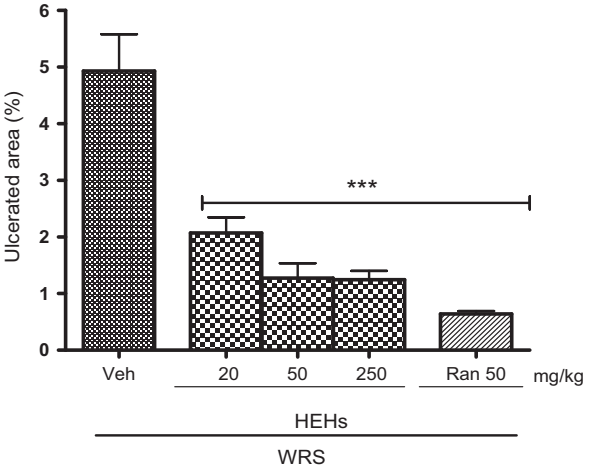

Fig. 2. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), ranitidine (Ran, 50 mg/kg p.o.) and vehicle (Veh, distilled water) on gastric ulcer lesions induced by water restrain stress in rats. Each column represents Mean ± S.E.M. of 5–6 animals. One way-ANOVA followed by Student–Newman–Keuls. $^{***}p < 0.001$ vs. vehicle.

3.2.1. Histopathological evaluation of gastric ulcers induced by WRS

Histopathological evaluations of the gastric tissues in WRS-ulcerated rats revealed extensive damages to the gastric mucosa manifested as haemorrhagic damage, submucosa oedema, loss of epithelial cells and inflammatory cell infiltration when compared with the normal control Fig. 3A and Table 1.

Overall pre-treatment with HEHs (Fig. 3C–E) one hour to WRS-ulcer induction demonstrated better gastroprotection as shown by the reduced or absence of epithelial cell loss, sub-mucosal oedema and inflammatory cells infiltration, at the highest dose used when compared with the vehicle control group (Fig. 3 and Table 1). The gastroprotection was more profound at the maximal dose (250 mg/kg) with attenuations of haemorrhagic damage, oedema, epithelial cell loss and inflammatory cell infiltration by 90.5%, 94.3%, 100% and 94.3% ($p < 0.01$) respectively. The standard drug, ranitidine was also equally active in protecting against WRS-induced lesions having produced 80.8%, 83.3%, 90.5% and 89% inhibitions in the respective histopathological parameters evaluated.

3.3. Gastric ulcer induced by piroxicam

Intragastric administration of piroxicam to rats caused various degrees of macroscopically evident mucosal damage (Fig. 4), with the gastric ulcer index of the vehicle treated being 13.96 ± 2.87. Pre-treatment with HEHs at doses of only 50 and 250 mg/kg significantly inhibited gastric mucosal damage index by 47.5% ($p < 0.05$) and 88.2% ($p < 0.001$) respectively. Ranitidine, the standard drug attenuated, in a more intense fashion, the gastric ulcerations producing 99.6% ($p < 0.001$) inhibition.

3.4. Anti-ulcerogenic mechanisms

3.4.1. Evaluation of antisecretory activity

As seen in Table 2, treatment with HEHs (20, 50 and 250 mg/kg) had no effect on the free and total acidity, but reduces significantly the juice volume by 45.6%, 50.7%, and 55.8% respectively. Omeprazole (20 mg/kg), the standard drug, reduced intensely total acidity, and the gastric juice volume by 43.7% ($p < 0.05$) and 87.5% ($p < 0.01$), respectively. Likewise, omeprazole caused a 2-fold increase ($p < 0.001$) in the pH of the gastric juice when compared with the control (Table 2).

3.4.2. Determination of gastric mucus in the EtOH/HCl ulcer in mice

Oral administration of EtOH/HCl resulted in significant depletion of mice gastric mucus. Pre-treatment with HEHs (20 and 50 mg/kg) and carbenoxolone significantly ($p < 0.01$) prevented mucus depletion, increasing it by 153%, 126% and 240%, respectively. The mucogenic effect of HEHs decreases with the increase in the dose in such a way that the highest dose (250 mg/kg) tested in this study had no significant effect on mucus production (Fig. 5).

3.4.3. Gastric ulcer induced by acidified ethanol in animals pre-treated with L-NAME, glibenclamide, indomethacin and yohimbine

Administration of EtOH/HCl to the vehicle group produced characteristic lesions in the form of straight haemorrhages in the glandular portion of the gastric mucosa. Treatment with HEHs (20 mg/kg) significantly ($p < 0.001$) attenuated gastric lesions induced by EtOH/HCl with 62.7% inhibition in comparison with the vehicle (Fig. 6B).

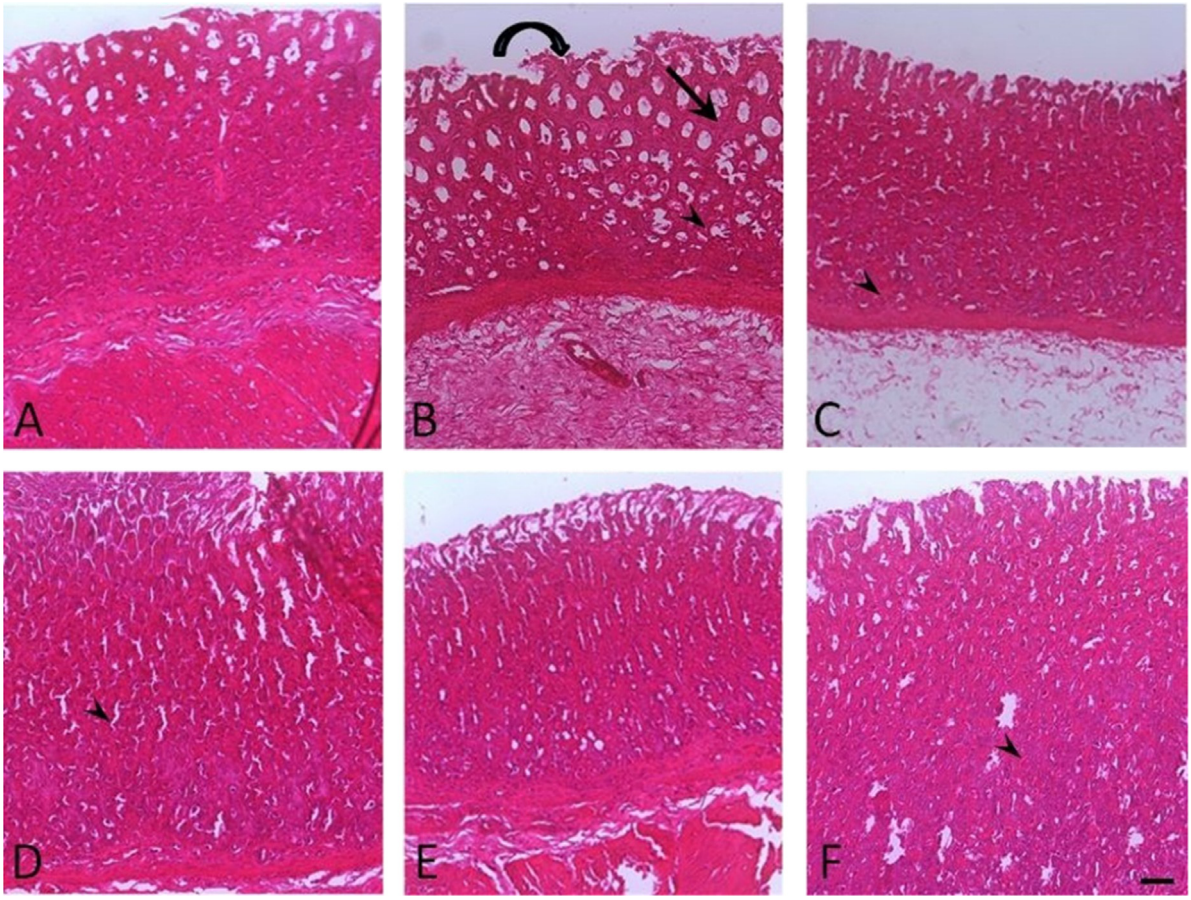

Fig. 3. Histopathological analysis indicating the effect of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs) on gastric mucosa lesions induced by water restrain stress. A) Sham, B) animals treated with the vehicle + WRS, C–E) animals treated with HEHs at 20, 50 and 250 mg/kg respectively F) Animals treated with ranitidine 50 mg/kg. Curved arrow indicates epithelial damage, arrow indicates oedema and arrowhead indicates inflammatory cells. The quantitative results are presented in Table 2. Stain: HE. Barr: 50 μm.

Table 1
Protective effect of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg) and ranitidine (50 mg/kg) on water immersion restraint stress-induced histological lesions.

Experimental group ($n=6$)	Haemorrhagic damage (score 0–4)	Oedema (score 0–4)	Epithelial loss (score 0–4)	Inflammatory cells (score 0–4)	Total (scores 16)
Normal group	0	0	0	0	0
Vehicle	3.50 (2–4)	3.00 (2–4)	1.80 (1–3)	3.00 (2–4)	11.30 (8–14)
HEHs (20 mg/kg)	2.80 (2–3)	2.80 (2–3)	1.70 (1–3)	1.70 (1–3)	9.00 (7–11)
HEHs (50 mg/kg)	0.83 (0–2)*	0.83 (0–2)	0.33 (0–1)	0.17 (0–1)**	2.16 (0–4)*
HEHs (250 mg/kg)	0.33 (0–1)**	0.17 (0–1)**	0 (0)**	0.17 (0–1)**	0.67 (0–2)**
Ranitidine (50 mg/kg)	0.67 (0–2)*	0.50 (0–2)*	0.17 (0–1)*	0.33 (0–1)*	1.67 (0–5)*

Data shown are medians with minimum and maximum scores shown in brackets. Kruskal–Wallis nonparametric test followed by Dunn's test.
* $p<0.05$ and ** $p<0.01$ vs. vehicle.

Significant but partial reversal of the gastroprotective effect of HEHs was observed with yohimbine, glibenclamide, indomethacin and L-NAME pre-treatments with 22.9%, 10.4%, 31.4% and −11.1% inhibitions, respectively, in comparison to the vehicle group (Fig. 6A and B).

3.4.4. Evaluation of effect of HEHs on antioxidant enzymes in mice with EtOH/HCl ulcer

3.4.4.1. Evaluation of myeloperoxidase activity. As shown in Fig. 7, in mice with EtOH/HCl-ulcer, gastric mucosal MPO activity in the vehicle treated group increased significantly ($p<0.05$) with 1.8-fold higher mucosal MPO than the sham control. Pre-administration of HEHs (50 and 250 mg/kg) and carbenoxolone (Cbx, 100 mg/kg) significantly ($p<0.05$) attenuated increase in the gastric mucosal MPO activity when compared to the vehicle control group.

3.4.4.2. Evaluation of catalase activity. Catalase activity in the normal mice stomach was 9.22 μmol H_2O_2 utilised per min per g of protein. EtOH/HCl treatment caused a 74% reduction in the catalase activity. Compared to the ethanol group, pre-treatment with HEHs (20, 50 and 250 mg/kg) and carbenoxolone (100 mg/kg) significantly augmented the activity of catalase (Fig. 8).

4. Discussion

Although the practice of herbal medicine is an old tested system of primary health care, there is a growing need to explore the vast potential of the medicinal plants through pharmacological evaluations. As part of our drive towards this end, we investigated the gastroprotective effects of HEHs in experimental rodents.

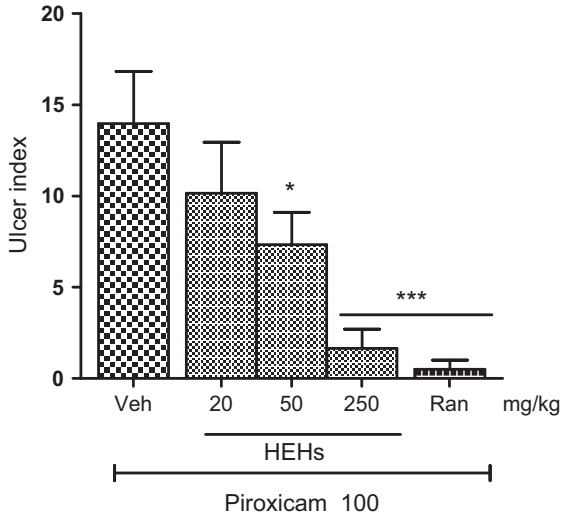

Fig. 4. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), ranitidine (Ran, 50 mg/kg p.o.) and vehicle (Veh, destilled water) on gastric ulcer lesions induced by piroxicam 100 mg/kg in rats. Each column represents Mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student–Newman–Keuls. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. vehicle.

Table 2
Effect of intraduodenal administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, id.), omeprazole (20 mg/kg id.) and vehicle (distilled water) on gastric juice parameters in rats.

Treatment	pH (units)	$[H^+]$ (mEq/mL/4 h)	Gastric juice volume (mL)
Vehicle	2.05 ± 0.25	4.21 ± 1.56	1.38 ± 0.29
HEHs 20 mg/kg	2.56 ± 0.12	2.02 ± 0.52	0.75 ± 0.15*
HEHs 50 mg/kg	2.03 ± 0.28	2.64 ± 0.62	0.68 ± 0.06*
HEHs 250 mg/kg	2.89 ± 0.39	2.51 ± 0.61	0.61 ± 0.10*
Omeprazole 20 mg/kg	4.61 ± 0.92***	0.84 ± 0.46*	0.48 ± 0.11**

Results are in Mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student–Newman–Keuls.

$^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. vehicle.

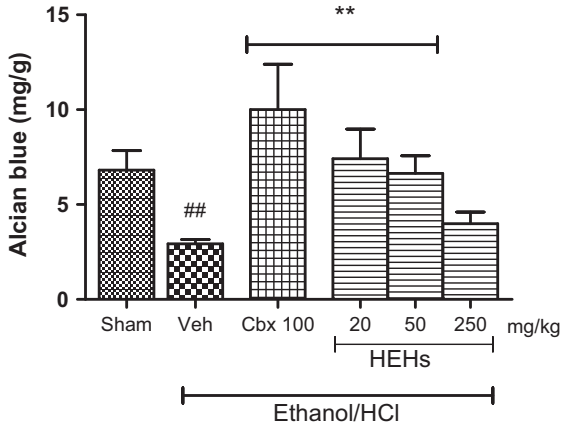

Fig. 5. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), carbenoxolone (Cbx,100 mg/kg p.o.) and vehicle (Veh, distilled water) on Alcian blue binding to free gastric mucus from acidified ethanol-ulcerated mice. Sham group was subjected only to fasting. Results are in Mean ± S.E.M. of 6 animals. One-way ANOVA followed by Student–Newman–Keuls. $^{\#\#}p < 0.01$ vs. sham and $^{**}p < 0.01$ vs. vehicle.

The aetiology of peptic ulcer is multifactorial and therefore research into new anti-ulcer agents requires the use of models that takes into consideration of these different aetiological factors. Animal

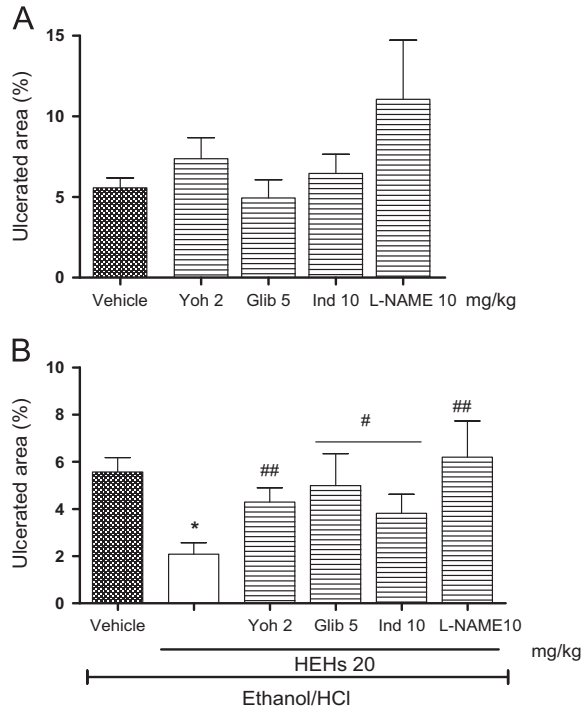

Fig. 6. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20 mg/kg) in animals pre-treated with yohimbine (Yoh, 2 mg/kg ip.), glibenclamide (Glib, 5 mg/kg po.), indomethacin (Ind, 10 mg/kg p.o.) and N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 10 mg/kg ip.) on (Fig. 7A). Results are in Mean ± S.E.M. of 5–6 animals. One-way ANOVA followed by Student–Newman–Keuls. $^{*}p < 0.05$ vs. vehicle, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. HEHs.

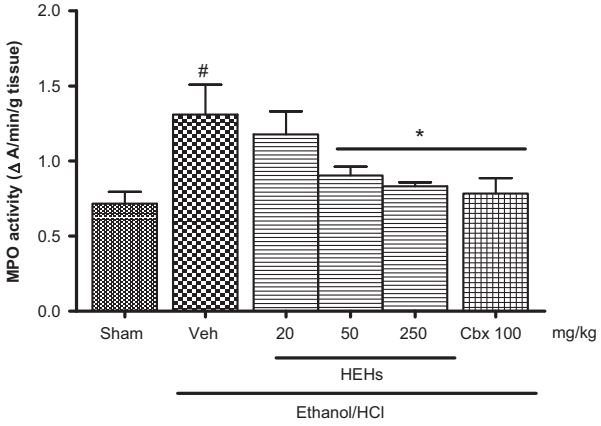

Fig. 7. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), carbenoxolone (Cbx, 100 mg/kg p.o.) and vehicle (Veh, distilled water) on myeloperoxidase activity in acidified ethanol-ulcerated mice. Sham group was subjected only to fasting. Results are in Mean ± S.E.M. of 5–6 animals. One-way ANOVA followed by Student–Newman–Keuls. $^{\#}p < 0.05$ vs. sham and $^{*}p < 0.05$ vs. vehicle.

models of any human disease should aim to mimic the human condition as much as possible (Lee, 2000; Jaggi et al., 2011). In this sense we employed 3 acute gastric ulcer models. Among the principal aetiological factors associated with peptic ulcer are the alcohol consumption, the use of NSAIDs drugs and emotional stress (Vimala and Gricilda Shoba, 2014).

We commenced the present investigation into the folkloric claim of *H. sacarolha* use in the treatment of gastric ulcer with EtOH/HCl ulcer, since ethanol induces ulcer by diverse mechanisms (Adinortey et al., 2013). Besides, alcohol can produce acute inflammatory reactions which is central to most ulcer diseases (Dixon, 2000; Andersen

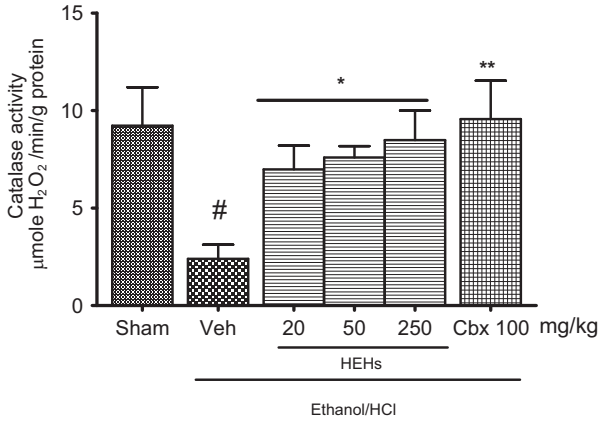

Fig. 8. Effect of orogastric administration of the hydroethanolic extract of *Helicteres sacarolha* leaves (HEHs, 20, 50 and 250 mg/kg, p.o.), carbenoxolone (Cbx, 100 mg/kg p.o.) and vehicle (Veh, distilled water) on catalase activity in acidified ethanol-ulcerated mice. Sham group was subjected only to fasting. Results are in Mean ± S.E.M. of 5–6 animals. One-way ANOVA followed by Student–Newman–Keuls. #$p < 0.05$ vs. sham, *$p < 0.05$ and **$p < 0.01$ vs. vehicle.

et al., 2005; Jia et al., 2007; Beserra et al., 2011) and therefore basic information can be gathered from this model.

The choice of the doses used in this experiment was based on a pilot study as well as the results of toxicity studies that showed that repeated treatment with dose with 250 mg/kg for 30 days had no toxic effects on the treated animals (Balogun et al., 2014).

HEHs, at all doses tested, effectively protects the gastric mucosa of the mice in dose dependent manner. Ethanol is also known to stimulate inflammation through imbalance between pro-inflammatory cytokines and anti-inflammatory cytokines, in addition to production of free radicals that may help propagate inflammatory conditions (Beserra et al., 2011). We therefore hypothesised that HEHs may be acting through inhibition of inflammatory processes that lead to the gastric ulcerations by possibly enhancing the mucosal defence like mucus production and protective antioxidant enzymes and attenuation of inflammatory infiltration of the neutrophils. We investigated the veracity of this hypothesis by assessing its mucogenic and modulation of antioxidant enzymes.

Mucus barrier may be the first line of defence of the gastric mucosa against ulcerogenic insults (Jain et al., 2007; Wallace, 2008). Our result demonstrates that mucus production accounts in part for the protective effect of HEHs. However, this mechanism alone could not account for it protective effect as the highest dose tested demonstrated no effect on the mucus production (Fig. 5).

Furthermore, significant reduction in infiltration of neutrophils by HEHs was confirmed with inhibition in the MPO activity in the EtOH/HCl-ulcer, while it replenished the depleted catalase activity, one of the enzymes involved in the scavenging of free radicals (Ohta and Nishida, 2003; Li et al., 2012). Inhibition of the MPO activity correlated well with the histopathological findings that revealed significant reductions in the infiltrations of inflammatory cells into the gastric mucosa. These results further confirm the anti-inflammatory nature of HEHs in these ulcer models.

Serious stress can induce organ injuries or contribute to diseases, such as gastric ulcers, hypertension and diabetes among others. WRS-ulcer model mimics the clinical acute gastric ulcerations caused by trauma, surgery or sepsis (Xie et al., 2005) and have been exploited to study pathogenic mechanisms of stress related diseases as well as useful therapeutic interventions (Ueyama et al., 1998; Bagchi et al., 1999; Lee, 2000; Guo et al., 2012). We therefore further explored the gastroprotective effect of HEHs using WRS-ulcer model in rats.

Our data shows that HEHs effectively suppresses WRS-induced ulcer by inhibiting gastric ulcerations, infiltration of inflammatory cells into the gastric mucosa, reduction or prevention of epithelial cell loss as confirmed by histological analysis. The aetiology of stress-induced ulcer may involves the effects of hypothalamic–pituitary–adrenal axis activation on healing, altered blood flow, or cytokine mediated impairment of mucosal defences (Alhazzani et al., 2012; Levenstein et al., 2014).

The major limitation of clinical application of NSAIDs are serious side-effects such as induction of acute haemorrhagic erosions, potentiation of gastric ulcerogenic response to various stimuli, exaggeration of colitis and impairment of healing of pre-existing ulcers (Brzozowski and Konturek, 2008). Gastrointestinal injury caused by NSAIDs occurs both by topical and systemic effects. The systemic effect is mediated principally by blocking prostaglandin synthesis through inhibition of the cyclooxygenase (COX) enzymes, COX-1 and COX-2. We therefore evaluated the gastroprotective action of HEHs in this ulcer model. HEHs effectively attenuated ulcerations due to NSAID piroxicam. Thus, HEHs may be acting by increasing the production of mucosal prostanglandins (Musumba et al., 2009).

In an attempt to evaluate the possible role of gastric secretion in HEHs protective action we utilised pylorus ligation model in rats. HEHs caused reduction in the volume of gastric acid secretion. Besides reduction in the volume of the gastric secretion, no significant effect was observed on the pH and concentration of HCl indicating that gastric acid secretion modulation does not have relevant role in the gastroprotective mechanism of action of HEHs. The incapacity of HEHs in interfering in hydrochloric acid secretion in the gastric juice may be desirable in some situations, since gastric acid secretion prevents microbial growth and hypergastrinaemia. Moreover, gastric ulcer patients as a group, sometimes exhibit normal, decreased basal or stimulated acid production, and therefore, gastric mucosal defence is the most important in this case (Schubert and Peura, 2008).

α_2-Adrenoreceptors that are widely distributed in the gastrointestinal tract, play crucial role in presynaptic modulation of transmitter release, and therefore in the regulation of gastrointestinal functions (Zádori et al., 2011). Pre-treatment with yohimbine, a non-selective α_2-adrenoreceptor antagonist partially reversed the protective effect of HEHs which indicates the involvement of α_2-adrenoreceptors. The study of Zádori et al. (2011) confirmed the participation of both α_{2B}- and α_{2C} -adrenoreceptor subtypes in the mediation of centrally induced gastroprotection in mice. It is therefore plausible that HEHs may be acting on these receptors in conferring its gastroprotective effect in this model.

PGs are known to inhibit mast cell activation and leucocyte adherence to the vascular endothelium, stimulate mucus and bicarbonate secretion, increase mucosal blood flow and accelerate ulcer healing (Tarnawski et al., 2013). It is possible that the gastroprotective effect of HEHs is therefore partly modulated by its modulation of PGs production.

The participation of K^+_{ATP}-channel in HEHs protective effect was evaluated with glibenclamide pre-treatment. Glibenclamide significantly, although partially, reversed the gastroprotective effect of HEHs and suggests that, at least in part, K^+_{ATP}-channel activation is involved in HEHs gastroprotective effect. Peskar et al. (2002) suggested that the endogenous PGs act as activators of K^+_{ATP}-channels, and that this mechanism mediates, at least in part in the gastroprotection. It is therefore understandable that pre-treatment with indomethacin attenuates HEHs protective action, as shown in Fig. 6. However, further studies are necessary to prove this hypothesis, which is beyond the scope of the present study.

To investigate the influence of endogenous NO on the gastroprotective effects of HEHs, mice were pre-treated with inhibitor of nitric oxide synthase (NOS), L-NAME, an analogue of L-arginine, which is hydrolysed to produce L-nitro arginine and thereby inhibits NOS activity. Nitric oxide (NO) is known to have a protective effect on the gastrointestinal tract. Preclinical studies have demonstrated that NO participates in maintaining gastric mucosal integrity, inhibiting

leucocyte adherence to the endothelium and neutrophil aggregation, and repair of NSAID-induced damage. In addition, NO is a vasodilator and mediates gastric blood flow (Lanas, 2008; Rozza et al., 2013). Our data demonstrates that endogenous NO production modulates the gastroprotective effect of HEHs.

Recent report by our research group demonstrates the presence of relatively high content of phenolics and flavonoids with HPLC analysis confirming ellagic acid, morin and naringin as part of constituents identified in HEHs (Balogun et al., 2014). In the referred study, ellagic acid (EA) accounts for 6% of HEHs. EA is a known phenolic compound with wide array of pharmacological activities such as anti-inflammatory and gastroprotective effects including in vivo antioxidant properties, and it has been found in many plant extracts as part of their active principles (Solon et al., 2000;Vattem and Shetty, 2005; Rogerio et al., 2008; Beserra et al., 2011; Huang et al., 2011). Morin is a flavonoid that is found in many fruits and herbs and it is used as a food additive because of its antioxidant activity (Cho et al., 2006). It also possesses anti-inflammatory property (Bellik et al., 2012). Naringin, also a flavonoid, is known to possesses gastroprotective properties among others (Schmeda-Hirschmann and Yesilada, 2005). The gastroprotective and antioxidant actions of HEHs may therefore be modulated, at least in part, by the combined action of these and other yet unidentified constituents.

5. Conclusion

The gastroprotective effect of HEHs seems to have multiple components in its action. HEHs may be protective against ulcers by increasing the endogenous defensive factors such as PGs, NO and mucus, stimulation of the central and or peripheral α-2 receptors of as well as activation K^{+}_{ATP}-channels. It inhibited gastric secretion volume without affecting secretion of HCl. Additionally, it increases CAT activity and inhibited infiltration of neutrophils into the gastric mucosa. The gastroprotective effect of HEHs on the acute gastric ulceration models observed in this study support the use in popular medicine of HEHs and suggests a potential clinical application in treating gastrointestinal injury due to ethanol, NSAIDs and stress.

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