Trop J Nat Prod Res, May 2022; 6(5):745-753 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Mechanisms Involved in the Antiulcer Activity of *Capparis atamisquea* Polar Leaves Extracts

Felix F. Taboada[1], Natalia C. Habib[2], Susana B. Genta[1]*

[1]*Instituto de Biología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán and Instituto Superior de Investigaciones Biológicas (INSIBIO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Chacabuco 461, San Miguel de Tucumán, Argentina*
[2]*Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina*

ARTICLE INFO

Article history:
Received 06 April 2022
Revised 26 April 2022
Accepted 06 May 2022
Published online 04 June 2022

ABSTRACT

Capparis atamisquea Kuntze (atamisqui) is used in northern Argentina to treat digestive disorders, however, scientific information on its potential therapeutic application has not been established. The study was designed to evaluate the gastroprotective effect and possible involvement mechanism of 5% infusion and 10% hydroalcoholic *Capparis atamisquea* leaf extracts in experimental model of acute gastric ulceration. Ethanol-induced gastric ulcer model was performed in adult Wistar rats followed by macroscopic and histological analysis, gastric mucus content quantification, mucosal antioxidant system study, myeloperoxidase activity determination and nitric oxide and prostaglandin E2 content. The *in vitro* antioxidant activities of both extracts were also determined. Pre-treatments with both extracts caused a decrease in ethanol-induced gastric injuries at a macroscopic and microscopic level. A significant increase in gastric mucus content was also observed. The level of nitric oxide and prostaglandin E2 in the gastric mucosa increased significantly in the pre-treatment groups compared to the ulcer control group. These results show the participation of these mediators in the gastroprotective effect. In addition, both extracts showed moderate *in vivo* and *in vitro* antioxidant activity. These results contribute to validate the use of this native plant which represents a natural alternative for gastric ulcer prevention.

Keywords: Antiulcer activity, Atamisqui, Gastroprotective mechanisms, Polar extracts.

Introduction

In recent years, interest in alternative therapies and the therapeutic use of natural products has increased especially those derived from plants.[1] The approach for drug development from plant resources depends on the purpose. Thus, different strategies will result in a herbal medicine or in an isolated active compound. The most common way to select a suitable plant for pharmacological testing is a careful observation of their use in folk medicine in different cultures, whereas the preparation procedure may give an indication of the best extraction method.

Capparis atamisquea Kuntze which is synonymous with *Atamisquea emarginata* (common name atamisqui) is a species belonging to the Capparaceae family. It has simple, alternate leaves, glabrous dark green on the upper surface, densely pubescent and greyish on the underside. They emit an unpleasant odor, as well as their flowers and fruits,[2] which are popularly known as smelly grass.

This plant species is distributed in Mexico and in southern South America (Bolivia, Chile and Argentina). Particularly, in Argentina it grows spontaneously as bushes in a vast region, mainly in the xerophilous forests of the Chaco western phytogeographic district and in the Monte phytogeographic province.[3] The wide distribution is favored by migratory birds using routes where atamisqui abounds. Therefore, this plant species provides food in summer and contributes in this way to the dissemination of the plant which colonizes new territories each year.

Native inhabitants obtain the different parts of these species from specimens that grow wildly. However, it is a plant which can be easily cultivated from seed or cuttings and could contribute greatly to the preservation of natural biodiversity and the environment.

Popular medicine in northern Argentina mentions a variety of medicinal uses of atamisqui leaves, such as, anti-inflammatory, anti-helminthic, and particularly those used to treat digestive and gastric disorders.[4] Until now, only few biological activities belonging to other species of the genus *Capparis* were scientifically validated.[5] Scientific information on the potential therapeutic application of atamisqui has not been found yet.

Chemical composition is another aspect which has been scarcely explored in the *Capparis* species, and particularly in *Capparis atamisquea*. Kaempferol, iso- rhamnetin and quercetin were the main flavonoid aglycone found in leaf material extracted with 80% methanol.[6] It is important to notice that flavonoids quercetin and kaempferol have been the center of several studies about their gastroprotective properties in several experimental models.[7,8,9]

Although the etiology of gastric ulcer is complex, an imbalance between aggressive and protective factors of mucosa is involved.[10]

The current pharmaceutical market offers drugs which have beneficial therapeutic properties but are scarce and also generate adverse effects and/ or pharmacological interactions.[11] These considerations justify the study of the potential therapeutic value of the species *Capparis atamisquea* Kuntze to prevent/cure gastric ulceration. Consequently, this study was designed to evaluate the gastroprotective activity of two *Capparis atamisquea* leaf extracts in an experimental model of acute gastric ulceration.

*Corresponding author. E mail: susana.genta@fbqf.unt.edu.ar
Tel: +54 0381 4107214

Citation: Taboada FF, Habib NC, Genta SB. Mechanisms Involved in the Antiulcer Activity of *Capparis atamisquea* Polar Leaves Extracts. Trop J Nat Prod Res. 2022; 6(5):745-753.

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Materials and Methods

Plant Material and extraction process

The Leaves of *Capparis atamisquea* Kuntze were collected in September 2019 from Silípica, Santiago del Estero, Argentina, located at 28°06′29″S, 64°08′49″W, 170 m.a.s.l. Voucher specimens (LIL-615921) are deposited in the herbarium of "Fundación Miguel Lillo", San Miguel de Tucumán, Tucumán, Argentina.

The plant leaves were dried in an oven at 40 ºC and ground to 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.[12] 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 -20ºC before being tested. Before each experiment, the extracts were dissolved in distilled water and given to rats orally.

Quantitative phytochemical analysis

The Folin-Ciocalteu method was used to determine the total phenolic and tannins contents in both extracts with slight modifications as were described by Taboada *et al*.[13] Gallic acid was used as a standard while tests were carried out in triplicate and the result was expressed as mg gallic acid equivalents (GAE)/g dry extract.

Flavonoid content determination was based on the formation of a flavonoid-aluminum complex according to Taboada *et al*.[13] Flavonoids amount was expressed as mg quercetin equivalents/g dry extract and all tests were carried out by triplicate.

Free radical scavenging activity: in vitro assay

The free radical scavenging capacity of the 5% I and 10% HE of *C. atamisquea* leaves was evaluated by the decrease in 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorbance accordingly to Blois.[14] EC_{50} values, the sample concentration necessary to decrease the initial DPPH concentration by 50%, were calculated by nonlinear regression analysis of the plotted depuration % vs. sample concentration using SPSS® statistic software. All assays were performed by triplicate.

Antilipoperoxidative activity

The antilipoperoxidative activities of 5% I and 10% HE samples were evaluated using β–carotene–linoleate system, as described by Kumar *et al*.[15] Quercetin and BHT were used as reference antioxidants.

All samples were assayed in triplicate. The percentage of antioxidant activity (% AA) was calculated in relation to BHT, which was the sample with the highest antilipoperoxidative activity in this test and it was considered 100%.

Animals

Adult male Wistar rats were selected for all the experiments aged from 8 to 12 weeks (weight 200 ± 20 g) The animals came 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 weren´t any identified contaminants in the food or water that could interfere with the results of the study. The individual rat was considered the experimental unit within the study. The total number of animals used in this work was 102. To minimise confounding factors treatment and measurement orders was randomized.

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) and ARRIVE guidelines 2.0. 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). We selected a small sample size because the gastroprotective effect of *C. atamisquea* leaf extracts was evaluated *in vivo* for the first time in the present study, and therefore, the initial intention was to gather basic evidence regarding the effect of these natural products.

Evaluation of the gastroprotective effect

Ethanol-induced gastric ulcer model.

The protocol was performed as follows: 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 rats per group, a total number of 48) using a computer based random order generator. Then, each group was given three different doses of 5% I or 10% HE (75, 150 and 300 mg/kg b.w.) orally as mentioned above. The untreated ulcer group received distilled water, whereas sucralfate (100 mg/kg b.w.) was administered orally 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 given absolute ethanol (6 ml/kg b.w.) orally to induce gastric ulcers. For each animal, two different investigators were involved as follows: a first investigator (NH) administered the treatment based on a randomization table. This investigator was the only person aware of the treatment group allocation. A second investigator (FT, unaware of treatment) was responsible for the ethanol administration, and performed the microscopic, macroscopic and biochemical procedures.

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 and opened, rinsed with cold PBS and photographed for analysis.

The degree of gastric mucosal damage calculated to establish the minimum protective dose of 5% I and 10% HE of atamisqui leaves was evaluated by gross pathology, counting the number of ulcers per stomach. The ulcer area (mm^2) was measured using ImageJ 1.48d software (National Institutes of Health, USA) and expressed as a percentage (%) of the 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 at 4% phosphate-buffered formaldehyde for 24 h. Then, the samples were washed, dehydrated with alcohol, cleared in xylene and embedded in paraffin in a hot air oven (56°C). The paraffin blocks were cut into 5 µm thick sections with a microtome.

The tissue sections obtained were deparaffinized and stained with hematoxylin and eosin, Alcian blue or Periodic Acid–Schiff (PAS) dyes for histopathological examination through the photomicroscope light (NIKON-fluophot).

A qualified observer, unaware of the identity of the specimens, was chosen to perform all histopathological procedures in order to avoid any certain bias.

Determination of the adherent gastric mucus

The mucus content attached to the gastric wall was determined according to Taboada *et al*.[13] The procedure was as follows: 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 colored solution obtained, 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 and inflammatory parameters
Preparation of gastric mucosa homogenates
After evaluating the ulcer percentage, the gastric mucosa of each experimental group was scraped off, weighed and homogenized at 4°C in 200 mM sodium phosphate buffer, pH 7.4, containing 1mM EDTA and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 5 µg/ml pepstatin and 5 µg/ml aprotinin) finally obtaining 10% (p/v) aqueous homogenates.
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 4ºC for 15 min. The resulting supernatant was used to evaluate malondialdehyde (MDA) levels and catalase (CAT) activity. Protein estimation was carried out by Lowry et al.[16] method using bovine serum albumin as a standard.

Determination of MPO activity
Myeloperoxidase activity was measured to assess neutrophil infiltration as described by Bradley et al.[17] with modifications. The pellet obtained as previously described in section 2.6.5 was resuspended in 50 mM phosphate buffer, pH 6, containing 0.5% hexadecyltrimethylammonium bromide, and sonicated for 1 min in an ice bath. The samples were centrifuged at 600 g for 20 min at 4ºC and the supernatants were diluted 1:30 with 50 mM phosphate buffer, pH 6, containing 0.167 mg/ml O-dianisidine dihydrochloride 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 25ºC. Enzymatic activity was expressed as U/mg protein.

Determination of reduced glutathione (GSH) levels
GSH in stomach homogenate was measured as described by Ellman using the 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent.[18] GSH levels were calculated using a calibration curve performed with the GSH 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 Beuge and Aust.[19] MDA reacts with thiobarbituric acid to give a red species absorbing at 535 nm. The MDA concentration of the sample was calculated with an extinction coefficient of $1.56x10^5\ M^{-1}\ cm^{-1}$. The results were expressed as nmoles of MDA formed/mg protein.

Determination of CAT activity
CAT activity was measured according to the modified method of Aebi.[20] 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 $0.043M^{-1}\ cm^{-1}$. One units of CAT was defined as nmoles H_2O_2 destroyed/min at 25 ºC.

Determination of nitric oxide (NO) content
Gastric mucosal NO production was estimated indirectly as nitrite concentration according to the method of Goshi *et al*.[21] NO content (pmol/g tissue) was quantified indirectly as total nitrite by the use of Griess reagent which reduces nitrate into nitrite. The pink azo-dye produced by the reaction of nitrite with sulfanilic acid followed by the subsequent coupling with N-(1-naphthyl)-ethylene diamine was measured colorimetrically at 540 nm.

Determination of prostaglandin E_2 (PGE_2) content
Gastric mucosal PGE_2 levels were determined in stomach homogenates using a commercial ELISA kit from ThermoFischer according to the manufacturer´s instructions. The absorbances were measured at 405 nm using a microplate ELISA reader (BioTek© ELx50). The results were expressed as pg of PGE_2/ mg protein.

Ethanol-induced gastric lesions in NEM, L-NAME and indomethacine-pretreated rats
To elucidate the possible pharmacological mechanism(s) in the gastroprotective effect of *C. atamisquea* leaf extracts against absolute ethanol-induced gastric ulceration, Wistar rats in 9 groups (n = 6, resulting in a total number of 54 animals) were pretreated with the sulfhydryl compounds blocker NEM (10mg/kg b.w, i.p.), the NO synthase inhibitor L-NAME (70 mg/kg b.w, i.p.) and the COX inhibitor indomethacin (10 mg/kg b.w, i.p.). All pretreatments were given 30 min before atamisqui infusion (150mg/kg b.w, orally) or hidroalcoholic extract (150mg/kg b.w, orally) treatment. Sucralfate (100mg/kg b.w, orally) was used as positive control. One hour later, each animal received 6 ml/kg b.w. of absolute ethanol, orally. The stomachs were removed 1 hour after ethanol administration, and the gastric mucosal lesions were evaluated as described previously.[22]

Statistical analysis
All the data were expressed as Mean ± SD and analysed 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 statistically significant.

Results and Discussion

Phytochemical evaluation of 5% infusion and 10% hydroalcoholic extract of the atamisqui leaf.
Two aqueous preparations were obtained from the atamisqui leaves, a 5% I and a 10% HE. The extraction process result was 12.2% and 12.4% of powdered extract (w/w of plant material) for the 5% I and 10% HE, respectively. Phytochemical evaluation of 5% I indicated a total phenolic compound content of 17.95 ± 0.15 mg/g (gallic acid equivalent per gram of dry extract), whereas the total content of flavonoids was 12.10 ± 0.52 mg/g (quercetin equivalent per gram of dry extract). In contrast, the 10% HE showed a significantly lower ($P<0.05$) content of total phenolic compounds (11.17 ± 0.70 mg gallic acid equivalent/g) and flavonoids (2.33 ± 0.16 mg quercetin equivalent/g) compared to the 5% I. Both extracts presented a moderate flavonoid and total phenolic compounds content but none of these extracts showed the presence of tannins. Unlike this, other studies showed that methanolic extracts of atamisqui had a high content of flavonoids such as iso- rhamnetin and kaempferol.[6] This difference could be explained taking into account the different solvent and extraction process used by these authors.

In vitro antioxidant activity of 5% infusion and 10% hydroalcoholic extract of atamisqui leaves
Based on the results of the previous section, we have determined the *in vitro* DPPH radical scavenging activity of both the 5% I and the 10% HE in order to assess their biological activities in a preliminary way. Indeed, while the sample concentration of the quercetin (the positive control for this assay) necessary to decrease the initial DPPH concentration by 50% (EC_{50} values) was 1.7 ± 0.1 µg/mL, the EC50 values were 265.4 ± 13.3 µg/mL for the 5% I and 176.7 ± 8.8 µg/mL for the 10% HE. These results were significantly higher compared to quercetin ($P<0.05$) and to other plant extracts[23], indicating a poor radical scavenging ability of both extracts of atamisqui leaves, despite the fact that the content of flavonoids and total phenolics compounds was higher for the first extract. This difference could be explained by considering that the DPPH scavenging reaction medium is ethanolic, so the phytoconstituents of 10% HE would dissolve better.
Additionally, the *in vitro* antilipoperoxidative capacity of the atamisqui extracts was studied using the betacarotene bleaching assay. The 5% I showed a percentage of lipid antioxidant activity (28.00 ± 1.30%) significantly higher ($P<0.05$) than the one obtained for 10% HE (22.10 ± 0.90%). However, both extracts evidenced a significantly lower ($P<0.05$) percentage of antioxidant activity compared to positive control quercetine (84.96 ± 4.25%). This finding could perhaps be explained by the higher content of phenolics and flavonoids compounds in 5% I, or due to the aqueous medium in

which the beta-carotene bleaching assay is carried out. These results have a relative impact so as far as it is concerned; this is the first study that demonstrates the antioxidant activity of two extracts from atamisqui leaves. Only a high antilipoperoxidative activity for the monofloral atamisqui honey was proved.[4]

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

Determination of the lowest effective dose

The lowest dose of 5% I and 10% HE with effective gastric protection were determined using an ethanol-induced gastric ulcer model. The *in vivo* activity was achieved with a pretreatment at a minimum dose of 150 mg/kg b.w. of both extracts of *C. atamisquea* leaves. As shown in Table 1, the ulcer percentage (%) was significantly lower ($P<0.05$) (0.87 ± 0.04 and 1.65 ± 0.07%, respectively) compared to the untreated ulcer group, showing a Protection percentage of about 80%. It is interesting to notice that the minimum effective dose for both preparations have a strong correlation with the use of leaves for the preparation of tea in the Argentinian traditional medicine (500 mL of medicinal tea daily)[24], which would support its ethnopharmacological use. The subsequent experiments were performed using only the lowest effective dose of both extracts (150 mg dry extract /kg b.w.) based on these results.

Macroscopic analysis

On a comparative basis, oral administration of ethanol caused an intense hemorrhagic damage, evidenced by high severity scores (Table 1) and confluent ulcerative lesions in a large area (Figure 1b1).

In contrast, oral pre-treatment with 5% I reduced the number and severity of the ulcer, as well as the ulcer percentage compared to the untreated ulcer group. It is very interesting to notice that these observations were reflected in a high protection percentage of gastric mucosa (88.98 ± 4.51%), without a significant difference with the positive control group pre-treated with sucralfate (100 mg/kg b.w.).

Table 1: Effect of pretreatments with 5% infusion or 10% hydroalcoholic extract of *C. atamisquea* leaves on the stomach gross pathology in an ethanol induced ulcer model in rats.

Treatments	Number of ulcers/stomach	Severity of gastric lesion	Ulcer Percentage (%)	Protection (%)
Vehicle (untreated normal group)	0.00	0.00	0.00	-
Etanol 100° (untreated ulcer group)	5.33 ± 0.27	2.00 ± 0.10	7.91 ± 0.40	0.00
Sucralfate (positive control drug) (100 mg/kg)	0.33 ± 0.02[a]	0.67 ± 0.03[a]	0.38 ± 0.02[a]	95.14 ± 4.76[a]
Atamisqui 5 % I (75 mg/kg)	3.50 ± 0.18[a,b]	1.25 ± 0.06[a,b]	5.48 ± 0.27[a,b]	30.66 ± 1.53[a,b]
Atamisqui 5 % I (150 mg/kg)	1.00 ± 0.05[a,b]	0.67 ± 0.03[a]	0.87 ± 0.04[a,b]	88.98 ± 4.51[a]
Atamisqui 5 % I (300 mg/kg)	1.33 ± 0.07[a,b]	0.50 ± 0.02[a,b]	0.82 ± 0.04[a,b]	89.60 ± 4.48[a]
Atamisqui 10% HE (75 mg/kg)	5.50 ± 0.28[b]	1.50 ± 0.08[a,b]	6.19 ± 0.25[a,b]	21.71 ± 1.89[a,b]
Atamisqui 10% HE (150 mg/kg)	1.67 ± 0.08[a,b]	0.67 ± 0.03[a]	1.65 ± 0.07[a,b]	79.19 ± 3.96[a,b]
Atamisqui 10% HE (300 mg/kg)	2.00 ± 0.1[a,b]	0.67 ± 0.03[a]	1.48 ± 0.07[a,b]	81.29 ± 4.06[a,b]

Data is 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.

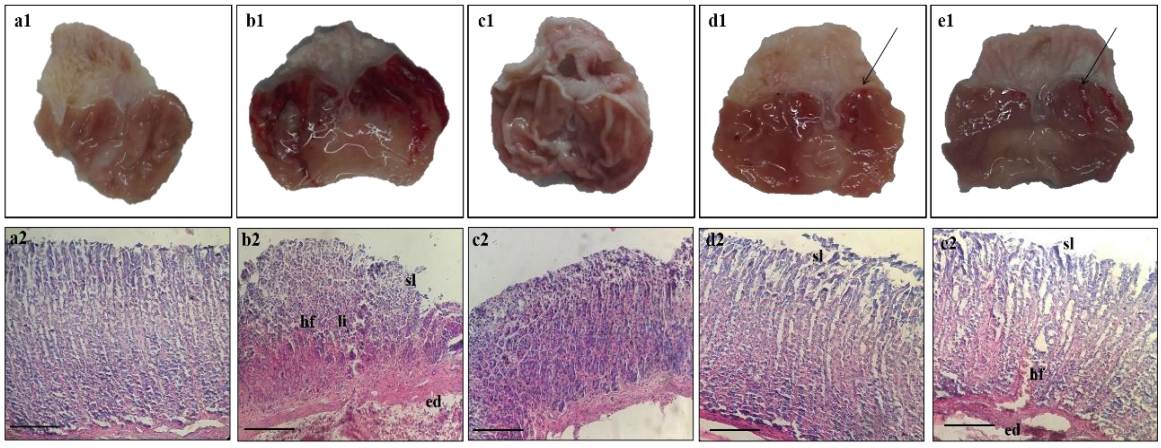

Figure 1: Macroscopic appearance of stomachs inner surface and histological analysis of the gastric wall in all experimental groups.
Macroscopic appearance of stomachs inner surface and histological analysis of the gastric wall (Hematoxylin&Eosin staining) in all experimental groups. (a1): Normal control rats. (b1): Untreated ulcer rats (only ethanol administration). (c1): Sucralfate (100 mg/kg) pretreated rats. (d1): 5% I of *C. atamisquea* leaves pretreated rats. (e1): 10% HE of *C. atamisquea* leaves pretreated rats. (a2): Stomach of normal rat showing normal histological structure of the mucosa and submucosa layers. (b2): Stomach of ethanol treated rat showing focal ulceration with surface mucosa loss (sl), hemorrhagic focus (hf), leukocyte infiltration (li) and submucosal edema (ed). (c2): Stomach of sucralfate (100 mg/kg) pretreated rats showing conserved histological structure of the mucosa (d2): 5% I (150 mg/kg) pretreated rats showing intact histological structure of the deeper mucosa with surface mucosa loss. (e2): 10% HE (150 mg/kg) pretreated rats showing surface mucosa scarcely attacked and small hemorrhagic focus. Scale bars = 25µm. I: Infusion, HE: Hydroalcoholic extract.

Oral pre-treatment with 10% HE was also able to decrease tissue damage. As shown in Table 1, a significantly lower (P<0.05) ulcer percentage was observed compared to the untreated ulcer group. This result indicated an acceptable protection percentage of gastric mucosa (79.19 ± 3.96%), although significantly lower (P<0.05) compared to the sucralfate control group. Figures 1d1 and 1e1 show the macroscopic appearance of the stomach inner surface of 5% I and 10% HE treated groups. Only few ulcer spots and streaks were observed, with a similar aspect to the positive control group pretreated with sucralfate (Figure 1c1). The action of sucralfate can be explained considering their ability to bind to proteins to form a barrier over the gastric epithelium, thus preventing the entry of the damaging agent.[25] Perhaps, both extracts of atamisqui can produce gastroprotection by the same route. All the macroscopic analyses indicate that both extracts of *C. atamisquea* leaves were effectively protected the gastric mucosa against the harmful agent at a dose of 150 mg/kg.

Histopathology of the Gastric Tissues

Sections from gastric wall samples were stained with hematoxylin/eosin, PAS and Alcian blue dye in order to go in depth in the study of Gastroprotective activity of *C. atamisquea* leaf extracts. The microscopic appearances of the gastric tissues from all experimental groups are presented in Figures 1 and 2.

Normal control group of rats showed normal gastric histological architecture both on the epithelial surface and in the submucosal layer (Figure 1a2). In contrast, the rats of the ethanol-induced ulcer control group showed extensive gastric injury with surface epithelial destruction, loss of mucosa layer patches and hemorrhagic points. The mucosa layer showed diffuse leukocyte infiltration, while the sub-mucosa layer had extensive edema (Figure 1b2). In contrast, the rats pre-treated with sucralfate (100 mg/kg) showed a near-normal gastric architecture (Figure 1c2), comparable to normal rats.

Consistent with the macroscopic findings, pre-treatment of the animals with 5% I contributed to preserving the normal histology of the entire gastric wall, showing only surface epithelial flaking (Figure 1d2). The infusion demonstrated comparatively better protection of the gastric mucosa than 10% HE. In fact, the gastric wall of the animals pre-treated with 10% HE showed scarcely attacked mucosa with a small hemorrhagic focus and scarce submucosal edema (Figure 1e2). In conclusion, both extracts of atamisqui leaves showed significant protective action at microscopic level. As shown in Figure 2d1 and 2e1 the pre-treatment with both extracts of atamisqui leaves resulted in a significant staining with PAS. This dye marks mucin-like glycoproteins, particularly the neutral mucins, which are secreted by superficial cells of the gastric epithelium to form the protective mucus layer.

In addition, positive Alcian Blue staining was observed in the lumen of the gastric pit, corresponding to acid glycosaminoglycans. In the pre-treated groups with 5% I or 10% HE a mucus layer was detected to be positive for Alcian Blue (Figures 2d2 and 2e2). The positive control drug, sucralfate, showed a slightly more intense staining with both PAS and Alcian Blue, than in the groups pre-treated with 5% I or 10% HE (Figures 2c1 and 2c2).

Gastric wall mucus

Another key factor in gastroprotection is the mucus barrier. In the ethanol induced ulcer model, this harmful agent caused a large loss of adherent mucus compared to the normal control group (P<0.05) (Figure 3). Surprisingly, the oral pre-treatment with 5% I significantly increased (P< 0.05) the mucus content in the gastric wall when compared to the ulcer control group. The pre-treatment with 10%HE was also able to increase significant amounts of adherent gastric mucus, but to a lesser degree than infusion (P<0.05). However, both results were significantly lower (P<0.05) than those obtained in the positive control group pre-treated with sucralfate (Figure 3). This control drug prevents depletion generated by ethanol of both acid mucins stained by alcian blue and neutral mucins stained by PAS. Meanwhile, atamisqui extracts produced a similar effect, although with lesser intensity compared to sucralfate. These findings could be related to a mucus secretagogue effect generated by the flavonoids from the extracts; since some of them are known to be responsible for increased mucus secretion.[26]

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

Oxidative stress is strongly involved in the development of ethanol-induced gastric injury. It is known that ROS produced by activated leukocytes triggers mucosal damage via lipid peroxidation and depletion of the antioxidant defenses, such as GSH.[27] As shown in Table 2, ethanol is a damaging agent that caused significant GSH depletion in the gastric mucosa homogenates (P<0.05) of the ulcer control group compared to normal untreated animals. Furthermore, oral pre-treatments with 5% I and 10% HE were able to maintain GSH values only at half the values corresponding to the normal control group and the positive control group. It is known that some phenolic compounds are capable of enhancing the activity of glutathione

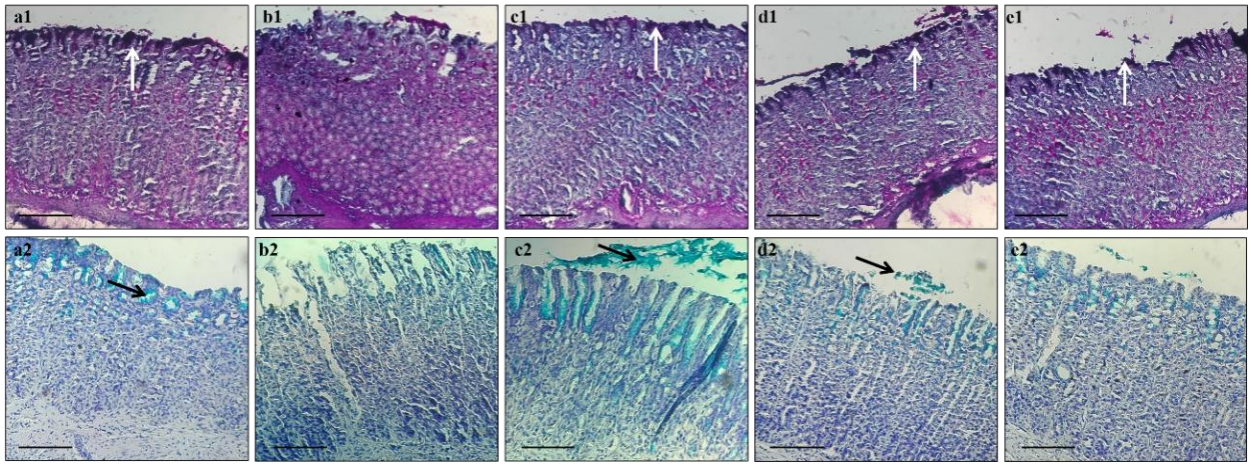

Figure 2: Histochemical analysis of gastric mucosa stained with PAS (Periodic Acid Shiff) and Alcian Blue dye.
Histological analysis of gastric mucosa stained with PAS and Alcian Blue dye. (a1): Normal control group: surface mucous cells strongly stained with PAS. (b1): Untreated ulcer group: the PAS reaction was reduced in surface cells. (c1): Positive control group treated with sucralfate (100 mg/kg): the PAS reaction remained in surface cells. (d1): Treated group with 5% Infusion (150 mg/kg) and (e1): Treated group with 10% hydroalcoholic extract (150 mg/kg): the PAS reaction remained in surface cells. (a2): Normal control group: the mucus was strongly stained in a mucus layer and in the gastric pit. (b2): Untreated ulcer group: the Alcian blue stain appeared reduced in surface cells. (c2): Positive control group (sucralfate, 100 mg/kg) the mucus remained on the surface of cells and in pits. (d2): Treated group with atamisqui leaves 5% I (150 mg/kg) and (e2): Treated group with atamisqui leaves 10% HE (150 mg/kg). White arrows mark positive mucus stain with PAS and black arrows mark mucus stained with Alcian Blue. Scale bars = 25µm. I: Infusion, HE: Hydroalcoholic extract.

reductase to provide a stable supply of GSH.[28] Further studies are required to link the chemical profile of the extracts with the effect on the enzymatic activity of glutathione reductase.

In addition, we determined the amount of malondialdehyde in gastric mucosa homogenates as a lipoperoxidation product. Rats which were treated with ethanol exhibited a significant increase (P<0.05) in gastric mucosa MDA levels. Additionally, we observed that pre-treatment with either 5% I or 10% HE significantly decreased MDA tissue values compared to untreated animals. Even so, those effects were significantly less than those showed by the sucralfate (Table 2). Comparing both extracts, the antilipoperoxidative activity resulted higher for 5% I than for 10% HE.

Catalase is an important component of the antioxidant defense system in the gastric mucosa, which protects it against ROS excess.[29] Under our *in vivo* experimental conditions, ethanol caused a significant increase (P<0.05) in the CAT activity with respect to normal values (Table 2), similar to the report by Zakaria et al.[30] Interestingly, the animals pretreated with 5% I or 10% HE demonstrated significant lower values (P<0.05) of CAT activity compared to the control ulcer animals, whereas sucralfate pre-treatment produced still much lower values in the enzyme activity. This finding is interesting, since it would account for the effect of extracts as modulator of antioxidant defenses.

Neutrophil infiltration. MPO activity

In the experimental model of gastric ulceration used in this study, ethanol caused an inflammatory effect with the subsequent neutrophil influx into the gastric mucosa. We have evaluated the MPO activity as a biochemical marker of neutrophil infiltration, finding a significant increase in the ulcer control group compared to the untreated normal group (P<0.05). As shown in Table 2, a significant reduction (P<0.05) in MPO activity was observed in pre-treated animals with both 5% I or 10% HE, compared to the ulcerated untreated group. Interestingly, the pre-treated groups achieved similar values to the untreated normal group. In comparison, the pretreatment with sucralfate also decreased significantly (P<0.05) the MPO value, even more than those the extracts values. In agreement with the microscopic observation, this finding represented a significant attenuation effect in neutrophil influx into the gastric mucosa caused by the extracts as was demonstrated in some plant products with recognized gastroprotective activity.[31,32]

The above results can indicate that the atamisqui extracts break the vicious cycle of neutrophil infiltration and the consequent generation of reactive oxygen species (ROS), producing both an antioxidant and anti-inflammatory activity in the gastric mucosa.

In summary, we show that the antioxidant activity of atamisqui extracts is mainly based on the inhibition of leukocyte infiltration. But even so, the antioxidant activity of both atamisqui extracts is poor and limited if we compare it to other plant extracts.[23] which leads us to analyze the participation of other mechanisms in the gastroprotective effect of atamisqui extracts.

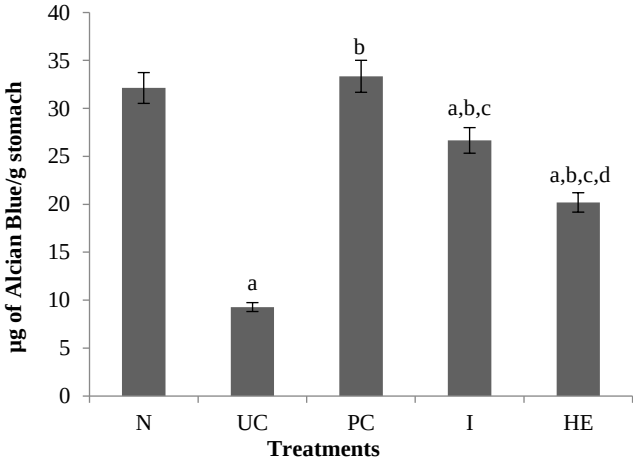

Figure 3: Effects of 5% infusion and 10% hydroalcoholic extract from the leaves of *C. atamisquea* on the production of adherent gastric mucus in an ethanol induced ulcer model in rats.

Data is presented as the means ± SD for n=6 rats per group. [a]P<0.05 vs. untreated normal group, [b]P<0.05 vs. ulcer control group, [c]P<0.05 vs. sucralfate (positive control drug) treated group, [d]P<0.05 vs. atamisqui 5% I treated group. N: normal group (receives vehicle), UC: ulcerated control group (receives ethanol 100º), PC: positive control group (receives sucralfate 100mg/kg), I: infusion, HE: hydroalcoholic extract.

Table 2: Effect of *C. atamisquea* leaves extracts in gastric mucosal oxidative stress and inflammatory markers on the ethanol induced ulcer model in rats: glutathione (GSH) level, malondialdehyde (MDA) level and catalase activity (CAT), mieloperoxidase activity (MPO), nitric oxide content (NO) and Prostaglandin E_2 (PGE_2).

Treatments	GSH (µg GSH/mg protein)	MDA (pmol /mg protein)	CAT (U/mg protein)	MPO (U/mg protein)	NO (pmol/mg protein)	PGE_2 (pg/mg protein)
Vehicle (untreated normal group)	251.64 ± 12.58	0.97 ± 0.05	291.41 ± 14.57	0.70 ± 0.03	0.45 ± 0.04	1227.92 ± 61.69
Ethanol 100° (untreated control ulcer group)	11.29 ± 0.56[a]	102.51 ± 5.13[a]	568.11 ± 28.41[a]	2.01 ± 0.10[a]	0.08 ± 0.01[a]	154.52 ± 7.72[a]
Sucralfate (positive control drug, 100 mg/kg)	237.36 ± 11.87[b]	0.57 ± 0.03[a,b]	151.38 ± 7.57[a,b]	0.48 ± 0.02[a,b]	0.49 ± 0.04[b]	1293.38 ± 64.67[b]
Atamisqui 5% I (150 mg/kg)	125.86 ± 4.70[b,c]	36.64 ± 1.40[a,b,c]	373.43 ± 18.97[a,b,c]	0.71 ± 0.04[b,c]	0.21 ± 0.02[a,b,c]	738.37 ± 36.93[a,b,c]
Atamisqui 10% HE (150 mg/kg)	135.74 ± 5.60[a,b,c,d]	38.79 ± 1.69[a,b,c,d]	405.91 ± 20.54[a,b,c,d]	0.73 ± 0.04[b,c]	0.20 ± 0.02[a,b,c]	647.77 ± 32.39[a,b,c,d]

Data is presented as the means ± SD for n=6 rats per group. [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. atamisqui 5% I treated group. I: infusion, HE: hydroalcoholic extract.

Evaluation of gastroprotective mediators in the gastric mucosa: NO and PGE_2

Nitric oxide is known as a gastroprotective mediator with a powerful vasodilator effect that helps oxygen and nutrients´ arrival and sweeps away harmful agents by increasing blood flow in the gastric mucosa. It is also capable of modulating mucus and bicarbonate secretion.[33] In addition, synthesis of prostaglandins by cyclooxygenase (COX) enzymes is also a decisive factor in gastroprotection. Specifically, PGE_2 can increase mucus and bicarbonate secretion, decrease HCl and pepsinogen, inhibits neutrophil recruitment and activation, in addition to its vasodilator effect.[34] The present study demonstrated that the level of both gastroprotective mediators, NO and PGE_2, in the gastric mucosa were significantly reduced in the ulcer control group ($P<0.05$) compared to normal untreated animals (Table 2). Interestingly, data reveals that pre-treatments with both, 5% I and 10% HE were able to significantly increase ($P<0.05$) both, the NO and PGE_2 levels compared to the ulcer control group and the analysis of the data indicated no significant difference in NO levels in the group pretreated with I 5% compared to 10% HE ($p>0.05$). The flavonoids present in atamisqui extracts could be responsible for the observed effect since other authors have evidenced the modulator action of these same compounds on NO synthesis.[27]

It is important to highlight that the levels of PGE2 in the gastric mucosa were higher in animals receiving a pre-treatment with I 5% compared to a 10% HE pre-treatment. Nevertheless, the content of PGE2 in the sucralfate pre-treated group and in the normal untreated group were significantly higher ($P<0.05$) than those obtained by pre-treatments with 5% I and 10% HE.

Rol of sulfhydryl compounds, NO synthase and COX of the gastric mucosa in the gastroprotective effect of 5% I and 10%

Under our experimental conditions, we observed that a pre-treatment with NEM did not affect the previously demonstrated gastroprotective effect of 5% I and 10% HE. As shown in Table 3 both extracts of atamisqui significantly reduced ($P<0.05$) the ulcer percentage compared to the ulcer control group, even with blocked sulfhydryl compounds.

On the other hand, pre-treatment with L-NAME, a non-specific inhibitor of NO synthase, produced a significant decrease ($P<0.05$) of the gastroprotective effect of both, 5% I and 10% HE. This result was evidenced by significantly high ulcer percentages in the animals pretreated whit L-NAME and atamisqui extracts, compared to the rats' groups without inhibition of NO synthase (Table 3).

The pre-treatment with indomethacine, a non-specific COX inhibitor, significantly reduced ($P<0.05$) the gastroprotective effect observed previously with 5% I and 10% HE.

These findings could indicate the strong participation of NO and PGs in the antiulcer activity of both extracts.

In a similar way to the animals pretreated with L-NAME and atamisqui extracts, the groups received indomethacine and 5% I or 10% HE showed a significant increase ($P<0.05$) in the ulcer percentages, compared to the results obtained without COX inhibition (Table 3).

As shown in Table 3, NEM, L-NAME and indomethacine were able to reduce ($P<0.05$) the gastroprotective effect of sucralfate.

Seven *Capparis* species growing in Argentina were investigated for their leaf flavonoid aglycone pattern,[6] indicating that kaempferol and isorhamnetin are the main flavonoids in *C. atamisquea* Kuntze leaves. On the other hand, a study on the eicosanoids release by human gastric mucosa showed that 1 µg/ml of kaempferol was able to enhance PGE release which as we know plays an important role in the gastroprotective activity.[35] Since we have not yet characterized the flavonoids profile in the tested atamisqui extracts, we believe that their ability to reduce the damage caused by ethanol in the gastric mucosa could be largely due to the presence of these compounds in the extracts while their protective effects are mediated by PGs and NO. In fact, we hypothesize that the flavonoids may be one of those responsible for this action.

Table 3: Effects of infusion and hydroalcoholic extract from the leaves of *C. atamisquea* on the gastric lesions induced by ethanol in rats (n = 6) pretreated with N-ethylmaleimide (NEM), N-nitro-L-arginine methyl ester (L-NAME), or Indomethacine.

Pretreatment (i.p.)	Treatment (o.p.)	Ulcer percentage (%)
Saline	-	7.91 ± 0.40
	Sucralfate (100 mg/kg)	0.38 ± 0.02[a]
	Atamisqui 5% I (150 mg/kg)	0.87 ± 0.04[a]
	Atamisqui 10% HE (150 mg/kg)	1.65 ± 0.07[a]
NEM (10 mg/kg)	-	7.68 ± 0.38
	Sucralfate (100 mg/kg)	3.69 ± 0.18[a]
	Atamisqui 5% I (150 mg/kg)	0.96 ± 0.07[a]
	Atamisqui 10% HE (150 mg/kg)	1.53 ± 0.07[a]
L-NAME (70mg/kg)	-	7.6 ± 0.33
	Sucralfate (100 mg/kg)	1.22 ± 0.06[a]
	Atamisqui 5% I (150 mg/kg)	1.02 ± 0.05[a]
	Atamisqui 10% HE (150 mg/kg)	1.88 ± 0.07[a]
Indomethacine (10 mg/kg)	-	7.64 ± 0.38
	Sucralfate (100 mg/kg)	1.05 ± 0.05[a]
	Atamisqui 5% I (150 mg/kg)	2.44 ± 0.12[a]
	Atamisqui 10% HE (150 mg/kg)	2.62 ± 0.13[a]

Data is presented as the means ± SD for n=6 rats per group. [a]$P < 0.05$ vs. untreated group in each pretreatment. I: infusion, HE: hydroalcoholic extract.

Conclusion

The results showed that two polar extracts of the atamisqui leaves exert significant *in vivo* gastroprotective activity, despite their limit *in vitro* and *in vivo* antioxidant activity. The findings also demonstrated that the beneficial effect of atamisqui extracts largely dependent on the participation of mediators such as NO and PGs. The findings

presented have validated the popular uses of this native plant and places it as a natural alternative for the prevention of gastric ulcers.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We wish to thank J. Hayman for her proof-reading. This research was funded by Scientific Research Council of National University of Tucumán (Grant code: PIUNT 2018 D-615).

References

1. Newman DJ and Cragg GM. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J Nat Prod. 2020; 83:770-803.
2. Boelcke O. Plantas vasculares de la Argentina nativas y exóticas. 2nd edn. (Hemisferio Sur, Buenos Aires, 1992. 334p.
3. González DS and Gutiérrez MC. Plantas de los Salares del Chaco Arido. 1st edn. (Gutierrez DS, La Rioja, 2014. 45p.
4. Reynoso MA, Medina FA, Vera NR. Therapeutic potential of organic unifloral atamisqui honey (*Atamisquea emarginata*): in vitro and in vivo biological evaluation. Int J Mod Pharm Res. 2020; 4(4):221-230.
5. Nazar S, Hussain MA, Khan A, Muhammad G, Tahir MN. Capparis decidua Edgew (Forssk.): A comprehensive review of its traditional uses, phytochemistry, pharmacology and nutrapharmaceutical potential. Arab J Chem. 2020; 13(1):1901-1916.
6. Pelotto JP, Del Pero Martínez MA. Flavonoid aglycones from Argentinian Capparis Species (Capparaceae). Biochem Syst Ecol. 1998; 26:577-580.
7. De Barros M, da Silva LM, Boeing T, Somensi LB, Cury BJ, Burci LM, Santin JR, Andrade SF, Monache FD, Cechinel-Filho V. Pharmacological reports about gastroprotective effects of methanolic extract from leaves of Solidago chilensis (Brazilian arnica) and its components quercitrin and afzelin in rodents, Naunyn Schmiedebergs Arch Pharmacol. 2016; 389(4):403-417.
8. Beber AP, de Souza P, Boeing T, Somensi LB, Mariano LNB, Cury BJ, Burci LM, da Silva CB, Simionatto E, de Andrade SF, da Silva LM. Constituents of leaves from Bauhinia curvula Benth. exert gastroprotective activity in rodents: role of quercitrin and kaempferol. Inflammopharmacol. 2018; 26(2):539-550.
9. Da Luz BB, De Oliveira AF, Maria Ferreira D, Dallazen JL, Cipriani TR, de Souza LM, Werner MFP, Chemical composition, antioxidant and gastrointestinal properties of Sedum dendroideum Moc & Sessé ex DC leaves tea infusion, J Ethnopharmacol. 2019; 1:141-151.
10. Lu H, Graham DY. New development in the mechanistic understanding of peptic ulcer diseases. Drug Discov Today Dis Mech. 2006; 3(4):431-437.
11. Sheen E and Triadafilopoulos G. Adverse effects of long-term proton pump inhibitor therapy, Dig Dis Sci. 2011; 6:931-950.
12. Farmacopea Argentina, 7th edn. 1050 Formas Farmacéuticas, 2003. Available from: http://www.anmat.gov.ar/webanmat/fna/pfds/Farmacopea_Argentina_2013_Ed.7.pdf Accessed 15 Dec 2021.
13. Taboada FF, Habib NC, Genta SB. Gastroprotective Effect of *Acacia aroma* Gill. ex Hook. and Arn. Leaves Extracts in Gastric Ulcer Models in Rats. Int J Pharm Investig. 2019; 9(4):187-194.
14. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181:1199-200.
15. Kumar S, Yadav A, Yadav M, Yadav JP. Effect of climate change on phytochemical diversity, total phenolic content and in vitro antioxidant activity of Aloe vera (L.) Burm.f, BMC Res Notes. 2017; 10:60.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1):265-275.
17. Bradley PP, Christensen RD, Rothstein G. Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood. 1982; 60(3):618-622.
18. Ellman GC. Tissue sulfhydryl groups. Arch Biochem Biophy. 1959; 82:70-77.
19. Beuge JA and Aust SD. Microsomal lipid peroxidation. Methods Enzymol. 1978; 52:302-310.
20. Aebi H. Catalase *in vitro*. Methods Enzymol. 1984; 105:121-123.
21. Goshi E, Zhou G, He Q. Nitric oxide detection methods *in vitro* and *in vivo*. Med Gas Res. 2019; 9(4):192-207.
22. Breviglieri E, Mota da Silva L, Boeing T, Somensi LB, Cury BJ, Gimenez A, Cechinel Filho V, de Andrade SF. Gastroprotective and anti-secretory mechanisms of 2-phenylquinoline, an alkaloid isolated from *Galipea longiflora*. Phytomed. 2017; 25:61-70.
23. Francik S, Francik R, Sadowska U, Bystrowska B, Zawiślak A, Knapczyk A, Nzeyimana A. Identification of Phenolic Compounds and Determination of Antioxidant Activity in Extracts and Infusions of Salvia Leaves. Materials (Basel). 2020; 13(24):5811.
24. Alvarez MA. Yungas. In: Pharmacological Properties of Native Plants from Argentina. Springer, Cham. 2019. 34p.
25. Hollander D, Tarnawski A. The protective and therapeutic mechanisms of sucralfate. Scand J Gastroenterol Suppl. 1990; 173:1-5.
26. Mota KS, Dias GE, Pinto ME, Luiz-Ferreira A, Souza-Brito AR, Hiruma-Lima CA. Flavonoids with gastroprotective activity. Molecules. 2009; 14(3):979-1012.
27. Mousa AM, El-Sammad NM, Hassan SK, Madboli AENA, Hashim AN, Moustafa ES, Bakry SM, Elsayed EA. Antiulcerogenic effect of Cuphea ignea extract against ethanol-induced gastric ulcer in rats. BMC Compl Altern Med. 2019; 19:345.
28. Ochiai T, Soeda S, Ohno S, Tanaka H, Shoyama Y, Shimeno H. Crocin prevents the death of PC-12 cells through sphingomyelinase–ceramide signaling by increasing glutathione synthesis. Neurochem Int. 2004; 44(5):321-330.
29. Alirezaei M, Dezfoulian O, Neamati S, Rashidipour M, Tanideh N, Kheradmand A. Oleuropein prevents ethanol-induced gastric ulcers via elevation of antioxidant enzyme activities in rats. J Physiol Biochem. 2012; 68(4):583-592.
30. Zakaria ZA, Balan T, Mamat SS, Mohtarrudin N, The LK, Salleh MZ. Mechanisms of gastroprotection of methanol extract of Melastoma malabathricum Leaves, BMC Complement Altern Med. 2015; 15:135.
31. De Araújo ERD, Guerra GCB, Araújo DFS, de Araújo AA, Fernandes JM, de Araújo Júnior RF, da Silva VC, de Carvalho TG, Ferreira LS, Zucolotto SM. Gastroprotective and Antioxidant Activity of *Kalanchoe brasiliensis* and *Kalanchoe pinnata* Leaf Juices against Indomethacin and Ethanol-Induced Gastric Lesions in Rats. Int J Mol Sci. 2018; 19(5):1265.
32. Lian YZ, Lin IH, Yang YC, Chao JC. Gastroprotective effect of *Lycium barbarum* polysaccharides and C-phycocyanin in rats with ethanol-induced gastric ulcer. Int J Biol Macromol. 2020 ;165:1519-1528.

33. Laine L, Takeuchi K, Tarnawski A. Gastric mucosal defense and cytoprotection: bench to bedside. Gastroenterol. 2008; 135:41-60.
34. Saremi K, Rad SK, Tayeby F, Abdulla MA, Karimian H, Majid NA. Gastroprotective activity of a novel Schiff base derived dibromo substituted compound against ethanol-induced acute gastric lesions in rats. BMC Pharmacol Toxicol. 2019; 20(1):13.
35. Goel RK, Maiti RN, Tavares IA. Role of endogenous eicosanoids in the antiulcer effect of Kaempferol. Fitoterapia. 1996; 6:548-552.