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Gastroprotective Effect of *Acacia aroma* Gill. ex Hook. and Arn. Leaves Extracts in Gastric Ulcer Models in Rats

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ABSTRACT

Background: Peptic ulcer is a disease caused by imbalance between protective and aggressive factors in the gastric mucosa. *Acacia aroma* Gill. ex Hook. and Arn. (tusca) is widely used in Argentinian's folk medicine for gastrointestinal disorders. The present study was designed to evaluate the protective effect of two *A. aroma* leaf extracts, 5% infusion and 10% hydroalcoholic extract in experimental models of acute gastric ulceration in rats. **Methods:** The gastroprotective action of the extracts was evaluated in absolute ethanol-induced gastric lesion models in adult Westar rats through macroscopic, histological and biochemical analyses of the stomach and gastric wall. We also determined the effects on the gastric juice parameters with a pylorus ligature model in rats. Evaluation of the acute toxicity of each extract was performed in adult normal Wistar rats. All results were statistically analyzed. **Results:** The lowest effective dose was 150 mg/Kg for each extract. Both of them showed a high protection of the mucosa against absolute ethanol. Pre-treatments with the extracts produced a significant increase in adherent mucus. These extracts showed a free radical scavenging capacity and they caused a significant reduction of the malondialdehyde, reduced glutathione levels, catalase and myeloperoxidase activities. The animals presented no signs of acute toxicity indicating a high safety margin for both extracts. **Conclusion:** This study shows for the first time the gastroprotective efficacy of 5% infusion and 10% hydroalcoholic extract of *A. aroma* leaves, highlighting their benefits as a prophylactic approach to maintain the integrity of the gastric mucosa.
Key words: Acute toxicity, Antioxidant activity, Gastroprotection, Tusca, Preclinical studies.

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INTRODUCTION

Gastric ulcer is a deep necrotic lesion involving the entire thickness of the mucosa and the muscular is mucosa and forms a cavity surround by acute or chronic inflammation.[1,2] This is a pathological disorder with increasing incidence and prevalence worldwide, the lifetime risk for developing a peptic ulcer being approximately 10%. Several aggressive factors are involved in the complex etiology of gastric ulcer, the most common ones being non-steroidal anti-inflammatory drugs and alcohol consumption, emotional distress, *Helicobacter pylori* infection, inflammatory mechanisms and higher free radical generation.[3]

Alcohol consumption has also been commonly linked to gastric mucosal lesions including gastritis and gastric ulcer. Ethanol could induce injury through multiple pathways, producing inflammatory/necrotic reactions and oxidative stress, which in turn reduces the defense barrier and damages the tissue.[4]

Regardless of etiology, the injured gastric mucosa has numerous repair mechanisms that contribute to the resolution of the lesions. The first line of defense is a layer of mucin-rich mucus and bicarbonate. The epithelial surface provides a defensive barrier both by producing mucus and through its tight junctions. In addition, a migration process regulated by several growth factors restores the damaged region. The intrinsic antioxidant defense, especially through Glutathione (GSH), can be effective in controlling oxidative stress, whereas the proper blood flow in the submucosal layer can supply micronutrients and oxygen and also eliminates toxic metabolic products.[5]

In the clinical setting, the suppression of acid secretion through H2 receptor antagonists, proton-pump inhibitors and anti-cholinergic drugs are the main treatment strategy in both the healing and protection of the gastric mucosa.[6] However, in recent years, much attention has been focused on improving the resistance of the stomach mucosa to ulcerative agents and studying the possibility that the quality of protection against harmful substances such as drugs or alcohol may be pharmacologically modulated.[7] In this context, pre-clinical studies in an ethanol-induced ulcer model are useful to define the efficacy of potential drugs with anti-ulcer activity. The gastric damage produced resembles acute peptic ulcers in humans and is independent of gastric acid secretion. It is a consequence of many interacting factors, each of which can be considered a potential therapeutic target, as was interpreted by Glavin and Szabo.[8]

In contrast, the model of pylorus-ligation induces peptic ulcers by accumulation of gastric acid in the stomach and serves to investigate the efficacy of drugs on gastric secretions.[9]

In the search for new therapies, plants have always been a prototypical source of chemical compounds showing activities consistent with their possible use in the treatment or prevention of gastric ulcers.[10]

The use of the ethno-botanical information in medicinal plant research allows the detection of potential sources of compounds with real activity. *Acacia aroma* Gill. ex Hook. and Arn. is an autochthonous species widely distributed in northern and central Argentina.[11] This plant (common name tusca), which belongs to the *Fabaceae* family, is used in folk medicine as a wound healer and antiseptic and in the treatment

of gastrointestinal disorders.[12] In addition, leaf and bark infusions have anti-inflammatory uses.[13] To date there are few studies that scientifically validate the plant's traditionally described therapeutic properties, although its antibacterial activity against Gram-negative and Gram-positive multi-resistant micro-organisms to antibiotic have already been demonstrated.[14]

Despite its frequent traditional use, which would account for its safety, there is little scientific information about its toxicity. Mattana *et al.*[15] showed that aqueous and ethanolic extracts of *A. aroma* were safe at the cell and genomic level; however, further *in vivo* studies are needed to confirm the absence of adverse and/or toxic reactions to ensure their safe use.

The most evident and best known secondary metabolites reported from various *Acacia* species widely distributed in the world are polysaccharides (gums) and several polyphenols, including condensed and hydrolysable tannins and also flavonoids.[16] Although no exhaustive phytochemical studies have been performed in *A. aroma* leaves, the presence of the above chemical compounds could indicate potential benefits against gastric mucosal injury generated by oxidative damage. However, to the best of our knowledge, there are no pre-clinical studies that evaluate the Gastroprotective effect of this medicinal plant in the literature. Therefore, the present study was designed to evaluate the protective activity of two *A. aroma* leaf extracts: infusion and hydroalcoholic extract, in experimental models of acute gastric ulceration. This work could contribute to establishing the basis for the use of extracts of this plant in joint treatments with substances recognized as aggressive to the gastric mucosa such as non-steroidal anti-inflammatory drugs.

MATERIALS AND METHODS

Plant Material

Leaves of *Acacia aroma* Gill. ex Hook. and Arn. used in this study were collected in September 2015 from Silípica, Santiago del Estero, Argentina, located at 28°06′29″S, 64°08′49"W, 170 m.a.s.l. Voucher specimens (LIL-615923) are deposited in the herbarium of "Fundación Miguel Lillo", San Miguel de Tucumán, Argentina.

The plant material (leaves) was dried under air stream in an oven at 40°C and ground to a powder. Two extracts, a 5% infusion (I) and a 10% hydroalcoholic extract (HE), were obtained from the dried plant material according to Farmacopea Argentina 7° Ed.[17] The extracts were filtered through Whatman filter paper N°4; the HE was concentrated under a vacuum at approximately 40°C and both of them were lyophilized. The dry extracts were kept at -20°C until use. Before each experiment, the extracts were dissolved in distilled water (vehicle) and given to rats by oral gavage at the established dose.

Quantitative phytochemical analysis

The total phenolic content in both extracts was determined by the Folin-Ciocalteu method with slight modifications. Briefly, 0.05 ml of a solution (1 mg/ml) of each extract was mixed with 0.2 ml 50% (v/v) Folin-Ciocalteu reagent (Sigma- Aldrich, Co. St. Louis, MO, USA) and 0.8 ml of 14.9% sodium carbonate solution. In order to complete a final volume of 2 ml, ethanol (for organic extracts) or water (for aqueous extracts) was added. After 20 min, the samples were read at 765nm in a spectrophotometer. Gallic acid was used as a standard. The result was expressed as mg Gallic Acid Equivalents (GAE)/g dry extract.

Flavonoid content determination in both extracts was based on the formation of a flavonoid-aluminum complex as was described by Chang *et al.*[18] Quercetin was used as a standard. The amount of flavonoids was expressed as mg quercetin equivalents/g dry extract.

The method used for tannins determination is based its property to precipitate proteins. At first the total phenolic compounds from each extract was determined with the Folin-Ciocalteu procedure described above. In order to remove tannins, BSA solution (0.2 M acetate buffer, pH 5, 0.17 M sodium chloride and 1 mg fraction V BSA- Sigma- Aldrich, USA) was added to the extract (1:1). After 15 min at room temperature, the mixture was centrifuged at 5000 g and an aliquot (50 µl) of the supernatant was used to determine total phenolic compounds using the Folin-Ciocalteu method. The amount of total tannins was obtained from the difference between the amount of phenolic compounds before and after the precipitation. Total tannin content was expressed as mg GAE/g dry extract.[19] All the determinations were carried out in triplicate.

Free radical scavenging activity: *In vitro* assay

The free radical scavenging capacity of the 5% I and 10% HE was evaluated by the decrease in 2,2-diphenyl-1- picrylhydrazyl (DPPH) absorbance accordingly to Blois.[20]

EC_{50} values were calculated by nonlinear regression analysis of the plotted DPPH depuration % vs. sample concentration using SPSS® statistic software. All assays were performed in triplicate.

Animals

Adult male Wistar rats aged 8 to 12 weeks (weight 200 ± 20 g) were selected for all the experiments. The animals were obtained from the colony bred at the INSIBIO (CONICET-UNT), Tucumán, Argentina and were acclimated for 7 days before the start of the experimental procedures.

The animals were housed in cages and the photoperiod (07:00 to 19:00 h), air exchange, temperature (22 ± 2°C) and relative humidity (60–70%) of the room were controlled. Rats were given free access to a powdered certified rodent diet obtained from a commercial source (Standard Food-Asociacion de Cooperativas Argentinas-S.E.N.A.S.A. No. 2706). Water was also available *ad libitum*. There were no known contaminants in the food or water that could interfere with the results of the study.

All animal handling and procedures complied with the current laws of Argentina (Ethical Framework of Reference for Biomedical Research in laboratory animals, Resol. D N° 1047 annex II, 2005). The current protocol was approved by the Institutional Committee of Animal Care and Use of the Facultad de Bioquímica, Universidad Nacional de Tucumán (N°0015-2017). Every effort was made to reduce the number and suffering of experimental animals.

Evaluation of the gastroprotective effect

Gastroprotective effect of Acacia aroma was studied using ethanol-induced gastric ulcer model. The rats were placed in individual cages with raised floors and fasted for 18 h with free access to 5% sucrose solution instead of water in order to avoid coprophagy. The animals were randomly assigned to eight groups (n=6). Six groups were treated with a specific dose each one 75, 150 and 300 mg/kg body weight (b.w.) of 5% I or 10% HE. The untreated ulcer group received vehicle (distilled water), whereas the standard drug sucralfate (100 mg/kg b.w.) was orally administered to rats in the positive control group. In addition, the untreated normal group received only the same volume of distilled water instead of ethanol. After 60 min, all groups were orally given absolute ethanol (6 ml/kg b.w.) to induce gastric ulcers. Animals were euthanized 1 h after ethanol administration with an intraperitoneal (i.p.) overdose of ketamine/xylazine (150:5 mg/kg b.w.).Their stomachs were immediately dissected, opened and rinsed with cold PBS.

The degree of gastric mucosal damage to establish the minimum protective dose of 5% I and 10% HE was evaluated by gross pathology, counting the number of ulcers per stomach. The ulcer area (mm^2) was measured using Image J 1.48d software (National Institutes of Health, USA) and

expressed as a percentage (%) of total area (ulcer percentage). The severity of gastric lesion was calculated according to a 0-3 scoring system: 0: Normal mucosa, 0.5: red mucosa, 1: spot ulcer, 1.5: hemorrhagic lines, 2: 3 or more confluent hemorrhagic lines, 3: 5 or more ulcers). The protection percentage (%) of each assayed material was calculated using the following equation:

Protection % = [(ulcer% $_{\text{ethanol control}}$ – ulcer% $_{\text{treated group}}$) / ulcer% $_{\text{ethanol control}}$)] X 100.

The statistical comparison of the dose-response curve provided the lowest effective dose, which was used for the next analyses.

Microscopic examination of gastric damage

Gastric tissue samples from each experimental group were fixed in 4% phosphate-buffered formaldehyde for 24 hr. Then, the samples were washed, dehydrated with alcohol, cleared in xylene and embedded in paraffin in hot air oven (56°C). The paraffin blocks were cut into 5 µm thick sections with a microtome (Levitz 1401, Germany).

The tissue sections obtained were deparaffinized and stained with hematoxylin and eosin, Alcan blue or Periodic Acid-Schiff (PAS) dyes for histopathological examination through the light photomicroscope (NIKON, Japan). In order to avoid bias, a qualified observer unaware of the identity of the specimens performed all histopathological procedures.

Determination of the adherent gastric mucus

The mucus content attached to the gastric wall was determined according to the method previously described by Corne *et al.*[21] with modifications. Briefly, stomachs from each experimental group were weighed, quickly transferred to 1% (p/v) Alcian blue solution prepared with 0.16 M sucrose in 0.05 M sodium acetate, pH 5 and incubated for 2 h at room temperature. Then, the organs were carefully rinsed with the above sucrose solution. The dye attached to the gastric wall was extracted with a 3% (p/v) sodium dodecyl sulfate solution (10 ml/stomach). The resulting colored solution was centrifuged at 5000 rpm (8000 g) for 5 min and the absorbance of the supernatant was measured at 605 nm. The Alcian blue (µg) extracted per gram of wet organ was then calculated using a calibration curve of Alcian blue (6.26-100.00 µg).

Biochemical estimations of oxidative stress and inflammatory parameters

Preparation of gastric mucosa homogenates

To obtain 10% (p/v) aqueous homogenates, the gastric mucosa of each experimental group were scraped off, weighed and homogenized at 4°C in 200 mM sodium phosphate buffer, pH 7.4, containing 1mM EDTA and the following protease inhibitors 2 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 5 µg/ml pepstatin and 5 µg/ml aprotinin (Sigma, USA).

After homogenization, one fraction was centrifuged at 9000 g for 15 min at 4°C and the resultant pellet was used to determine myeloperoxidase (MPO) activity, while another homogenate fraction was centrifuged at 18000 g at 4°C for 15 min. The resulting supernatant was used to evaluate malondialdehyde (MDA) levels and catalase (CAT) activity. Protein estimation was carried out by the method of Lowry *et al.*[22] using BSA as a standard.

Determination of GSH levels

GSH in stomach homogenate was measured as described by Ellman[23] using the 5,5-dithiobis-(2-nitrobenzoic acid) (Sigma, USA) reagent. GSH levels were calculated using a calibration curve performed with the GSH (Sigma, USA) standard and expressed as µg GSH/mg protein.

Determination of lipid peroxides level (MDA)

Lipid peroxidation was assessed by determining the levels of MDA in supernatants of mucosa homogenate according to the spectrophotometric method of Buege and Aust.[24] The MDA concentration of the sample was calculated with an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The results were expressed as n moles of MDA formed/mg protein.

Determination of CAT activity

CAT activity was measured according to the modified method of Aebi.[25] Briefly, 500 µl of supernatants of mucosa homogenate (containing approximately 1.5 mg of protein) was added to 250 µl of 0.1 M phosphate buffer, pH 7, containing 100 mM H_2O_2. The rate of H_2O_2 decomposition was followed spectrophotometrically at 240 nm for 1 min. Enzyme activity was expressed as international Units (U)/mg protein and was calculated using a molar extinction coefficient of $0.043 M^{-1}.\ cm^{-1}$. One units of CAT was defined as nmoles H_2O_2 destroyed/min at 25°C.

Determination of MPO activity

Myeloperoxidase activity was measured to assess neutrophil infiltration as described by Bradley *et al.*[26] with modifications. The pellet obtained as previously described above was resuspended in 50 mM phosphate buffer, pH 6, containing 0.5% hexadecyltrimethylammonium bromide (Biopack, Argentina) and sonicated for 1 min in an ice bath. The samples were centrifuged at 600 g for 20 min at 4°C and the supernatants were diluted 1:30 with 50 mM phosphate buffer, pH 6, containing 0.167 mg/ml O-dianisidine dihydrochloride (Sigma, USA) and 0.0005% H_2O_2. The change in absorbance at 460 nm (25°C) was measured at 1 min intervals for 3 min. One unit of MPO was defined as the µg of enzyme that degraded one micromole of peroxide per min at 25°C. Enzymatic activity was expressed as U/mg protein.

Model of gastric ulcer induced by pylorus ligation

This model was carried out to evaluate the effects of 5% I and 10% HE on gastric juice parameters. After 18 hr fasting, male Wistar rats were divided into four groups (n=6). The untreated group received vehicle (distilled water), whereas the positive control group was intraperitoneally administered the standard drug Omeprazole sodium (20 mg/kg b.w.). The treated groups orally received 5% I (150 mg/kg b.w.) or 10% HE (150 mg/kg b.w.). Sixty min after each treatment the rats were anesthetized with ketamine/xylazine (75/5 mg/kg b.w., i.p.) and the abdomen was opened, the stomach was exposed and the pyloric sphincter was ligated without causing any damage to the blood supply. All the animals were euthanized 4 hr later with an overdose of ketamine/xylazine (150:5 mg/kg b.w., i.p.). The stomach was removed and the gastric content was collected and their volume and its pH were determined. It was immediately centrifuged at 2000 g for 10 min. The supernatant was removed and titrated with 0.01 N NaOH using methyl orange as an indicator. The free acid concentration was expressed in mEq/L/100g b.w. The supernatant was also used for the determination of pepsin activity according to Anson.[27]

Acute toxicity studies

Acute oral toxicity of 5% I and 10% HE was studied with a single dose experiment. Healthy adult Westar rats were randomly divided into groups of six animals. Each group included three males and three females with a weight of 200 ± 20 g that were orally given a single dose of 5% I and 10% HE. The doses tested were 3.75, 7.50 and 15.00 g dry extract/kg b.w., representing 25, 50 and 100 times the effective Gastroprotective dose.

The control group received only the vehicle (distilled water). Rats were observed continuously for 1 hr after treatment to evaluate their behavior, neurologic and autonomic activity and toxic effects. Then, the controls

were carried out at 3 and 6 hr post-dose on day 0 and twice daily (morning and afternoon) thereafter for 14 days. Food consumption, faces and urine were also examined twice daily (morning and afternoon) during the 14 days after the single dose administration of the dry extract.

Statistical analysis

All the data were expressed as mean±SD and evaluated by one-way analysis of variance (ANOVA) followed by unpaired Student's *t*-test using the Statistical Package for the Social Sciences version 12.0 (SPSS) program (SPSS Inc., Chicago, IL). Values of $p<0.05$ were considered as statistically significant.

RESULTS

Phytochemical evaluation of 5% infusion and 10% hydroalcoholic extract of tusca leaves

The yield of the 5% I was found to be 8.4% of powdered extract (w/w of plant material), whereas the 10% HE yield 14.9% of powdered extract (w/w of plant material).

The total phenolic compounds and tannin quantified in 5% I were found to be 94.61±0.32 and 24.50 mg gallic acid equivalent/ g of dry extract, respectively. The total content of flavonoids was 82.08 mg/g (quercetin equivalent per gram of dry extract). 10% HE showed an important content of total phenolic compounds (72.60±0.74 mg gallic acid equivalent/g) but especially of flavonoids (104.58±1.06 mg quercetin equivalent/g) and tannins (40.62±2.03 mg gallic acid equivalent/g dry extract) compared with the infusion.

In view of these results, we have demonstrated the *in vitro* DPPH radical scavenging activity of both the 5% I and the 10% HE. Indeed, the sample concentration of the infusion as well as the ethanol extract necessary to decrease the initial DPPH concentration by 50% (EC_{50} values) were 47.5±2.4 and 39.3±2.0 ug/ml, respectively. Unsurprisingly, quercetin, the positive control for this assay, showed an EC_{50} of 1.7±0.1 ug/ml, confirming its well-known ability for radical scavenging, whereas the EC_{50} values for the infusion or the ethanolic extract were significantly higher compared with quercetin ($p<0.05$).

Acute toxicity of *A. aroma* leaf extracts

Acute oral toxicity of 5% I and 10% HE was evaluated in normal healthy rats. No deaths or acute toxic effects (changes in behavior or posture, presence of convulsions or occurrence of secretions) were reported within 14 days. All treatments were well tolerated and did not produce adverse nutritional effects.

No gastrointestinal symptoms such as diarrhea or constipation were observed at the doses assayed. Volume, pH and urine specific gravity were within normal ranges. No nitrites, protein or blood were detected in the urine samples of the animal groups treated with different doses of each extract throughout the 14 days of control. No obvious treatment-related alterations were seen in the stomach, liver or kidney weights when these were expressed in relation to body weight. (Data not shown).

In this experiment no evidence of acute toxicity was found at any dose level up to the highest doses tested (15 g dry extract/kg b.w., 100 times the effective dose). This does represent the non-observed-adverse-effect level (NOAEL), indicating that the safety margin of both extracts is high.

Evaluation of the *in vivo* effects of 5% infusion and 10% hydroalcoholic extract of *A. aroma* leaves on ethanol-induced gastric ulcer

Determination of lowest effective dose

As shown in Table 1, under our experimental conditions, effective gastric protection was achieved with pretreatment at a minimum dose of 150 mg/kg b.w. of both 5% I and 10% HE. The ulcer percentage was significantly lower (1.41±0.11 and 2.16±0.07%, respectively) compared to the untreated group ($p<0.05$), suggesting an interesting protection against the harmful agent. Taking into account the results presented in this section, the subsequent experiments were performed using only the lowest effective dose (150 mg dry extract /kg b.w.).

Table 1: Effect of *A. aroma* leaves extracts on the stomach in an ethanol induced ulcer model.

Treatments	Number of ulcers/ stomach	Severity of gastric lesion	Ulcer Percentage (%)	Protection (%)
Vehicle	0.00	0.00	0.00	-
Ethanol 100°	5.33±0.27	2.00±0.10	7.91±0.40	0.00
Sucralfate (100 mg/kg)	0.33±0.02[a]	0.67±0.03[a]	0.38±0.02[a]	95.14±4.76
Tusca 5 % I (75 mg/kg)	4.98±0.09[b]	2.00±0.08[b]	6.55±0.98[b]	17.2±0.86[a,b]
Tusca 5 % I (150 mg/kg)	1.33±0.07[a,b]	1.00±0.05[a,b]	1.41±0.11[a,b]	82.20±4.11[a,b]
Tusca 5 % I (300 mg/kg)	0.54±0.03[a,b]	0.98±0.04[a,b]	0.67±0.07[a,b]	91.5±4.58[a]
Tusca 10% HE (75 mg/kg)	5.01±0.09[b]	2.00±0.10[b]	7.20±0.10[a,b]	9.00±1.40[a,b]
Tusca 10% HE (150 mg/kg)	1.67±0.08[a,b]	1.00±0.05[a,b]	2.16±0.07[a,b]	72.65±3.63[a,b]
Tusca 10% HE (300 mg/kg)	1.49±0.10[a,b]	1.00±0.10[a,b]	2.00±0.13[a,b]	74.7±1.70[a,b]

Data are presented as the means ± SD for n=6 rats per group. [a]$p<0.05$ vs. untreated ulcer group, [b]$p<0.05$ vs. sucralfate treated group. I: infusion, HE: hydroalcoholic extract.

Macroscopic analysis

In order to determine the gastroprotective efficacy of the evaluated treatments, gross signs of damage were examined first. Oral administration of ethanol caused severe hemorrhagic gastritis with high severity scores [Table 1]. As shown in Figure 1, ulcerative lesions cover a large area, revealing extensive damage to the gastric mucosa. Interestingly, oral pretreatment with 10% HE was able to reduce the extent of the ulcerated area, showing a significantly lower ulcer percentage compared with the untreated ulcer group**.** This is reflected in an important protection percentage of gastric lesions (72.65±3.63%).

Similarly, oral pretreatment with 5% I significantly decreased the extension of ulcerated tissue, showing lower ulcer percentage and a high protection percentage of gastric lesion (82.20±4.11%) in a significantly higher range than with the ethanolic leaf extract ($p<0.05$). Although the extracts of tusca leaves were able to protect the gastric mucosa against the harmful agent ($p<0.05$), this effect was lower than that of sucralfate at a dose of 100 mg/kg.

As shown in Figure 1 and e only few fields of hyperemia were observed, with a similar aspect to the positive control group treated with sucralfate [Figure 1].

A- E: Macroscopic analysis. (A): Normal control rats. (B): Untreated ulcer rats (only ethanol administration). (C): Sucralfate (100 mg/kg) pretreated rats. (D): 5% I pretreated rats. (E): 10% HE pretreated rats.

A1- E1: Histological analysis. (A1): Stomach of normal rat showing normal histological structure of the mucosa and submucosa layers. (B1): Stomach of ethanol treated rat showing focal ulceration with surface mucosa loss (sl), hemorrhagic focus (hf), leukocyte infiltration (li) and submucosal edema (ed). (C1): Stomach of sucralfate (100 mg/kg) pretreated rats showing intact histological structure of the mucosa. (D1): 5% I (150 mg/kg) pretreated rats showing intact histological structure of the mucosa with scarce submucosal edema. (E1): 10% HE (150 mg/kg) pretreated rats showing only surface mucosa scarcely attacked.

Scale bars = 25μm. Hematoxylin and Eosin staining. I: infusion of *A. aroma* leaves, HE: hydroalcoholic extract of *A. aroma* leaves. Full page width.

Microscopic analysis

Sections from gastric wall samples of the different animal groups were stained with hematoxylin/eosin, Periodic Acid–Schiff (PAS) and Alcian blue dye. Normal control rats receiving the vehicle showed normal mucosal architecture with intact epithelial surface and submucosa layers [Figure 1]. In contrast, the administration of ethanol caused a severe gastric injury with surface epithelial cell destruction, loss of the surface mucosa layer and hemorrhagic points. The mucosa and sub-mucosa layers showed also diffuse leukocyte infiltration and extensive edema in the second layer [Figure 1]. Interestingly, treatment of the animals with the infusion or the hydroalcoholic extract of tusca leaves contributed to preserve the normal histology of the whole gastric wall, showing a scarcely attacked mucosa, scant inflammatory cell invasion and scarce submucosal edema [Figure 1]. These effects were very similar to those of sucralfate [Figure 1] representing a significant protective action.

All the above pretreatments resulted in a significant staining intensity with PAS dye for mucin-like glycoproteins [Figure 2], particularly of neutral mucins, which are secreted by superficial cells of the gastric epithelium to form the protective mucus layer.

In addition, sections from gastric wall stained with Alcian blue dye showed a conserved mucus layer and mucus in the gastric pit of groups pretreated with sucralfate and 5% I [Figure 2] in a similar way to the normal control group. In the 10% HE pretreated group a layer of mucus was detected, although slightly lesser than in the group treated with infusion and mainly distributed in the gastric pit [Figure 2].

A- E: PAS dye. (A): Normal control group: surface mucous cells were strongly stained with PAS. (B): Untreated ulcer group: The PAS reaction was reduced in surface cells. (C): Positive control group treated with sucralfate (100 mg/kg): the PAS reaction remained in surface cells. (D): Treated group with 5% I (150 mg/kg) and (E): Treated group with 10% HE (150 mg/kg). Arrows mark positive mucus stain with PAS.

A1- E1: Alcian blue dye. (A1): Normal control group: the mucus was strongly stained in a mucus layer and in the gastric pit. (B1): Untreated ulcer group: the Alcian blue stain appeared reduced in surface cells. (C1): Positive control group (sucralfate, 100 mg/kg) the mucus remained on the surface of cells and in pits. (D1): Treated group with tusca leaves 5% I (150 mg/kg) and (E1): Treated group with tusca leaves 10% HE (150 mg/kg). Arrows mark mucus stained with Alcian Blue.

Scale bars = 25μm. I: infusion of *A. aroma* leaves, HE: hydroalcoholic extract of *A. aroma* leaves. Full page width.

Determination of gastric wall mucus

As shown in Table 2 an oral pretreatment with tusca leaf infusion caused a significant increase (3.35 times) in the amount of adherent acid mucin-like glycoproteins compared with the non- pretreated group. As expected, sucralfate enhanced mucus production (3.59 times) and there were no significant differences between the groups treated with sucralfate or with tusca infusion ($p>0.05$). On the other hand, ethanolic extract pretreatment was not equally effective, showing a significant increase in adherent mucus (2.02 times) compared with the non-pretreated group but to a lesser degree than infusion or sucralfate ($p<0.05$).

Neutrophil infiltration. MPO activity

MPO activity, an index of neutrophil infiltration, was significantly increased in the ethanol-treated group compared with the untreated normal group ($p<0.05$). This result would indicate a substantial neutrophil influx into the gastric mucosa in response to the ethanol injury. As shown in Table 2, this inflammatory effect was markedly attenuated by pretreatment with 5% I or 10% HE evidenced by a significant reduction (65 and 62%, respectively) in MPO activity as compared to the ulcerated untreated group ($p<0.05$). Comparatively, the sucralfate pretreated group also showed a strongly reduction in gastric MPO activity (76%) ($p<0.05$).

Evaluation of gastric mucosal oxidative stress markers in ethanol-treated rats

Ethanol is a damaging factor that promotes significant GSH depletion in the mucosa ($p<0.05$) compared with normal untreated animals. Interestingly, oral pretreatment with tusca leaf infusion was able to maintain GSH levels at values similar to the normal control group and to the sucralfate treated group. In contrast, pretreatment with hydroalcoholic extract of tusca leaves was less effective, showing lower GSH levels than those determined in the groups treated with infusion or sucralfate ($p<0.05$). Despite this significant difference, it should be noted that both pretreatments contributed to maintain GSH levels in the gastric tissue [Table 2].

Ethanol treated rats exhibited a significant increase ($p<0.05$) in gastric mucosa MDA levels. We observed that pretreatment with either 5% I or 10% HE significantly decreased MDA tissue accumulation promoted by ethanol [Table 2]. In the same way as was observed with GSH levels, tusca infusion pretreatment was significantly more effective ($p<0.05$) than alcoholic extract in preventing lipid peroxidation.

Table 2: Effects of *A. aroma* leaves extracts on mucus content, oxidative stress markers and neutrophil infiltration.

Treatments	Mucus (µg of Alcian Blue/g stomach)	GSH (µg GSH/mg protein)	MDA (pmol /mg protein)	CAT (U/mg protein)	MPO (U/mg protein)
Vehicle	32.13±1.61	251.64±12.58	0.97±0.05	291.41±14.57	0.70±0.03
Ethanol 100°	9.28±0.46	11.29±0.56[a]	102.51±5.13[a]	568.11±28.41[a]	2.01±0.10[a]
Sucralfate (100 mg/kg)	33.35±1.67[a]	237.36±11.87[b]	0.57±0.03[a,b]	151.38±7.57[a,b]	0.48±0.02[a,b]
5% I (150 mg/kg)	31.13±1.56[a]	253.94±12.70[b]	27.92±1.40[a,b,c]	225.38±11.27[a,b,c]	0.74±0.04[b,c]
10% HE (150 mg/kg)	18.81±0.94[a,b,c]	111.94±5.60[a,b,c,d]	32.43±1.62[a,b,c,d]	370.77±18.54[a,b,c,d]	0.78±0.04[a,b]

Data are presented as the means ± SD for n=6 rats per group in an ethanol induced ulcer model. [a]$p<0.05$ vs. normal group, [b]$p<0.05$ vs. control ulcer group, [c]$p<0.05$ vs. sucralfate control group, [d]$p<0.05$ vs. tusca 5% I treated group. I: infusion, HE: hydroalcoholic extract, GSH: glutathione, MDA: malondialdehyde, CAT: catalase, MPO: myeloperoxidase.

Table 3: Effects of *A. aroma* leaves extracts on gastric secretion parameters.

Treatments	Gastric juice volume (ml)	pH	Free Acid (mEq/L/100 g bw)	Pepsin activity (µM tyrosine/ml)
Vehicle	4.5±0.01	1.0	43.2±2.2	16.32±0.82
Omeprazole (20 mg/kg)	1.5±0.01[a]	5.0	0.0[a]	2.85±0.14[a]
Tusca 5% I (150 mg/kg)	2.5±0.01[a,b]	1.0	34.3±1.7[a,b]	12.28±0.61[a,b]
Tusca 10% HE (150 mg/kg)	4.5±0.01[b,c]	1.0	33.3±1.7[a,b]	11.53±0.58[a,b]

Data are presented as the means ± SD for *n*=6 rats per group in a pylorus ligation model. [a]$p<0.05$ vs. negative control group, [b]$p<0.05$ vs. omeprazole (positive control drug) treated group, [c]$p<0.05$ vs. tusca 5% I treated group. I: infusion, HE: hydroalcoholic extract, bw: body weight.

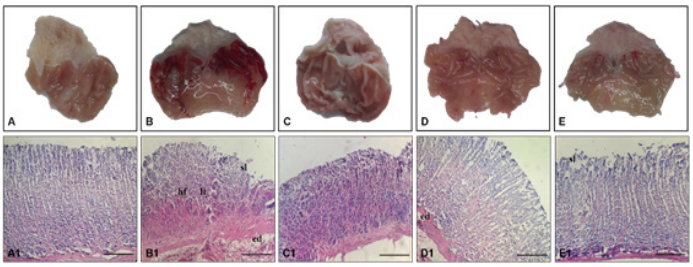

Figure 1: Macroscopic appearance and histological analysis of the rat´s stomachs.

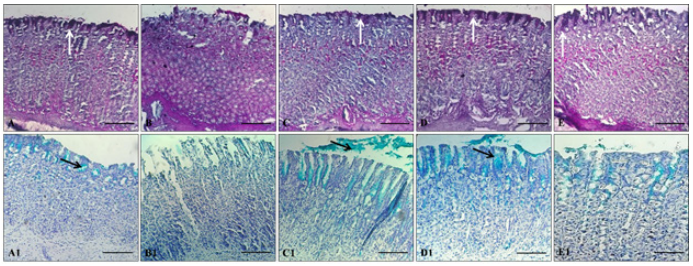

Figure 2: Histochemical analysis of gastric mucosa stained with PAS (Periodic Acid Shiff) and Alcian Blue dye.

It is interesting to note that the animals pretreated with 5% I or 10% HE showed significantly lower values ($p<0.05$) of CAT activity compared to control ulcer animals, whereas sucralfate pretreatment showed still much lower values in the enzyme activity.

Evaluation of the *in vivo* effects of 5% infusion and 10% hydroalcoholic extract of *A. aroma* leaves on rat pylorus ligation model

We studied the effect of 5% I and 10% HE on gastric juice parameters such as volume, pH and free acid using a rat pylorus ligation model. Although treatment with the infusion caused a significant decrease in the volume of gastric juice and free acid content compared with untreated animals, it did not change their pH. Similar results were obtained when treating the animals with 10% HE, but with no volume reduction [Table 3]. Only the group treated with omeprazole (an effective anti-secretory drug) at a dose of 20 mg/kg displayed a significant decrease in the volume of gastric juice accompanied by a drastic decrease in free acid and a rise in pH.

The activity of pepsin in the gastric juice of the animals treated with 5% I or 10% HE was significantly lower ($p<0.05$) than in the untreated animals; however, it was not reduced to the levels found in the animals treated with omeprazole, as shown in Table 3.

DISCUSSION

Acacia aroma Gill. ex Hook. and Arn. is a medicinal plant with extensive use in folk medicine[13] and the present work highlights the gastroprotective effects of two extracts from its aerial part using ethanol-induced gastric ulcer models in rats.

The phytochemical analysis of 5% I and 10% HE showed a rich content of chemicals species such as flavonoids and tannins. Flavonoids from several plants have a significant gastroprotective activity in experimental models of gastric ulceration, mainly due to their cytoprotective, antisecretory and antioxidant properties.[28] Tannin-containing herbs have been proposed to treat mild gastric ulcers because of the ability of these compounds to form complexes with proteins of cell membranes and mucus itself and also because of their antioxidant capacity.[29] Given these evidences, we focused on the antiulcerogenic potential of the tusca leaf extracts using an ulcer induced experimental model in rats.

The starting point of this study was the determination of the protective effectiveness of tusca extracts on the gastric mucosa subjected to a harmful agent. Under our experimental conditions, absolute ethanol has the ability to damage the gastric mucosa to a large extent, as shown by macroscopic and microscopic analyses. Interestingly, 5% I and 10% HE prevented mucosa injury when administered orally in rats. The minimum effective dose for both preparations was 150 mg/kg.

Increased mucus secretion by gastric mucosal cells seems to be important in acute protection.[30] It should be emphasized that our experimental model implies the use of a single effective dose of each vegetal extract as a pretreatment in experimental animals. Under this condition, we demonstrated an effective increase in mucus content in animals pretreated with 5% I or 10% HE, similar to the effect of sucralfate. It is likely that enhanced secretion and release of mucus could contribute to an intraluminal dilution of damaging agents, it being a rapid strategy to prevent mucosal injuries, as proposed by Szabo.[7] We think that the increased mucus content noted in pretreated rats could be attributed to the presence of flavonoids in the extracts, a plant compound with recognized mucus secretagogue activity.[28]

We also studied the effect of 5% I and 10% HE on gastric juice parameters using a pylorus ligation model in rats.[31] The treatments with both tusca extracts had just a little antisecretory effect and cause a small change in the free acid concentration, with a slight decrease in pepsin activity. Taking the above results together, it would be possible to affirm that the tusca extracts strengthened the mucosal barrier with a slightly reduction of gastric acidity. These findings are similar to the effect of most of the available bismuth salts,[32] which supports the gastroprotective concept of the tusca preparations evaluated in the present work.

The presence of gastric ulcers has been associated with an increase in MPO activity, a marker of neutrophil infiltration.[33] Under our experimental conditions, ethanol-induced mucosal injury was accompanied by inflammatory cell infiltration and a consequent increase in MPO activity. Interestingly, rats pretreated with tusca extracts showed no gastric inflammation and normal levels of MPO activity. We think that these effects could be important to break the vicious cycle that exists between infiltration of inflammatory cells and the formation of Reactive Oxygen Species (ROS), as was observed in several studies.[34]

Oxidative stress results from an imbalance between the generated ROS and the available antioxidant capacity. The involvement of sequential ROS-mediated induction of lipid peroxidation in the pathogenesis of gastric injury has been widely confirmed, the infiltrating inflammatory cells being a major source of ROS production.[34] In this regard, an effective protector of gastric mucosa should increase antioxidant defenses and/or eliminate free radicals.

Flavonoids and all polyphenols in general, have a free radical scavengers potential, mainly in organs exposed to high concentrations thereof such as the gastrointestinal tract.[35] In this work it is possible to suggest that, at least in part, the gastroprotective effect elicited by tusca extracts could be associated with both, the *in vitro* ability to scavenge free radicals and the flavonoid content.

It is well established that several polyphenol compounds enhance the activities of glutathione reductase and other enzymes, contributing to a stable GSH supply.[36] We observed a significant preservation of the gastric mucosal GSH levels in animals pretreated with 10% HE but particularly with 5% I. Thus, it is possible to suggest that the above treatments increase GSH availability to reduce intrinsic oxidative stress after ethanol administration.

CAT is a key antioxidant enzyme to defend cells against the damaging effects of oxygen free radicals.[37] The data presented here indicates a lesser CAT activity in the gastric mucosa of animals pretreated with 5% I or 10% HE compared with rats receiving only ethanol. This leads us to suggest that the free radical scavenging capacity of extracts and their positive effects on GSH levels could allow the gastric mucosa to overcome the oxidative effect of alcohol with no need of increased CAT activity. Ethanol-induced oxidative stress evidenced by high levels of MDA in gastric tissue, in a process probably dependent on neutrophil infiltration and activation.[38] However, the animals pretreated with both extracts of tusca leaves showed lower MDA levels. These findings reflect a protective effect against the oxidative damage caused by ethanol.

The pharmacological potential of a plant extract necessarily requires a toxicological analysis that justifies its safe use thereof. *A. aroma* is a plant often used in traditional folk medicine.[13] Although this fact could account for its safety, there is not scientific information about its toxicity. In the present work, we evaluated the acute toxic effect of 5% I and 10% HE in healthy male and female adult rats. The lack of toxic effects at all doses up to the highest one tested suggested that the lethal dose 50 of both extracts would be above 15 g /kg. Although this result allows us to suggest a high safety margin for the two extracts assayed, additional experiments are in progress to complete their toxicological profile.

CONCLUSION

This study shows for the first time the gastroprotective efficacy of 5% I and 10% HE as a pretreatment in an animal model of ethanol-induced gastric damage. The observed effect can be attributed to their antioxidant activity and neutrophil infiltration inhibition as well as to an increase in mucus secretion by gastric mucosal cells. Thus, the current study highlights the benefits of tusca extracts as a prophylactic approach to maintain gastric mucosal integrity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

ANOVA: Analysis of variance; **BSA:** bovine serum albumin; **CAT:** Catalase; **DPPH:** 2,2-diphenyl-1- picrylhydrazyl; **EDTA:** etilendiamino tetraacetic; **GAE:** Gallic acid equivalent; **GSH:** Glutathione; **HE:** Hydroalcoholic extract of tusca leaves; **I:** Infusion of tusca leaves MDA: Malondialdehyde; **MPO:** Myeloperoxidase; **NOAEL:** Non-observed-adverse-effect level; **PAS:** Periodic acid shiff; **PBS:** Phosphate buffer saline; **ROS:** Reactive oxygen species.

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