Antioxidant and gastroprotective effects of the ethyl acetate extract from *Stemodia maritima* L. in ethanol-induced gastric ulcer model

Efeito antioxidant e gastroprotetor do extrato acetato de etila de *Stemodia maritima* L. em modelo de úlcera gástrica induzida por etanol

Efecto antioxidant y gastroprotector del extracto de acetato de etilo de *Stemodia maritima* L. en el modelo de úlcera gástrica inducida por etanol

Abstract

*Stemodia maritima* L., is a shrub of the Plantaginaceae family, with some biological activities already described, such as: larvicide, antimicrobial, and anti-inflammatory. Thus, the objective of this work was to evaluate the antioxidant, and gastroprotective activities of the ethyl acetate extract from *S. maritima*. The phytochemical profile was investigated through the quantification of total phenolic compounds, flavonoids, and CCD analysis. The toxicity of the extract was performed through cell viability using L929 line cell, and acute toxicity by the OECD Guide 423. The antioxidant activity was analyzed by the methods of reduction of the ferric ion (FRAP), total antioxidant activity (TAA), and the gastroprotective activity by the absolute ethanol-induced gastric ulcer model, with analysis of NO, MDA, GSH and MPO levels in the stomach tissues. In the phytochemical profile it was possible to identify the presence of flavonoids, triterpenes, steroids, mono, and sesquiterpenes. The extract was not cytotoxic against L929.
lineage, maintaining cell viability above 70% at the doses tested, and in acute toxicity it did not show physiological changes indicative of toxicity compared to the control group. The extract presented antioxidant activity of 157.3 ± 9.7 mg equivalent of Trolox/g of extract in the FRAP method, and 50.0 ± 1.1% by TAA. The ethyl acetate extract of *S. maritima*, at the doses tested, reduced the ulcerative lesion index compared to the injured control group, increased the levels of NO and GSH, and was able to decrease the concentrations of MDA and MPO, enhancing their gastroprotective activity.

**Keywords:** Antiulcer; Phenolic compounds; Cytotoxicity; Oxidative stress.

**Resumen**

*Stemodia maritima* L., es un arbusto de la familia Plantaginaceae, con algunas actividades biológicas ya descritas, como: larvicida, antimicrobiana y anti-inflamatoria. Portanto, el objetivo de este trabajo fue evaluar las actividades antioxidante y gastroprotecora del extracto acetato de etila de *S. maritima*. El perfil fitoquímico se analizó a través de la dosificación de compuestos fenólicos totales, flavonoides y análisis por CCE. La toxicidad del extracto fue feita por meio da análise de viabilidade celular com linhagem L929 e a toxicidade aguda pelo Guia OECD 423. A atividade antioxidante foi analisada pelos métodos de redução do íon férrico (FRAP) e atividade antioxidante total (AAT), e a atividade gastroprotecora pelo modelo de úlcera gástrica induzida por etanol absoluto, com avaliação dos níveis de NO, MDA, GSH e MPO nos tecidos dos estômagos. No perfil fitoquímico foi possível identificar a presença de flavonoides, triterpenos, esteroides, mono e sesquiterpenos. O extracto não foi citotóxico frente à linhagem L929, mantendo a viabilidade celular acima de 70% nas doses testadas, e na toxicidade aguda não apresentou alterações fisiológicas indicativas de toxicidade em comparação com o grupo controle. O extracto apresentou atividade antioxidante de 157,3 ± 9,7 mg equivalente de Trolox/g de extracto no método de FRAP, e de 50,0 ± 1,1% pelo AAT. O extracto acetato de etila de *S. maritima*, nas doses testadas, reduziu o índice de lesão ulcerativa em relação ao grupo controle lesionado, aumentou os níveis de NO e GSH e foi capaz de diminuir as concentrações de MDA e MPO, acentuando sua atividade gastroprotecora.

**Palavras-chave:** Antiúlcera; Compostos fenólicos; Citotoxicidade; Estresse oxidativo.

**Resumen**

*Stemodia maritima* L., es un arbusto de la familia Plantaginaceae, con algunas actividades biológicas ya descritas, tales como: larvicida, antimicrobianas y antiinflamatorias. Así que, el objetivo de este trabajo fue evaluar las actividades antioxidantes y gastroprotectoras del extracto de acetato de etila de *S. maritima*. El perfil fitoquímico se investigó a través de la dosificación de compuestos fenólicos totales, flavonoides y análisis por CCE. La toxicidad del extracto se realizó a través de la viabilidad celular con L929 y toxicidad aguda utilizando la Guía 423 de la OCDE. La actividad antioxidante se analizó mediante los métodos de reducción del ion férrico (FRAP) y la actividad antioxidante total (AAT), y la actividad gastroprotectora por el modelo de úlcera gástrica inducida por etanol absoluto, con evaluación de niveles de NO, MDA, GSH y MPO de los tejidos estomacales. En el perfil fitoquímico fue posible identificar flavonoides, triterpenos, esteroides, mono y sesquiterpenos. El extracto no fue citotóxico contra el linaje L929, manteniendo la viabilidad celular por encima del 70% en las dosis probadas y en la toxicidad aguda no mostró cambios fisiológicos que indicaran toxicidad en comparación con el control. El extracto presentó una actividad antioxidante de 157,3 ± 9,7 mg equivalentes de Trolox/g de extracto en el método FRAP y de 50,0 ± 1,1% por AAT. El extracto acetato de etila de *S. maritima*, a las dosis probadas, redujo el índice de lesión ulcerosa en relación con el grupo control lesionado, aumentó los niveles de NO y GSH y fue capaz de disminuir las concentraciones de MDA y MPO, destacando su actividad gastroprotectora.

**Palabras clave:** Antiúlcera; Compuestos fenólicos; Citotoxicidad; Estrés oxidativo.

1. Introduction

The gastric ulcer, which appears in the stomach mucosa, presents as inflammatory or necrotizing lesions causing pain, bleeding, obstruction, and even perforations that can progress to death. Currently, gastric ulcers affect about 10% of the world population, and are the target of scientific investigations due to this frequency, and concern in clinical practice (Lanas & Chan, 2017; Farrag et al., 2019). Gastric ulcer occurs due to an imbalance between aggressive and protective factors of the gastric mucosa. Among aggressive factors, can be described endogenous and exogenous factors, such as high secretion of gastric acid, and pepsin, inhibition of mucosal cell proliferation, reduced blood flow, suppression of prostaglandins, excessive alcohol intake habits, stress, *Helicobacter pylori* infection, and prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs). Factors related to protection include bicarbonate and mucus secretion, gastroprotective prostaglandin biosynthesis, nitric oxide, adequate tissue blood microcirculation, and antioxidant agents. The imbalance between aggressive and protective factors triggers to oxidative stress by the formation of oxidizing substances, increasing the production of pro-inflammatory cytokines,
reducing blood flow and cell viability, and promoting the appearance of gastric lesions (Cook & Guyatt, 2018; Kavitt et al., 2019).

Treatments for gastric ulcers mainly include histamine H2-receptor antagonist drugs, proton pump inhibitors, and antacids. However, they are not fully effective methods of cure, as they act only to relieve symptoms, and there is a high rate of relapse after treatment interruption, which may also be related to serious side effects (Kavitt et al., 2019).

The use of medicinal plants to treat various disorders is an ancient practice inherited over several generations, thus, is an increasingly explored source. In addition to medicinal plants known for their pharmacological properties, it is common to carry out pre-clinical research on plants or their metabolites as potential therapeutic sources in the treatment of diseases, or selected as candidates for new drugs (Safavi et al., 2015).

*Stemodia maritima* L. is a shrub of the Plantaginaceae family, common in northeastern Brazil, where it can be found in marshy regions by the name of “matruz-bravo”, “melosa” or “meladinha” (Rodrigues et al., 2010; Moreira & Bragança, 2011). Chemical studies report the presence of flavonoids, diterpenes and polyphenols, and some related biological activities, such as larvicidal (Arriaga et al., 2007), antioxidant, antimicrobial (Silva et al., 2014), and anti-inflammatory (Texeira et al., 2017). There are also reports of antiviral, and cytotoxic activities of analogues derived from crenatoside and stemodin, respectively, compounds that can be found in *S. maritima* (Chen et al., 2016; Russell et al., 2011).

Despite these data, there are no reports of specific studies for gastric ulcer inflammation with *S. maritima* L. extract or its bioactive metabolites. Therefore, this work aimed to investigate the in vivo gastroprotective and antioxidant activities of the ethyl acetate extract of *S. maritima* leaves (AcSm), analyze the phytochemical profile and toxicity, as well as contribute for enriching the scientific community, as our group is a pioneer in the study of gastroprotection of this species.

2. Methodology

This study is an experimental scientific investigation, the research project was approved by the Ethics Committee in the Use of Animals at the Federal University of Pernambuco – UFPE, under protocol 134/2019.

2.1 Animals

Swiss mice (*Mus musculus*), female (25-30 g), and Wistar rats (*Rattus norvegicus*), male (250-280 g), from the Animal Facility of the Department of Antibiotics of UFPE, Campus Recife, Pernambuco, Brazil were used. The animals were kept under appropriate conditions, following the guidelines of the National Council for the Control of Animal Experimentation (NCCAE), and the method to perform euthanasia was an anesthetic overdose: Ketamine (0.2 mL/100 g + 0.2 mL/100 g overdose) and xylazine (0.1 mL/100 g + 0.1 mL/100 g overdose) intraperitoneal route (i.p.).

2.2 Obtaining the plant extract

The leaves of *S. maritima* (794 g) were collected in the municipality of Boqueirão - PB (coordinates: latitude -7.4824 and longitude -36.1223) and botanical identification was performed at the Herbarium of the Agronomic Institute of Pernambuco (IPA) by specialist Olivia Cano, with the number of catalog 92093. Ethyl acetate extract (AcSm) was obtained by exhaustive maceration, under agitation and at room temperature, for three cycles with solvent change every 72 hours. The solvent was totally removed with the aid of a rotary evaporator at low pressure to obtain the AcSm extract.
2.3 Phytochemical profile

2.3.1 Thin layer chromatographic (TLC) analysis

AcSm was weighed and dissolved in methanol at a concentration of 5 mg/mL. As the fixed phase, F254 silica gel plates were used and as mobile phases specific eluting and developer systems for flavonoids, cinnamic derivatives, phenylpropane glycosides (AcOEt-HCOOH-AcOH-H2O (100:11:11:27 v/v), standards: quercetin, rutin and chlorogenic acid, NEU reagent - ethylborilamoester acid); for triterpenes and steroids (Toluene:AcOEt (90:10 v/v), standard: β-sitosterol, Lieberman & Burchard reagent), mono and sesquiterpenes (Toluene:AcOEt (97:3 v/v), standard: timol, sulphuric anisaldehyde reagent), for alkaloids (AcOEt-HCOOH-AcOH-H2O (100:11:11:27 v/v), pattern: pilocarpin, Dragendorff reagent), and for condensed proanthocyanidins and leucoanthocyanidin (AcOEt-HCOOH-AcOH-H2O (100:11:11:27 v/v), standard: catechin, vanillin/HCl reagent).

2.3.2 Dosage of total phenolic compounds

According to the methodology proposed by Li et al. (2008), the dosage of total phenolic compounds in AcSm was determined using the Folin-Ciocalteu reagent, in triplicate. This test is based on the formation of a blue complex, by reduction of the reagent by the phenolic hydroxyls present in the sample. AcSm (1 mg) was added to 1 ml of Folin-Ciocalteu reagent (1:1 v/v) and the mixture was allowed to stand (protected from light) for 3 minutes. Then, 0.8 ml of 7.5% sodium carbonate was added and the mixture was incubated for 120 minutes in a dark environment. Absorbances were read in a spectrophotometer at 765 nm. Gallic acid (0 – 500 mg/L) was used to calibrate the standard curve and the results were expressed in milligrams equivalent of gallic acid (mg EAG)/g of extract.

2.3.3 Flavonoid dosage

Flavonoid content was quantified by the technique described by Pekal & Pyrzynska (2014), where 100 µL of AcSm, blank (solvent used to dilute AcSm) or quercetin concentrations were added to 0.05 mL of aluminum chloride reagent (2%) and 0.05 mL of sodium acetate (1M). Another blank was performed for each sample (0.1 mL of sample + 0.1 mL of distilled water), to subtract the absorbance value of the diluted samples, to avoid false positives. The samples were shaken and kept at room temperature for 10 minutes, then absorbances were measured at 425 nm and the total flavonoid content in the extracts was expressed as quercetin equivalent (mg EQ/g extract).

2.4 In vitro antioxidant activity

2.4.1 Ferric ion reducing activity (FRAP)

The FRAP assay was performed according to Benzie & Strain (1996). The stock solution was prepared with 300 mM acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s triazine) solubilized in 40 mM HCl, and 20 mM FeCl3 solution. The working solution was prepared with 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL FeCl3 (10:1:1), and incubated for 5 min at 37 °C. A volume of 10 µL of AcSm at a concentration of 1 mg/mL was mixed with 290 µL of FRAP reagent, and left to stand for 15 minutes at 37 °C in the dark. Subsequently, absorbances were measured at 593 nm. Quercetin was used as an antioxidant reference, and a standard curve with Trolox (0 – 2000 µg/mL) was obtained. The results were expressed in equivalent mg of Trolox/g of extract (ET/g).

2.4.2 Total antioxidant activity (TAA)

The total antioxidant activity was measured according to Prieto et al. (1999). The 100 µL volume of AcSm at the concentration of 1 mg/mL was mixed with 1 mL of the phosphomolybdenum solution (600 mM sulfuric acid, 28 mM sodium...
phosphate and 4 mM ammonium molybdate). Samples were incubated at 95 °C for 90 minutes. After returning to room temperature, absorbances were measured at 695 nm against a blank (1 mL of solution and 100 µL of sample dilution solvent). Quercetin was used as a reference control and TAA was expressed in relation to the ascorbic acid standard and calculated by the formula:

\[
\text{TAA} (%) = \frac{\text{Sample absorbance} - \text{White absorbance}}{\text{Standard Absorbance} - \text{White Absorbance}} 
\times 100
\]

2.5 Cell viability assay

AcSm was submitted to cell viability colorimetric assay, based on the mitochondrial activity of cells by the reduction of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Mosmann, 1983). Fibroblasts of the L929 lineage were used, these were treated with AcSm at concentrations from 1.56 to 50 µg/mL for 72 hours and kept in an oven at 5% CO₂. After 72 h of incubation, 25 µL of MTT (5 mg/mL) was added and after 3 h, the culture media with the MTT was aspirated and 100 µL of DMSO was added. Absorbance was measured in a microplate reader at a wavelength of 560 nm.

2.6 Acute toxicity

AcSm acute toxicity was performed according to OECD Guide 423 (OECD, 2001). The mice were divided into two groups according to their single-dose treatment: control (aqueous solution of Tween 20 at 1%) and AcSm 2,000 mg/kg (by gavage) and behavioral parameters were observed during the first hours after treatment. Feed/water consumption and body mass gain were measured daily until the 14th day. After the last analysis, the animals received a dose of anesthetic, and blood was collected by cardiac puncture for biochemical and hematological analysis. After euthanasia, livers, kidneys, and spleen were dissected for macroscopic analysis of signs of toxicity.

2.7 Absolute ethanol-induced gastric injury

The rats were randomly divided into 6 groups (n=6), fasted for 18 h and then treated with their respective substances: Healthy control - HC (no ethanol administration, treated with aqueous solution with Tween 20 at 1%), Injured control – IC (received absolute ethanol and was treated with aqueous solution with Tween 20 at 1%), Lansoprazole (30 mg/kg), AcSm 25, 50 and 100 mg/kg through gavage. After 1 hour of treatment, the animals were induced to gastric injury with the administration of absolute ethanol (4 mL/kg through gavage). One hour after this administration, the rats were euthanized and their stomachs removed, washed with saline solution, opened by the greater curvature, and photographed. Ulcerative Lesions Index (ULI) calculations were performed using computerized planimetry (Software ImageJ) (Morimoto et al., 1991).

2.8 Determination of nitric oxide (NO) levels

The concentration of nitrite in the stomach homogenate (100 mg/mL in 150 mM phosphate buffer, pH 7.4) was used as an index of NO production through the Griess reaction, where 50 µL of the sample was incubated for 10 min with 50 µL of Griess solution, protected from light. Absorbance was measured at a wavelength of 560 nm using a microplate reader and the concentration of nitrite was determined by comparing the absorbance of the sample to a standard curve for sodium nitrite (Green et al., 1981).
2.9 Determination of malondialdehyde (MDA) levels

The degree of lipid peroxidation was estimated by determining MDA levels through the thiobarbituric acid reactive substances test (TBARS), using the method described by Draper & Hadley (1990). Tissues were homogenized (100 mg/mL) in 150 µM phosphate buffer (pH 7.4). After homogenization, the samples were mixed with 1 mL of 10% trichloroacetic acid and centrifuged at 10000 rpm/15 min/4 ºC. Then, the supernatant was incubated with 500 µL of 1.2% thiobarbituric acid. The mixture was brought to the boiling water bath (95 ºC/30 min). After the samples had cooled down, they were placed in a 96-well plate and read using a microplate reader (535 nm). The results were expressed in nmol of MDA/mg tissue.

2.10 Determining Reduced Glutathione Levels (GSH)

The determination of GSH concentration is based on the reaction of Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), with the free thiol, resulting in a mixed disulfide plus 2-nitro-5 acid thiobenzoic, whose measurement of the reaction product formed is made by spectrophotometric reading. For the quantification of GSH, the glandular portion of the stomachs was homogenized in EDTA 0.02 M (1 mL/100 mg of tissue). An aliquot of 40 µL of this homogenate + 50 µL of distilled water + 10 µL of trichloroacetic acid 50% was stirred and centrifuged at 5000 rpm/15 min/4 ºC. Then, 60 µL of the supernatant was collected, and 25 µL of Tris buffer 0.4 M plus 20 µL of DTNB 0.01 M were added. After 1 minute of the reaction, the color reading was performed in a microplate reader at 412 nm (Sedlak & Lindsay, 1968).

2.11 Determination of myeloperoxidase levels (MPO)

The stomachs were homogenized (1 mL/100 mg of tissue) in a 0.5% hexadecyltrimethylammonium bromide (HTAB) solution. The myeloperoxidase activity was evaluated according to the protocol described by Bradley et al. (1982), by reacting the samples with 200 µL of reaction solution (o-dianisidine hydrochloride - 0.167 mg/mL, 50 mM sodium phosphate buffer, and 0.0005% hydrogen peroxide). After incubation, the reaction was read in a microplate reader at a wavelength of 450 nm.

2.12 Statistical analysis

Data were subjected to analysis of variance (one-way ANOVA), followed by Tukey's test. For cytotoxic activity, Bonferroni was used as a post-test, and for acute toxicity and antioxidants, the t-test was used. All numerical values were presented as mean ± standard deviation of the mean (SD) and p<0.05 values were considered significant. Analyzes were performed using the GraphPad Prism software, version 8.0 (San Diego, CA, EUA).

3. Results and Discussion

3.1 Phytochemical profile analysis

Analysis by CCD indicated the presence of flavonoids, triterpenes and steroids, mono and sesquiterpenes in AcSm (Table 1).
Table 1 - Classes of secondary metabolites found in AcSm via CCD.

| Secondary metabolites                      | AcSm |
|--------------------------------------------|------|
| Flavonoids                                 | +    |
| Phenylpropanoids                           | -    |
| Triterpenes and steroids                    | ++   |
| Mono and sesquiterpenes                    | ++   |
| Proanthocyanidins and leucoanthocyanidins  | -    |
| Anthocyanins and anthocyanidins            | -    |
| Hydrolyzable tannins                       | -    |

Subtitle: (-) absent; (+) weak; (++) medium; (+++) strong. Source: Authors.

These data corroborate previous chemical studies, where they found flavonoids and diterpenes in the composition of *S. maritima*. Other species of the genus also have phenolic compounds, steroid triterpenes and diterpenes abundance (Rodrigues et al., 2010; Silva et al., 2014).

After analysis by CCD, the content of total phenolic compounds and flavonoids was analyzed. AcSm presented, respectively, 71.25 ± 3.54 mg equivalent of gallic acid/g of extract and 6.97 ± 2.06 mg equivalent of quercetin/g of extract. Considerable levels of phenolic compounds are found in *Veronica spicata* (Dunkic et al., 2015), and Plantago lagopus (Harput et al., 2012), species that also belong to the Plantaginaceae family.

Flavonoids constitute the largest class of plant phenolics and can act as reducing agents, hydrogen donors, superoxide radical scavengers or metal chelating agents. As they are antioxidant substances, they are relevant compounds in several other biological activities, they can be divided into some classes, such as: flavonols, anthocyanins, isoflavonoids, flavanones, and flavones (Zakaria et al., 2016). Terpenes are another class of relevant compounds found in AcSm, they have a variety of activities, such as: anticancer, healing, antinociceptive, antimicrobial, anti-inflammatory, and gastroprotective activity (Choudhary et al., 2013; Chinedu & Ibrahim, 2017).

3.2 Evaluation of antioxidant activity

In the ferric ion reduction assay, AcSm presented 157.3 ± 9.7 mg ET/g of extract while quercetin presented 551.57 ± 17.49 mg ET/g of the extract. In the total antioxidant activity assay, the ascorbic acid standard is considered the compound that has 100% activity, the AcSm extract showed 50.0% ± 1.8 of total antioxidant activity, a value close to quercetin which was 62.4% ± 1.1 (Table 2).

The antioxidant activity observed can be explained by the presence of phenolic compounds and flavonoids present in the extract, knowing that these substances have a high potential for reducing free radicals due to their chemical structures, where there are hydroxyl groups conjugated with aromatic rings, responsible for reactions that make the free radicals present in the media stable (Liu et al., 2013). There are no records in the literature regarding the antioxidant activity of these methods for *S. maritima*, however, Silva et al. (2014) described the antioxidant activity, by the DPPH method, of two compounds isolated from *S. maritima*, stemodin and crenatoside, where stemodin was not able to significantly sweep the DPPH radical and crenatoside showed potential action, with scanning of 99% of the radical at a concentration of 100 µg/mL and IC<sub>50</sub> of 2.2 µg/mL.
Table 2 - Evaluation of the antioxidant activity of AcSm by the FRAP and TAA method.

| Extract | FRAP equivalent mg of Trolox (ET/g extract) | TAA (%) |
|---------|--------------------------------------------|---------|
| AcSm    | 157.3 ± 9.7 *                              | 50.0 ± 1.8 * |
| Quercetin | 551.6 ± 17.5                               | 62.4 ± 1.1 |

Subtitle: Results are expressed as mean ± standard deviation. Analyzed by t test, * indicate p< 0.0001 in relation to the quercetin group. Source: Authors.

3.3 Analysis of cell viability and acute toxicity

AcSm cytotoxicity was analyzed before proceeding to in vivo tests, although cell cultures do not undergo the same physiological processes as animals, this in vitro test is commonly used to simulate, precede or even eliminate in vivo tests, as it is sensitive, fast, safe, and able to quantify cell viability after the treatment of new compounds (Li et al., 2015; Hampshire & Gilbert, 2018). In Figure 1, can be seen that AcSm did not show significant cytotoxicity against the L929 lineage, at concentrations ranging from 1.56 to 50 µg/mL, maintaining cell viability, which indicates a certain safety for the start of in vivo testing.

Figure 1 - Effect of AcSm on the percentage of cell viability at concentrations from 1.56 to 50 µg/mL in fibroblast lineages (L929) using the MTT colorimetric method after 72 hours of treatment.

Subtitle: CTRL: control group – DMSO 1%; DOX: Doxorubicin group – 5 µg/mL; Results are expressed as mean ± standard deviation. One way ANOVA analysis of variance, followed by Bonferroni test. * indicate p< 0.0001 when compared the control group. Source: Authors.

After analyzing cell viability, the acute toxicity test was performed according to OECD 423 (2001). After the administration of substances in the AcSm group, the animals were observed to identify possible changes and signs indicative of toxicity. Initially, all three animals in the AcSm-treated group showed similar behavioral signs: agitation, piloerection, eyelid ptosis and excessive grooming. From the first hour, the animals were drowsy, but responded to touch.

During the 14 days of observation, the consumption of water, feed and body mass gain were evaluated, as shown in Table 3. There was no significant difference between the AcSm group and the control group. The reduction in body mass is a characteristic sign observed in most animals exposed to toxic substances, this is due to a variety of responses. Analyzing
changes in the animal's body mass during exposure to exogenous substances is an important indicator of toxicity (Sangeetha et al., 2013).

Organs reveal metabolic changes caused by toxic substances, the characteristics of macroscopic changes, together with mass are relevant physiological indicators (Vaghasiya et al., 2011). The mass analysis of the dissected organs also did not reveal significant alterations indicative of toxicity (Table 4).

**Table 3** - Effect of oral administration of AcSm and aqueous solution containing 1% Tween 20 (control) on body mass gain and feed and water consumption in female mice, during 14 days of observation.

| Parameters                  | Aqueous solution containing 1% Tween 20 | AcSm (2,000 mg/kg) |
|-----------------------------|----------------------------------------|-------------------|
| Feed consumption (g)        | 19.50 ± 2.10                           | 18.36 ± 1.59      |
| Water consumption (mL)      | 31.79 ± 2.69                           | 32.57 ± 2.79      |
| Body mass gain (g)          | 3.73 ± 0.64                            | 3.03 ± 0.25       |

Subtitle: Results are expressed as mean ± standard deviation (t test). Source: Authors.

**Table 4** - Effect of oral administration of AcSm and aqueous solution containing 1% Tween 20 (control) on organ mass (g) of female mice, during 14 days of observation.

| Organs (g)                     | Aqueous solution containing 1% Tween 20 | AcSm (2,000 mg/kg) |
|--------------------------------|----------------------------------------|-------------------|
| Liver                          | 2.43 ± 0.08                            | 2.24 ± 0.08       |
| Kidneys                        | 0.54 ± 0.12                            | 0.60 ± 0.12       |
| Spleen                         | 0.29 ± 0.06                            | 0.32 ± 0.03       |

Subtitle: The results are expressed as mean ± standard deviation (t test). Source: Authors.

Hematological parameters were also analyzed, such as: red blood cells, hemoglobin, hematocrit, leukocytes, platelets, neutrophils, lymphocytes, monocytes, and eosinophils. The biochemical parameters analyzed was: urea, creatinine, TGO, TGP and uric acid. There was no significant difference between animal treated with AcSm when compared to the control group. Corroborating Texeira et al. (2017), where they demonstrated that values obtained for TGO/TGP and creatinine had no difference between the group treated with ethanol extract of *S. maritima* over 11 days and the control group. In that same study, the animals did not show toxicological signs, adverse effects, or behavioral changes, there was also no change in body mass and wet organ weight.

According to the guide's recommendations, in this study, the test was repeated (n=3) under the same conditions and again there were no deaths. Thus, AcSm falls into category 5 (Lethal dose - LD₅₀ > 2,000 mg or not estimated) of Guideline 423 (OECD, 2001).

### 3.4 Evaluation of gastroprotective activity

An experimental model widely used for preclinical evaluation of agents with potential gastroprotective activity is ethanol-induced gastric injury, since ethanol has been considered one of the causes of gastric ulcers in humans, and has rapid acute action in rodents (Song et al., 2018; Kavitt et al., 2019). In our study, the injured control group, which received vehicle and then absolute ethanol, had 76.5% of the injured area, the mucosas showed hemorrhage and necrosis, corroborating with other data, where ethanol was used as an inducing agent of gastric injury (Simões et al., 2019).
Treatment with lansoprazole or AcSm doses significantly decreased ULI, all compared to the injured control. Lansoprazole preserved the mucosa and reduced 91% of lesions, while AcSm at doses of 25, 50 and 100 mg/kg showed a reduction in the injured area of 81, 85, and 86%, respectively. There was no significant difference between the doses tested (Figure 2). These results can also be seen through the macroscopic examination of the gastric mucosa, as shown in Figure 3.

These data corroborate the results obtained by Sousa et al. (2021), that observed the gastroprotective activity of the hexane extract of *S. maritima* leaves. Abud et al. (2012), described the gastroprotection of the methanol extract of *Plantago major* L., a species belonging to the same family as *S. maritima*, Plantaginaceae, and presenting similar compounds, such as polyphenols of the verbascoside type (Zubair et al., 2011).

**Figure 2** - Effect of oral administration of AcSm on gastric lesions induced by absolute ethanol in rats.

Subtitle: ULI - Ulcerative lesion index; IC - injured control; Lanso - lansoprazole 30 mg/kg. Values are expressed as mean ± SD. A one-way ANOVA followed by Tukey multiple-comparisons test. * indicate $p < 0.0001$ in relation to the IC group. Source: Authors.
Figure 3 - Macroscopic evaluation of the effect of AcSm on gastric lesions induced by absolute ethanol in rats.

Subtitle A: Healthy control; B: Injured control; C: Lansoprazole (30 mg/kg); D: AcSm (25 mg/kg); E: AcSm (50 mg/Kg); F: AcSm (100 mg/kg). Source: Authors.

The gastroprotective effect of natural products may be related to the production of nitric oxide, stimulation of the antioxidant system, production of mucus and bicarbonate, prostaglandins, regeneration of gastric epithelial cells or with the inhibition of neutrophil migration and infiltration (Ateufack et al., 2015; Zhang et al., 2019).

In this work, the levels of nitric oxide, malondialdehyde, reduced glutathione and myeloperoxidase in the stomach mucosa were analyzed, as parameters of inflammation and oxidative stress. All data are shown in Table 5.

Among the main mucosal protection factors are nitric oxide and enzymatic and non-enzymatic antioxidant defense systems (Sanchez-Mendonza et al., 2019). The NO produced by the activation of nitric oxide synthase constitutively is involved in regulating the integrity of the gastric mucosa, being able to reduce motility, regulate mucus production, blood flow, and acid secretion (Antonisamy et al., 2016; Czekaj et al., 2018). In our findings, the groups pretreated with AcSm (50 and 100 mg/kg) showed an increase of NO levels when compared to the injured control, remaining equivalent to the value of the healthy control group. Only the dose of 25 mg/kg did not get the same result.

GSH is the main non-enzymatic antioxidant that is involved in several cellular functions and participates in oxidative metabolism, including the neutralization of hydroperoxides and maintenance of the physiological condition of proteins in the sulphydryl groups (Beserra et al., 2011; Ali et al., 2020). When the animals were pretreated with AcSm at the three doses tested, there was an increase in GSH levels compared to the injured control, indicating a potential non-enzymatic antioxidant action.
Table 5 - Effect of AcSm on Nitric Oxide (NO), Malondialdehyde (MDA), Reduced Glutathione (GSH) and Myeloperoxidase (MPO) levels.

| Groups    | NO (µmol/g tissue) | MDA (nmol/mg of tissue) | GSH (µg/g of tissue) | MPO (U/mg of tissue) |
|-----------|-------------------|------------------------|---------------------|---------------------|
| HC        | 23.97 ± 1.23      | 11.36 ± 1.46           | 725.29 ± 45.92      | 0.18 ± 0.01         |
| IC        | 15.08 ± 2.45      | 20.50 ± 2.25           | 440.49 ± 52.67      | 0.56 ± 0.02         |
| Lanso     | 25.38 ± 2.36 *    | 10.47 ± 1.19 *         | 736.06 ± 46.10 *    | 0.21 ± 0.03 *       |
| AcSm 25   | 16.02 ± 2.40      | 10.18 ± 0.72 *         | 728.87 ± 62.94 *    | 0.26 ± 0.03 *       |
| AcSm 50   | 20.82 ± 1.79 *    | 11.47 ± 1.67 *         | 741.88 ± 51.41 *    | 0.24 ± 0.04 *       |
| AcSm 100  | 22.07 ± 1.69 *    | 10.60 ± 1.25 *         | 703.11 ± 66.56 *    | 0.22 ± 0.04 *       |

Subtitle: HC– healthy control; IC - injured control; Lanso - lansoprazole 30 mg/Kg. Values are expressed as mean ± SD. A one-way ANOVA followed by Tukey multiple-comparisons test. * indicate p<0.0001 in relation to the IC group. Source: Authors.

Still observing oxidative stress parameters, we report that MDA levels in the groups treated with AcSm significantly reduced compared to the injured control. MDA is the main reaction product with thiobarbituric acid, indicating lipid peroxidation and, consequently, oxidative stress (Gulcin, 2020). In this work, the reduction in the concentration of MDA is in accordance with the data on increased levels of NO and GSH, balancing the oxidant/antioxidant system and promoting protection of the gastric mucosa.

According to Silva et al. (2014), S. maritima is composed of stemodine-type diterpenes, and Russell et al. (2011) reported that stemodine analogues have lipid peroxidation reducing properties in in vitro assays. Therefore, the effect found in this assay may be related to the phytochemical composition of this species.

Myeloperoxidase is an enzyme found in neutrophil granules. In this MPO concentration assay, it is an indirect assessment of neutrophil migration into tissue, where neutrophils are associated with increased production of pro-inflammatory cytokines (Nicolau et al., 2017). Thus, MPO levels rise in inflammatory conditions, as shown by the result of ethanol induction in the injured control group, which presented an enzyme activity level of 0.56 ± 0.02 U/mg. In the present study, AcSm (25, 50 and 100 mg/kg) was able to reduce MPO levels in stomachs tissue when compared to the injured control, by reducing the migration of neutrophils into the tissue.

Similar findings to these have already been reported by our group, where the hexane extract of S. maritima reduced MPO levels and increased NO and GSH levels in a gastric protection assay (Sousa et al., 2021).

4. Conclusion

In conclusion, the results of the current study demonstrated that ethyl acetate extract from S. maritima extract alleviated ethanol-induced gastric mucosal injury in rats by mainly increasing nitric oxide, and reducing glutathione, malondialdehyde, and myeloperoxidase levels. The extract also showed antioxidant activity in vitro in the tested methods and did not show toxicity in the tested conditions.

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