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Original Article

Anti-inflammatory effect of the total flavonoid content of the hydroalcoholic extract of the leaves of *Senna alata* (L.) Roxb. in an experimental model of acute inflammation

[Efecto anti-inflamatorio del contenido total de flavonoides del extracto hidroalcohólico de las hojas de *Senna alata* (L.) Roxb. en un modelo experimental de inflamación aguda]

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Abstract

Context: Acute inflammation due to persistent stimulation of noxes evolves into chronic inflammation that contributes to the pathogenesis of asthma, arthritis, atherosclerosis, diabetes, aging, and cancer; therefore, it is justified to study plants with anti-inflammatory activity and fewer adverse effects.

Aims: To evaluate the potential anti-inflammatory effect of the total flavonoid content of the hydroalcoholic extract of the leaves of *Senna alata* (L.) Roxb (HELSA) in an experimental model of acute inflammation induced by carrageenan in rats.

Methods: The total flavonoid content (TFC) was determined by spectrophotometry at 510 nm, total polyphenolic content (TPC) by the Folin-Ciocalteu method, and antioxidant activity by two *in vitro* methods. The oral median lethal dose (LD_{50}) was determined by the logarithmic method, and acute inflammation was induced by 1% carrageenan in rats.

Results: The TFC was 1.63 ± 0.02 mg QE/100 mg and TPC was 22.90 ± 0.05 mg GAE/100 mg. Antioxidant capacity: DPPH IC_{50} 2.89 ± 0.04 µg/µL; ABTS 11.67 ± 0.03 mM TEAC/100 mg dry extract. Correlation relationship TFC/DPPH (r = -0.9941; R^2 = 0.9884), TFC/ABTS (r = 0.9971; R^2 = 0.9944), TPC/DPPH (r = 0.5694; R^2 = 0.3243) and TPC/ABTS (r = -0.5960; R^2 = 0.3553). The LD_{50} was 9602 mg/kg. A significant decrease in rat paw edema ($p<0.05$) was evident from the first hour, in contrast to the negative control. The highest percentage of anti-inflammatory inhibition (75.75%) was at 7 hours with a dose of 500 mg/kg of HELSA, being higher than the effect of ibuprofen (39.40%) and lower than dexamethasone (87.87%).

Conclusions: The total flavonoid content and polyphenols of the hydroalcoholic extract of the leaves of *S. alata* exhibit potential anti-inflammatory activity in a model of acute inflammation that should be examined with other experimental models to initiate clinical trials in humans.

Keywords: *Senna alata*; anti-inflammatory effect; flavonoids; biophenols; antioxidant activity *in vitro*.

Resumen

Contexto: La inflamación aguda por estimulación persistente de noxas evoluciona a inflamación crónica que contribuye a la patogénesis del asma, artritis, aterosclerosis, diabetes, envejecimiento, y cáncer; por ello, se justifica estudiar plantas con actividad antiinflamatoria y con menos efectos adversos.

Objetivos: Evaluar el potencial efecto antiinflamatorio del contenido total de flavonoides del extracto hidroalcohólico de las hojas de *Senna alata* (L.) Roxb (HELSA) en un modelo experimental de inflamación aguda inducida por carragenina en ratas.

Métodos: El contenido total de flavonoides (TFC) se determinó por espectrofotometría a 510 nm, contenido total polifenólico (TPC) por el método Folin-Ciocalteu y la actividad antioxidante por dos métodos *in vitro*. La dosis letal media oral (LD_{50}) se determinó por el método logarítmico y la inflamación aguda inducida por carragenina 1% en ratas.

Resultados: El TFC fue 1,63 ± 0,02 mg QE/100 mg y TPC fue 22,90 ± 0,05 mg GAE/100 mg. Actividad antioxidante: DPPH IC_{50} 2,89 ± 0,04 µg/µL; ABTS 11,67 ± 0,03 mM TEAC/100 mg de extracto seco. Relación de correlación TFC/DPPH (r= -0,9941; R^2 = 0,9884), TFC/ABTS (r= 0,9971; R^2 = 0,9944), TPC/DPPH (r = 0,5694; R^2 = 0,3243) y TPC/ABTS (r = -0,5960; R^2 = 0,3553). La DL_{50} fue 9602 mg/kg. Se evidenció disminución significativa del edema de la pata de rata ($p<0.05$) desde la primera hora, en contraste con el control negativo. El mayor porcentaje de inhibición antiinflamatoria (75,75 %) fue a las 7 horas con una dosis de 500 mg/kg de HELSA, siendo superior al efecto del ibuprofeno (39,40%) y menor a la dexametasona (87,87%).

Conclusiones: El contenido total de flavonoides y polifenoles del extracto hidroalcohólico de las hojas de *S. alata* exhiben potencial actividad antiinflamatoria en un modelo de inflamación aguda que debería examinarse con otros modelos experimentales para iniciar los ensayos clínicos en humanos.

Palabras Clave: actividad antioxidante *in vitro*; biofenoles; efecto antiinflamatorio; flavonoides; *Senna alata*.

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INTRODUCTION

Inflammation is a complex and necessary component of the immune system's response to endogenous and exogenous chemical, physical or biological noxes, and is characterized by clinical signs of flushing, heat, pain, and functional impotence (Germolec et al., 2018; Medzhitov, 2008; Silva et al., 2015). In the acute phase of the inflammatory response, leukocytes, plasma proteins, and blood derivatives migrate to the site of injury due to the presence of cytokines, chemokines, and acute phase proteins; this process could be sufficient to resolve tissue damage and eliminate infections (Medzhitov, 2008; Silva et al., 2015). However, inflammation can be persistent, due to prolonged exposure to noxa stimulation or due to an inappropriate reaction against the molecules themselves, generating chronic inflammation (Arulselvan et al., 2016; Germolec et al., 2018) that contributes to the pathogenesis of asthma, arthritis, atherosclerosis, diabetes, autoimmune diseases, aging and cancer (Germolec et al., 2018; Okin and Medzhitov, 2012; Silva et al., 2015). In addition to the chronic inflammatory process, there are other factors that induce cancer, including various viruses [high-risk human papillomavirus (HR-HPV), Epstein-Barr virus (EBV) and others], bacterium (*Helicobacter pylori*), allelic variants of genes *CYP1A1, CYP2C19, CYP2D6* and *CYP17* that encode their respective isoenzymes that biotransform procarcinogenic agents [polycyclic aromatic hydrocarbons (benzopyrene), arylamines, N-nitrosamines and dioxins] into reactive metabolites that produce DNA adducts (Alvarado et al., 2021; Wongpratate et al., 2020). Fig. 1 shows the inflammatory process that is generated in proinflammatory cells, in mast cells, it begins when Hageman factor Cβ (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) in two-second messengers, one is inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC) that phosphorylates phospholipase A_2 (PLA_2), which releases arachidonic acid (ARA) from the inner membrane. ARA is converted into prostaglandin G_2 (PGG_2) by the action of prostaglandin H synthetase 2 or cyclooxygenase 2 (COX_2); reduced glutathione (GSH) activates COX_2 and is oxidized to GSSG. Lipopolysaccharide (LPS) interacts with toll-like receptors (TLR_1), which couples to the signal translation protein TNF receptor-associated factor (TRAF) and recruits IRAK family kinases. At the same time, this complex activates signaling mediated by JNK, ERK, p38, Egr-1, and NF-κB, among others, inducing COX_2 genes that encode its enzymatic protein (An et al., 2002; Díaz-Muñoz et al., 2010). PGG_2 is transformed in two ways: due to the action of COX_3, PGG_2 is converted into PGE_2, generating fever in the preoptic area of the hypothalamus. By the action of hydroperoxidase, PGG_2 is converted into PGH_2, which is biotransformed by PGD_2 synthetase into PGD_2, which is responsible for bronchoconstriction, inhibition of platelet-activating factor (PAF) and vasodilation. At the same time, IgE stimulates FCεRI and stimulates mast cell granules to degranulate histamine, which contributes to vasodilation. While in the other proinflammatory cells, macrophages and monocytes, PGH_2 is biotransformed by PGE_2 synthetase into PGE_2, which generates vasodilation (causes edema due to extravasation of fluids and plasma proteins), erythema (redness) and pain when stimulating type C nerve fibers, these three signs they constitute the triad of inflammation (Alfranca et al., 2006; Chen et al., 2008; Crespo-Pardo and Taboada-Iglesias, 2021).

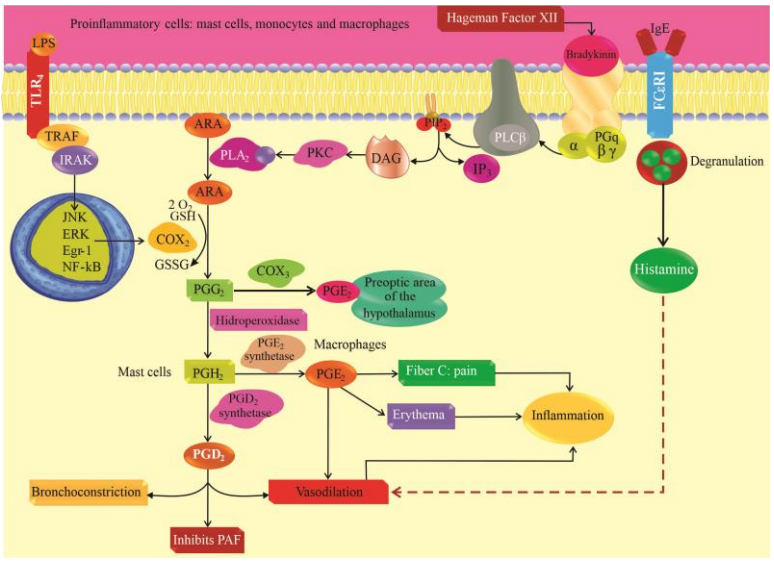

Figure 1. Molecular mechanism of acute inflammation.

Currently, synthetic drugs inhibit COX_2, modifying inflammatory processes. However, physiological COX_1 is also inhibited, inducing adverse effects such as cough, gastrolesivity, kidney dysfunction, and thrombosis (García Colmenero et al., 2018). For this reason, a line of research in green chemistry has been proposed to evaluate polyphenolic compounds from medicinal plants with potential antioxidant and anti-inflammatory activity with minimal adverse effects, and that inhibit reactive oxygen species (ROS) and nitrogen species (RNS), which they are involved in chronic diseases (Baroi et al., 2022; Carvajal, 2019). In this sense, one of the genera with anti-inflammatory properties is *Senna*, which has more than 350 species, such as shrubs, trees, and herbs, belonging to the legume family *Fabaceae* and the subfamily *Caesalpinioideae* (Eldemerdash et al., 2022; Ongchai et al., 2019). *Senna alata* (L.) Roxb. (ringworm cassia or candle bush), also known as *Cassia alata* (Oladeji et al., 2020). It is a shrub native to Ghana that has spread to Brazil, Peru, and other countries (Oladeji et al., 2020). Said bush is 1.83-3.66 m tall with waxy yellow erect spikes resembling thick candles before the individual flowers open (bisexual and zygomorphic); large, bilaterally symmetrical leaves with pinnate pairs, leaflets of 8 to 20, four pairs with a lanceolate shape and smooth margin; ovary with marginal placentation; the fruit is a winged pod and the seeds are small and square (Ibrahim and Osma, 1995; Idu et al., 2007). This species contains bioactive compounds such as saponins, tannins, phenols, cinnamic acid, alkaloids, 1,8-cineole, caryophyllene, limonene, α-selinene, β-caryophyllene, germacrene D, pyrazole-5-ol, methaqualone, steroids, isoquinoline, terpenoids (sitosterol, stigmasterol and campesterol), reducing sugars, fatty acids (oleic, palmitic and linoleic acids) (Idu et al., 2007; Liu et al., 2009; Oladeji et al., 2020); flavonoids (kaempferol 3-gentiobioside and kaempferol) (Farag et al., 2015; Yeong et al., 2022); vitamin E (2.04%) (Eldemerdash et al., 2022); and anthraquinones (alatinone, allatonal, rhein and aloe-emodin) (Abubakar and Haque, 2020; Liu et al., 2009). Rhein (1,8-dihydroxyanthraquinone-3-carboxylic acid) and aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-anthraquinone) exhibit antioxidant properties and anti-inflammatory activity (Abubakar and Haque, 2020; Lewis and Levy, 2011), by decreasing the production of interleukin-1β (IL-1β) and matrix metalloproteinases in articular chondrocytes (Panichayupakaranant et al., 2009; Tamura et al., 2001).

The SciELO, PubMed-NCBI, and ScienceDirect databases were searched for published studies of the extracts and polyphenolic compounds of *S. alata* in Peru, and it is evident that studies of this species are limited or scarce. In this sense, it is justified to carry out a study for three reasons: First, determine the total content of polyphenols and their antioxidant activity *in vitro*, given that this species is native to Ghana, which has adapted to Peru, mainly in the Pichanaqui district (jungle zone), and this jungle microclimate could influence its polyphenolic components and therefore its antioxidant and anti-inflammatory properties. Second, our results could contribute to revaluing the ethnomedicinal use of jungle communities, at the same time, it would serve as scientific evidence for health authorities to promote the rational use of this species. Third, through preclinical studies, the potential anti-inflammatory activity is evident, which will allow further study of said species to isolate the active metabolites, elucidate its chemical structure, and promote the phase 0 study, and if its anti-inflammatory activity is verified, promote the clinical trial in humans (Phase I, II, III and IV). Therefore, the objective was to evaluate the potential anti-inflammatory effect of the total flavonoid content of the hydroalcoholic extract of the leaves of *S. alata* in an experimental model of acute inflammation induced by carrageenan in Albino rats of the strain Holtzman to help generate scientific evidence on its bioactive properties.

MATERIAL AND METHODS

Type of study

Double-blind experimental phase study in small animals.

Reagents and standard

All chemicals and solvents used were reagent grade: distilled water, HPLC grade water, ethanol, and methanol (Beaker Brand, USA); sodium acetate, acetic acid, hydrochloric acid, sodium carbonate, ferric trichloride, and Folin-Ciocalteu reagent from the Merck (Germany); gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(ammonium 3-ethyl benzothiazolin-6-sulfonate (ABTS) Sigma-Aldrich (USA); physiological saline 9% (RS EN- 02537, Medifarma), ibuprofen Tab 400 mg (Fredol, RS EN-02090, Portugal), dexamethasone Tab 4 mg (Medicort, RS EN-02332, Medifarma), carrageenan (Sigma Chemical Co. St. Louis, MO, USA) and pentobarbital sodium (Halatal, Montana).

Sample and population of experimental animals

A random sampling of 36 *Rattus norvegicus* (albino rats) of the Holtzman strain females with an average weight of 210.55 ± 0.61 g was used. A random sampling of 48 *Mus musculus* (male albino mice)

Balb/c/CNPB with an average weight of 25.25 ± 0.10 g was used. The experimental animals were acquired from the Vivarium of the National Center for Biological Products (CNPB) of the National Institute of Health.

Ethics for handling experimental animals

The Research Ethics Committee of the National University San Luis Gonzaga of Ica approved this study through CEI-UNICA certificate Nº0004/04-2024. The study was carried out in strict compliance with national and international standards on the ethics and management of laboratory animals. After the experiment, all animals were euthanized with an overdose of sodium pentobarbital, intraperitoneally (100 mg/kg). The sacrificed animals were disposed of according to NTSN°144-MINSA/2018/DIGESA, Technical Health Standard "Comprehensive Management and Management of Solid Waste in Health Establishments, Medical Support Services and Research Centers" (Alvarado et al., 2022; Carrasco et al., 2013; Rojas et al., 2012).

Vegetable sample

The leaves of the shrub *Senna alata* (L.) Roxb. were collected in the Pichanaqui district (Geographically located between 10°55'29"S 74°52'36"W), altitude 525 m.a.s.l province of Chanchamayo, department of Junín, Peruvian jungle zone. The sample was identified by a specialist in taxonomy and deposited in the Herbarium of the Natural History Museum of the National University of San Marcos, Lima, Peru (certificate No. 11-USM-2018).

Extraction and isolation

The *S. alata* bushes were transported to the Instrumental Analysis laboratory of the Faculty of Pharmacy and Biochemistry of the National University San Luis Gonzaga of Ica. In the laboratory they were dried under shade for 15 days, and then the leaves were separated manually and cleaned with a fine-bristled brush. Subsequently, they were ground in a manual mill (IVYMEN JP SELECTA, YCW-010E, Spain) until a fine powder was obtained. 500 g of the fine powder was weighed (Sartorius model ED224S, Germany) and placed in a glass bottle, and 70% ethanol was immediately added, the proportion being 1 sample and 3 solvent (1:3). It was left to macerate for 10 days, protected from light, heat and at laboratory temperature; to accelerate the extraction of bioactive compounds, it was shaken for 30 min daily. The liquid extract obtained was filtered (filter paper No. 40), and evaporated (Heidolph model LABOROTA 4000 rotary evaporator, Germany) at reduced pressure and 38 ± 2°C to obtain a semisolid extract. Said extract was conditioned in an oven (Binder series 05-75803, Germany) at 40°C, obtaining a dry hydroalcoholic extract from the leaves of *S. alata*. The percentage yield was calculated from the weight of the fine leaf powder and the final weight of the dry extract, obtaining a yield of 4.47%. Subsequently, it was stored in an amber bottle at 4°C until experimental analysis *in vitro* and *in vivo* (Carrasco et al., 2013; Chávez et al., 2021; Rojas et al., 2012; Surco-Laos et al., 2023).

Total polyphenol content

Previously, a calibration curve of the gallic acid standard (Sigma-Aldrich) in 70 % alcohol was carried out in the range of 1-7.5 µg/mL. In a 5 mL tube, 0.1 mL (900 µg/mL) of hydroalcoholic extract and 0.45 mL of Folin-Ciocalteu solution (Merck, reagent ratio: HPLC grade water 1:2) were incorporated, homogenized and it was allowed to react for 5 min. After that time, 0.45 mL of 20 % sodium carbonate (Na_2CO_3) and HPLC grade water enough quantity for (eqf) 3 mL were added. The entire solution was immediately homogenized. It was allowed to react for 30 min at laboratory temperature and protected from light. The absorbances of the samples and the blank were read in triplicate at a wavelength of 760 nm (spectrophotometer Peak Instrumental, model C-7100, USA). The total polyphenolic content was expressed in mg of gallic acid (GAE)/100 mg of dry hydroalcoholic extract (Alvarado et al., 2023; 2024; Surco-Laos et al., 2022a; 2023).

Total flavonoid content

A quercetin calibration curve of 50-500 µg/mL was previously prepared. In a tube with a nominal capacity of 2 mL, 200 µL of the hydroalcoholic extract and 1000 µL of distilled water were added and homogenized; then, 75 µL of 5% sodium nitrite ($NaNO_2$) was added, homogenized again, and allowed to react for 5 min. After the previous reaction, 75 µL of 10% aluminum chloride ($AlCl_3$) was added, mixed, and allowed to react for 6 min, then 500 µL of 1 M sodium hydroxide (NaOH) was added. The entire mixture was homogenized and allowed to rest for 5 min. The absorbances of the samples were read in triplicate at a wavelength of 510 nm (spectrophotometer Peak Instrumental, model C-7100, USA). The total flavonoid content was expressed in mg of quercetin/100 mg of dry hydroalcoholic extract (Alvarado et al., 2024; Ramos-Escudero et al., 2012; Vega et al., 2017).

Antioxidant assay

DPPH radical scavenging assay

Several 3 mL tubes were identified, then the sample and reagents were added in the following order:

0.1 mL of the dilution of the hydroalcoholic extract, and 2.9 mL of DPPH solution, the mixture was homogenized. The tubes were protected from light and allowed to react for 30 min at laboratory temperature. The absorbances (abs.) of the samples and the blank (methanol) were read in triplicate at a wavelength of 517 nm (spectrophotometer Peak Instrumental, model C-7100 USA). The inhibition percentage (% Inh) was determined using the formula [1].

$$\text{Inhibition (\%)} = \frac{\text{Blank abs.} - \text{Sample abs.}}{\text{Blank abs.}} \times 100 \quad [1]$$

The IC_{50} was determined from a curve of % inhibition vs. µL of hydroalcoholic extract. Previously, the 100 mM DPPH (Sigma) solution was prepared in 80 % methanol (analytical grade, Beaker) and the absorbance was established between 0.9-1.1 at a wavelength of 517 nm (spectrophotometer Peak Instrumental, model C -7100 USA) (Alvarado et al., 2023; Ramos-Escudero et al., 2012; Surco-Laos et al., 2023).

ABTS radical scavenging capacity

ABTS reagent (2,2'-azino-bis-(3-ethyl benzothiazolin-6-ammonium sulfonate) was reacted with potassium persulfate ($K_2S_2O_8$) for 12 h at laboratory temperature and under the protection of light. In a tube with a nominal capacity of 3 mL, 10 µL of the hydroalcoholic extract and 990 µL of $ABTS^{\bullet+}$ radical solution were added and allowed to react at 37°C for 4 min. The absorbances of the samples and the ABTS initial reagent were read in triplicate at a wavelength of 734 nm (spectrophotometer Peak Instrumental, model C-7100 USA). The results were expressed as values of mM of Trolox equivalent antioxidant capacity (TEAC)/100 mg of dry extract by constructing a Trolox standard curve (Surco-Laos et al., 2022a).

Experimental animal management methodology

Albino rats (*Rattus norvegicus* strain Holtzman) and albino Balb/c/CNPB mice (*Mus musculus*) were conditioned in clean, ventilated propylene cages. The laboratory photoperiod conditions were 12 h of light/12 h of darkness and a temperature of 22 ± 2°C. They were provided with food according to their species and drinking water *ad libitum*. The animals were acclimatized for 7 days. After that time the experiment began (Chávez et al., 2021).

Acute toxicity assay

After 7 days of acclimatization, the *Mus musculus* were weighed at the beginning and end of the experiment, and then 6 groups were formed at random (n= 8), who were administered a single oral dose of the hydroalcoholic extract of the leaves of *S. alata* (HELSA). Previously, the limit test of the observation study was applied at 2000 mg/kg body weight (n = 8 animals) and was expected to produce some signs of toxicity without causing serious toxic effects or mortality within 24 h. Then, the trial was designed with doses of 500, 1000, 5000, and 7500 mg/kg of HELSA. Each experimental animal was recorded individually every 30 min during the first 4 h and subsequently every 1 h until the first 24 h. They were then observed daily twice a day (8:00 and 20:00 hours) for 14 days. The clinical variables observed included changes in the eyes, mucous membranes, integument (changes in skin and fur), nervous system (somatomotor activity, behavioral pattern, tremors, seizures, salivation, lethargy, sleep and coma), respiratory (presence of dyspnea) and digestive system (consistency of stool or diarrhea and changes in the eating pattern). Toxicity was determined from the description of visible signs of toxicity after the administration of HELSA, considering the variables imminent death, dying state, predictable death, and delayed death (Ahmad et al., 2022; Jatsa et al., 2018; OECD, 2001). The design of the HELSA Fixed Dose Acute Toxicity Test was as described in Table 1 as established by the Organization for Economic Cooperation and Development (OECD) in the Guidelines for the Testing of Chemicals (OECD TG), Test No. 423: Acute oral toxicity-Acute toxic class method.

Table 1. Design of the acute toxicity test of the hydroalcoholic extract of the leaves of *S. alata* (HELSA).

Groups	Plant drug/drug/placebo	Animal treatment
G-1	Distilled water	Distilled water (2 mL/kg body weight) was administered through an orogastric tube (negative control group).
G-2	HELSA 500 mg/kg.	HELSA was administered at a dose of 500 mg/kg body weight via an orogastric tube.
G-3	HELSA 1000 mg/kg.	HELSA was administered at a dose of 1000 mg/kg body weight via an orogastric tube.
G-4	HELSA 2000 mg/kg.	HELSA was administered at a dose of 2000 mg/kg body weight via an orogastric tube.
G-5	HELSA 5000 mg/kg.	HELSA was administered at a dose of 5000 mg/kg body weight via an orogastric tube.
G-6	HELSA 7500 mg/kg.	HELSA was administered at a dose of 7500 mg/kg body weight via an orogastric tube.

Protocol established by the Organization for Economic Cooperation and Development (OECD) in the Guidelines for the Testing of Chemicals (OECD TG), Test No. 423: Acute oral toxicity-Acute toxic class method (2021).

Table 2. Design of acute inflammation induced by 1% carrageenan and the anti-inflammatory effect of the hydroalcoholic extract of the leaves of *S. alata (*HELSA).

Groups	Plant drug/drug/placebo	Animal treatment
G-2 to G-6	Carrageenan 1%	0.1 mL of 1% carrageenan was injected into 6 groups identified as G-1, G-2, G-3, G-4, G-5 and G-6. Carrageenan was injected into the subplantar aponeurosis of the right hind paw of the rat.
G-1	Physiological saline	Physiological saline (2 mL/kg body weight) was administered to the negative control group (G-1) through an orogastric tube.
G-2	HELSA 50 mg/kg	HELSA was administered at a dose of 50 mg/kg body weight via an orogastric tube. The edema formed was measured at 0, 1, 3, 5, 7 hours using a digital micrometer.
G-3	HELSA 250 mg/kg	HELSA was administered at a dose of 250 mg/kg body weight via an orogastric tube. The edema formed was measured at 0, 1, 3, 5, 7 hours using a digital micrometer.
G-4	HELSA 500 mg/kg	HELSA was administered at a dose of 500 mg/kg body weight via an orogastric tube. The edema formed was measured at 0, 1, 3, 5, 7 hours using a digital micrometer.
G-5	Ibuprofen as a reference drug	Ibuprofen was administered at an oral dose of 120 mg/kg of experimental animal weight. The edema formed was measured at 0, 1, 3, 5, 7 hours using a digital micrometer.
G-6	Dexamethasone as a reference drug	Dexamethasone was administered at an oral dose of 2 mg/kg of experimental animal weight. The edema formed was measured at 0, 1, 3, 5, 7 hours using a digital micrometer.

Anti-inflammatory effect test

After 7 days of acclimatization, the female rats (*Rattus norvegicus* strain Holtzman) were weighed (n = 36), obtaining an average weight of 210.55 ± 0.61 g at the beginning of the experiment; Then 6 groups were formed at random (n = 6). Twelve hours before the experiment, food was removed, but with free access to water. The design of acute inflammation induced by 1% carrageenan and the anti-inflammatory effect of HELSA is described in Table 2 below (Meshram et al., 2015; Zaa et al., 2012).

Plantar edema (volume displacement) and control was measured using a digital micrometer (Series 293, Mitutoyo Brand, Japanese). Then, the difference in displacement between the irritated right paw (treated) and the left paw (untreated) was calculated. The percentage of inhibition (PI) was calculated at each time interval according to the formula [2].

$$PI = \frac{(Vt\text{-}Vo)\ control - (Vt\text{-}Vo)\ treated}{(Vt\text{-}Vo)\ control} \times 100 \quad [2]$$

Where: Vt: Average paw volume over a time interval, Vo: Average volume of the paw at zero hour.

Statistical analysis

The results were transcribed into an Excel spreadsheet, from where they were exported to perform the statistical analysis. The data were expressed as mean ± standard deviation (SD), the 95% confidence interval (95% CI), Pearson correlation coefficient, and one-way analysis of variance (ANOVA) were calculated. A $p<0.05$ was considered statistically significant. The GraphPad Prism 9 Statistical Software was used in version 9.1.2.

RESULTS AND DISCUSSION

After collecting, washing, and drying the leaves of *S. alata*, 500 g of dry powder (ground dry leaves) were weighed, macerated and subsequently, a semi-solid extract was obtained (concentrated in a rotary evaporator), which was incorporated into a plate of porcelain (previously weighed) and taken to a stove (Binder series 05-75803, Germany) for drying, then conditioned in a desiccator and weighed again obtaining a weight of 22.36 g of dry extract, which is equivalent to a percentage yield of 4.47% of dry extract of the species. The same one that was used for the *in vivo* and *in vitro* studies.

The percentages of HELSA inhibitions were determined (Table 3), and these values were used for making a DPPH curve constructed at various concentrations (µg/µL), and the concentration at which 50% of the DPPH free radicals were neutralized was found (IC_{50}).

Fig. 2 shows the relationship between the percentage of inhibition vs. concentration of the extract.

Table 4 describes the inhibition percentages determined by the different concentrations of the Trolox standard (µg/mL).

Fig. 3 shows the standard curve of Trolox at various concentrations (µg/mL); therefore, by the DPPH

method, 2.8 mg of the HELSA extract was equivalent to 170.4 µg/mL of Trolox.

Table 3. Percentage of inhibition of the hydroalcoholic extract of *S. alata* (HELSA) leaves by the DPPH method.

C (µg/µL)	Inhibition (%)	ABS (Mean ± SD)
0.38	1.83	0.968 ± 0.0035
0.75	11.50	0.873 ± 0.0032
1.50	24.70	0.714 ± 0.0040
2.99	50.70	0.495 ± 0.0023
3.49	61.60	0.339 ± 0.0055

C: extract concentration; ABS: mean values of the absorbances read from the samples; SD: standard deviation; White ABS = 0.986 at 515 nm; data represent (n = 3).

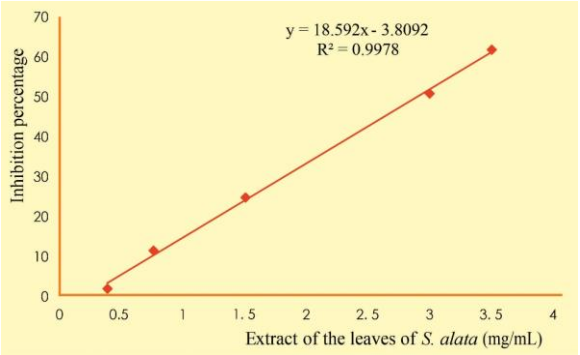

Figure 2. Percentage of inhibition vs. concentration of the HELSA extract.

Table 4. Percent inhibition of Trolox standard solutions by the DPPH method.

C (µg/mL)	Inhibition (%)	ABS (Mean ± SD)
30.5	2.23	0.964 ± 0.0045
41.0	5.38	0.933 ± 0.0030
82.0	19.90	0.790 ± 0.0200
164.0	48.70	0.506 ± 0.0030
246.0	75.30	0.243 ± 0.0040

C: Trolox concentration; ABS: means of the absorbances read from the Trolox dilutions at 517 nm; SD: standard deviation; Data represent (n = 3).

The total polyphenol content (TPC) and total flavonoid content (TFC) were evaluated at the concentration of 1.10 mg/mL of HELSA. The TPC was determined in mg gallic acid equivalents (GAE)/100 mg, and the TFC was quantified in mg quercetin equivalents (QE)/100 mg of dry hydroalcoholic extract in both cases. While the potential antioxidant activity of said hydroalcoholic sample was determined by two *in vitro* methods. Using the DPPH method, it was determined at a wavelength of 515 nm, observing discoloration of the solution (plant sample + DPPH radical), which indicates that the plant sample can capture free radicals (DPPH); It was found that the antioxidant capacity of the plant extract is 2.89 ± 0.04 µg/µL. In a previous study, it was described that the mechanism of action is due to the transfer of a hydrogen atom from the hydroxyl group of the polyphenolic compound to DPPH (HAT) to generate a stable compound (Alvarado et al., 2024), while Mendoza Isaza et al. (2020) indicates that polyphenolic compounds have the ability to capture reactive oxygen species (ROS).

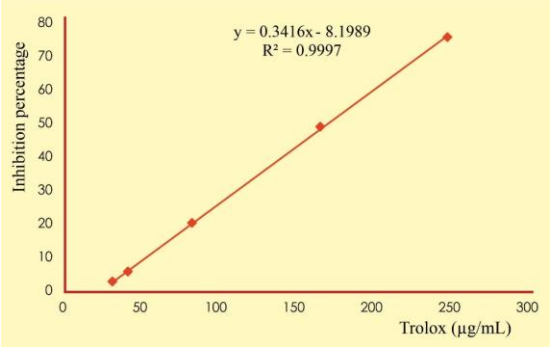

Figure 3. Percentage of inhibition vs. concentration of the Trolox standard.

By the ABTS method, extinction of the $ABTS^{\bullet+}$ cationic radical was observed (the solution was discolored) due to a reduction in absorbance at 730 nm. The uptake capacity of HELSA by the $ABTS^{\bullet+}$ cation was 11.67 ± 0.03 mM TEAC/100 mg of dry sample. Table 5 reports the estimation of the total content of polyphenols (TPC), flavonoids (TFC), and the antioxidant activity of the hydroalcoholic extract of the leaves of *S. alata* by two *in vitro* methods. A previous study carried out by Surco-Laos et al. 2022b on the ethanolic extract of the leaves of *S. nutans* reported that low IC_{50} values indicate high antioxidant power, and said activity would be due to its polyphenolic components.

Fig. 4A reports the ratio of total polyphenolic content (TPC: mg GAE/100 mg) and DPPH (IC_{50}) µg/µL of the HELSA, whose correlation is moderate and positive (r = 0.569), and its coefficient of determination (R^2 = 0.3243) indicates a 32.43% relationship at a linear level of both variables, this indicates that by increasing the amount of polyphenols, antioxidant activity increases. Fig. 4B describes the relationship between TPC (mg QE/g) /ABTS (mM TEAC/100 mg), which is moderate and negative (r= -0.596; R^2 = 0.3553) with a 35.53 % relationship at the linear level of both variables, this suggests that as one of the variables increases, the other decreases.

Table 5. Estimation of the total content of polyphenols, flavonoids, and antioxidant activity of the hydroalcoholic extract of the leaves of *S. alata*.

Assay	Mean ± SD	95%CI	#Reference compounds	ANOVA p<0.05
TPC (mg GAE/100 mg)	22.90 ± 0.05	0.0566	Gallic acid	5.79×10^{-11}*
TFC (mg QE/100 mg)	1.63 ± 0.02	0.0173	Quercetin	4.12×10^{-10}**
DPPH (IC_{50}, µg/µL)	2.89 ± 0.04	0.0397	Trolox	5.79×10^{-7}***
ABTS (mM TEAC/100 mg)	11.67 ± 0.03	0.0285	Trolox	4.90×10^{-11}****

TPC: total polyphenol content in mg gallic acid equivalent (mg GAE/g); TFC: total flavonoid content in mg quercetin equivalent (mg QE/g); TEAC: mM Trolox equivalent antioxidant capacity/g. 95 % CI: 95% confidence interval. *DPPH/TPC; **ABTS/TPC; ***DPPH/TFC; ****ABTS/TFC. #Means values (n = 3) of the calibration curve.

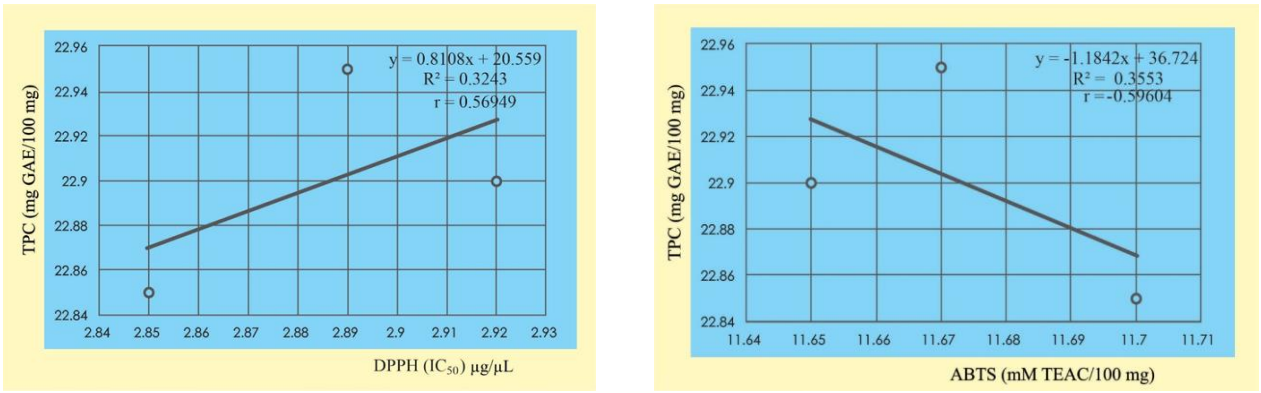

Figure 4. Pearson correlation coefficient of total polyphenol content (TPC)/antioxidant activity of DPPH **(A)** and TPC/ABTS **(B)**.

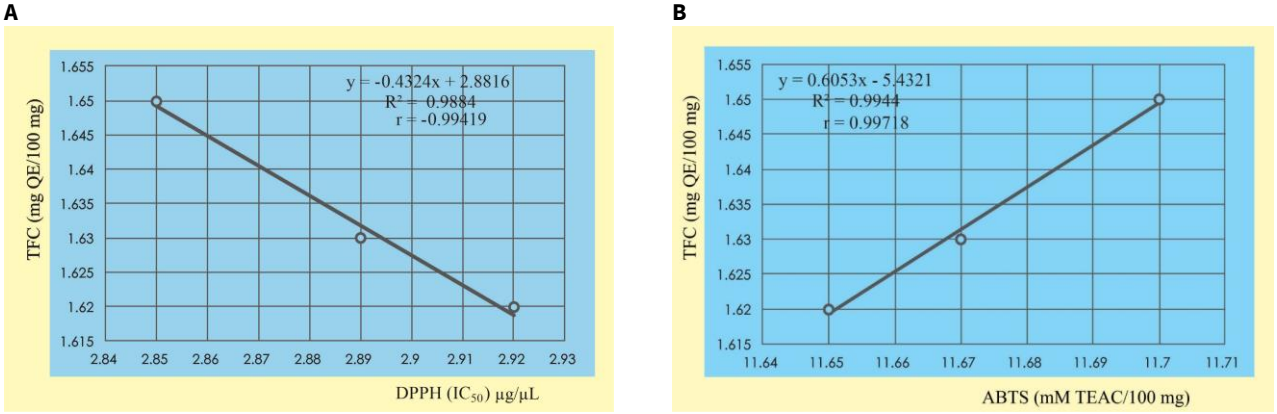

Figure 5. Pearson correlation coefficient of total flavonoid content (TFC)/antioxidant activity of DPPH **(A)**, and TFC/ABTS **(B)**.

Fig. 5A shows the strong inverse correlation (r= -0.994; R^2 = 0.9884) of the TFC (mg QE/100 mg) and the DPPH (IC_{50}) µg/µL assay; Fig. 5B indicates a strong and positive correlation (r = 0.997) between TFC (mg QE/100 mg)/ABTS (mM TEAC/100 mg) with a coefficient of determination (R^2 = 0.9944) indicating a 99.44% relationship at a linear level of both variables, that is, increasing the amount of flavonoids increases the antioxidant capacity. These results indicate the potential antioxidant activity of HELSA, which would be due to its polyphenolics compounds, especially flavonoids, as has been established in studies of the relationship between TFC/DPPH total flavonoid content and specifically with the TFC/ABTS correlation. Thabit et al. (2018) mention that the antioxidant activity of the *Senna* genus is correlated with the content of polyphenols and flavonoids, which includes catechins, proanthocyanidins, scutellarein, rutin, quercimerythrin, kaempferol glycosides, rhein, chrysophanol, aloe emodin, and fiscion. Another study conducted by Yeong et al. (2022) showed that the ethanolic extract of *S. alata* contains kaempferol 3-gentiobioside (4.27 mg/g dry extract), kaempferol (8.54 mg/g) and aloe emodin (0.86 mg/g). These fla-

vonoids are positively correlated with antioxidant activity.

The acute toxicity test was carried out at various doses of the HELSA, at doses lower than 2000 mg/kg. No changes in behavior or clinical alterations were observed, except that one experimental animal died after 90 min. At a dose of 5000 mg/kg, 3 mice died after 90 min, while 5 animals showed sedation and sleep between 90 and 120 min, then recovered during the first 6 hours; while, at the maximum dose of the experiment, 4 animals died, and the other 4 presented salivation, sleep, and diarrhea up to 120 min. After 120 min, no further toxicity or death was observed until 14 days into the experiment. When analyzing the percentage of mortality, 66.7% was observed at a dose of 7500 mg/kg of HELSA (Table 6 and Fig. 6A). A study conducted by Roy et al. (2016) indicates that at doses of 3000 mg/kg of *S. alata* leaves, no toxic effects were shown in physical, biochemical, and hematological parameters or in histopathological studies. Therefore, in the present study, it was not necessary to perform histopathological studies.

Table 6. Dose and percentage mortality of the hydroalcoholic extract of the leaves of *S. alata* in an acute toxicity test.

Study groups	Body weight (mean ± SD)		Dose (mg/kg)	Dead (n)	Live (n)	Accumulated deaths	Accumulated living	Total	Mortality (%)
	Initial weight	Final weight							
G-1	25.25 ± 0.13	27.18 ± 0.25	0	0	8	0	40	40	0
G-2	25.17 ± 0.06	27.13 ± 0.06	500	0	8	0	32	32	0
G-3	25.20 ± 0.10	27.16 ± 0.02	1000	0	8	0	24	24	0
G-4	25.20 ± 0.10	27.15 ± 0.03	2000	1	7	1	16	17	0.059
G-5	25.20 ± 0.10	27.14 ± 0.07	5000	3	5	4	9	13	0.308
G-6	25.22 ± 0.13	27.20 ± 0.10	7500	4	4	8	4	12	0.667

SD: standard deviation; n: number of *Mus musculus*. Mean of the body weight in g of the experimental animal.

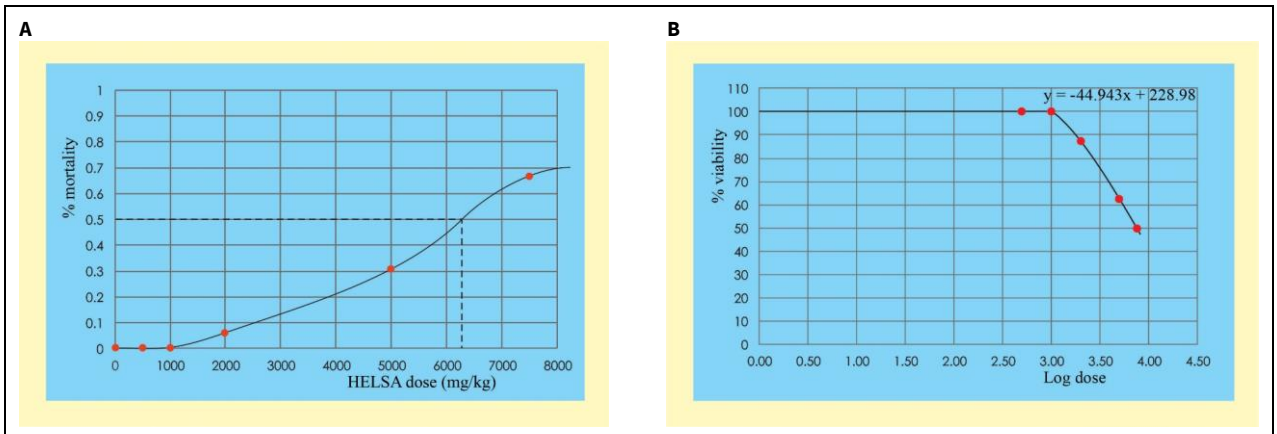

Figure 6. Percentage of mortality and viability to determine the mean lethal dose (LD_{50}).
(A) curve of the percentage of mortality versus the dose of HELSA (mg/kg); **(B)** curve of the percentage of viability versus the logarithmic dose.

Table 7. Percentage of viability and estimation of the mean lethal dose (LD_{50}) by the logarithmic method.

Study group	Dose (mg/kg)	*Mus musculus* (n)	Dead *Mus musculus* (90 min)	Arithmetic method % fatality	Logarithmic method to determine the mean lethal dose (LD_{50})		
					Log Dose	% viability	DL_{50}
G-6	7500	8	4	50	3.88	50	9602 mg/kg
G-5	5000	8	3	37.5	3.70	62.5	
G-4	2000	8	1	12.5	3.30	87.5	
G-3	1000	8	0	0	3.00	100	
G-2	500	8	0	0	2.70	100	
G-1	0	8	0	0			

G-1: control group; n: number of *Mus musculus*.

Table 8. Anti-inflammatory effect of the hydroalcoholic extract of the leaves of *S. alata* (HELSA) in an experimental model of acute inflammation.

Treatment	Body weight (g)		Anti-inflammatory effect (mean ± SD mm)				
	Initial weight	Final weight	0 h	1 h	3 h*	5 h	7 h
Physiological saline (2 mL/kg body weight)	210.55 ± 0.61	212.56 ± 0.64	0.15 ± 0.03	0.50 ± 0.02	0.46 ± 0.02	0.48 ± 0.04	0.48 ± 0.04
HELSA 50 mg/kg	210.21 ± 0.05	212.54 ± 0.62	0.16 ± 0.02	0.48 ± 0.02	0.41 ± 0.02	0.41 ± 0.04	0.38 ± 0.03
HELSA 250 mg/kg	210.56 ± 0.61	212.23 ± 0.08	0.15 ± 0.02	0.46 ± 0.03	0.36 ± 0.03	0.36 ± 0.03	0.29 ± 0.03
HELSA 500 mg/kg	210.52 ± 0.59	212.20 ± 0.06	0.15 ± 0.02	0.44 ± 0.02	0.43 ± 0.02	0.29 ± 0.04	0.23 ± 0.03
Ibuprofen 120 mg/kg	210.51 ± 0.52	212.51 ± 0.68	0.15 ± 0.03	0.46 ± 0.03	0.45 ± 0.02	0.41 ± 0.02	0.35 ± 0.04
Dexamethasone 2 mg/kg	210.52 ± 0.60	212.36 ± 0.21	0.16 ± 0.01	0.36 ± 0.04	0.31 ± 0.04	0.24 ± 0.02	0.20 ± 0.02

*ANOVA at doses of 50 and 250 mg of HELSA vs. ibuprofen (p=0.0306 and 0.0001, respectively) and not significant at doses of 500 mg/kg of HELSA vs ibuprofen (p=0.1135) at 3 hours; at 50 and 500 mg/kg of HELSA vs dexamethasone it was significant (p=0.0002 and p=9.13 x 10^{-5}, respectively) and at 250 mg/kg of HELSA vs. dexamethasone it was not significant (p=0.0664) at 3 hours.

Table 9. Anti-inflammatory effect and percentage of inhibition of the hydroalcoholic extract of the leaves of *S. alata*.

Treatment	Anti-inflammatory effect		Inhibition (%) (7 h)	ANOVA (p<0.05)
	0 h	7 h		
Physiological saline (2 mL/kg body weight)	0.15 ± 0.03	0.48 ± 0.04	0	
HELSA 50 mg/kg	0.16 ± 0.02	0.38 ± 0.03	33.33	0.0013*
HELSA 250 mg/kg	0.15 ± 0.02	0.29 ± 0.03	57.60	3.714 x 10^{-6}*
HELSA 500 mg/kg	0.15 ± 0.02	0.23 ± 0.03	75.75	7.029 x 10^{-5}*
Ibuprofen 120 mg/kg	0.15 ± 0.03	0.35 ± 0.04	39.40	0.0004
Dexamethasone 2 mg/kg	0.16 ± 0.01	0.20 ± 0.02	87.87	5.008 x 10^{-8}

*ANOVA between the different doses of HELSA vs. control (physiological saline solution).

Table 7 shows the treatment of the data at 90 min, and using the logarithmic method, the mean lethal dose (LD_{50}) was estimated at 9602 mg/kg, which is higher than the maximum dose of the present experiment. This indicates that HELSA would have a broad therapeutic index, which warrants further evaluation in other animal species and the conclusion that they are safe in order to evaluate their pharmacological effects.

Fig. 6B shows the curve of the percentage of viability versus the logarithmic dose to determine the mean lethal dose (LD_{50}).

A significant decrease in rat paw edema was evident at the three doses of HELSA compared to the control group (p<0.05). While at doses of 250 and 500 mg/kg of HELSA compared to ibuprofen (p=0.014 and 0.029, respectively) and dexamethasone (6.60 × 10^{-5} and 0.04, respectively), a significant anti-inflammatory effect was evident 7 hours after administration 1 % carrageenan (Table 8). At the dose of 50 and 250 mg of HELSA versus ibuprofen, an anti-inflammatory effect was evident (p=0.0306 and p=0.0001, respectively) and at the dose of 50 and 500 mg/kg of HELSA versus dexamethasone they were statistically significant (p=0.0002 and p=9.13 × 10^{-5}, respectively) after 3 hours of the experiment; However, no statistically significant effect was evident at a dose of 500 mg of HELSA compared to ibuprofen at 3 hours (p=0.1135), despite the fact that the maximum time (t_{max}) to reach plasma concentration is 1.5 hours (Shin et al., 2017). It is likely that the gastric emptying time and intestinal transit time were slow in the group of experimental animals, affecting the bioavailability of the drug (Cheng and Wong., 2020) because the 12-hour fast was controlled before starting the experiment.

Table 9 shows the effect and percentage (%) of HELSA inhibition. A significant decrease in rat paw edema was evident (p<0.05) at the three doses of HELSA vs. negative controls. A considerable percentage of anti-inflammatory inhibition (75.75 %) was observed at a dose of 500 mg/kg of HELSA compared to ibuprofen (39.40%) and dexamethasone (87.87%) at doses of 120 and 2 mg/kg, respectively, after 7 hours

after the administration of carrageenan, as seen in Fig. 5. In a previous study conducted by Patrick-Iwuanyanwu et al. (2011) it has been reported that the butanol extract of *S. alata* inhibits inflammation by 78.36%, ethyl acetate extracts 58.21% and methanol extract 20.89% at a dose of 100 mg/kg compared to indomethacin (79.59%) at a dose of 10 mg/kg after 4 h of administering carrageenan to the paw of the experimental animal. Our preliminary findings were made with a hydroalcoholic extract, and the final analysis of the anti-inflammatory effect was performed at 7 hours, unlike the study that analyzed the anti-inflammatory effect in different extracts at 4 hours. The anti-inflammatory effect was analyzed at 7 hours based on two pharmacokinetic parameters of ibuprofen that was used as one of the references. The half-life time ($t_{1/2}$) of ibuprofen is 2 h (Regueras et al., 2024; Shin et al., 2017), which indicates that in 4 half-life times, the next dose should be administered, that is, at 8 h, the minimum effective concentration is found; and the maximum time to reach the maximum concentration (t_{max}) is 1.5 h, indicating that the maximum plasma concentration has been reached (C_{max} = 24.1 mg/L) inhibiting cyclooxygenases 1 and 2 (COX-1 and COX-2) until 8 h (Shin et al., 2017). In a previous study conducted by Moriyama et al. (2003), it was demonstrated that the extract of *S. alata* or *Casia alata* leaves has an anti-inflammatory effect *in vitro* (rat peritoneal exudate cells) by inhibiting histamine, 5-lipoxygenase (5-LOX), COX-1 and COX-2. However, these results should be considered as part of scientific evidence for their potential anti-inflammatory effect, but they need to be validated with studies of the mean effective concentration (EC_{50}) of HELSA and the reference drugs to verify the effectiveness of the extract of the present study.

Fig. 7A shows the edema of the right paw of the rat induced by 1% carrageenan, and Fig. 7B shows the anti-inflammatory effect of HELSA at a dose of 500 mg/kg, and in Fig. 7C the anti-inflammatory effect of ibuprofen at a dose of 120 mg/kg is noted.

It is likely that the mechanism of action of the polyphenols and flavonoids in HELSA is related to the inhibition of histamine, serotonin, bradykinin, and COX_2, decreasing proinflammatory prostaglandins, due to the experimental model of edema induced by 1% carrageenan. Masresha et al. (2012) describe that 1% carrageenan induces localized inflammation that develops in two stages: the first stage begins 0 to 2.5 hours after injection and is characterized by the release of histamine, serotonin and bradykinin, and the second phase begins between 2.5 and 5 hours, a period in which COX_2 is expressed. Studies carried out by Coura et al. (2015) and Arega et al. (2023) describe that phase 2 of the inflammatory process begins between 2.5 and 5 hours after the injection of 1% carrageenan, inducing the overproduction of COX_2 that synthesizes proinflammatory prostaglandins (PGE_2 and PGD_2), at the same time, generating invasion of polymorphonuclear leukocytes (neutrophils).

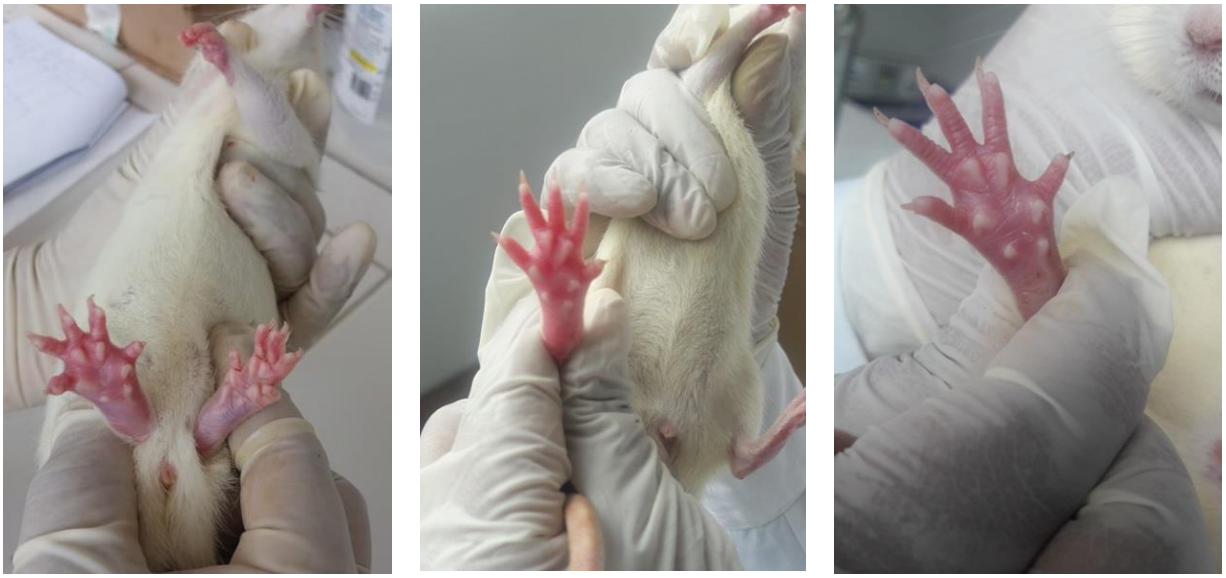

Figure 7. Edema induced by 1% carrageenan **(A)** and anti-inflammatory effect of the hydroalcoholic extract (500 mg/kg) of the leaves of *Senna alata* (L.) Roxb **(B)** and ibuprofen (120 mg/kg) **(C)**.
Photos of the experimental study.

Subsequently, Lewis and Levy (2011) demonstrated that the hexane extract of *S. alata* leaves at a dose of 500 mg/kg has an anti-inflammatory effect by significantly reducing (p=0.0032) the knee circumference (swelling) in CFA arthritic rats. It was observed that the normal structure of the cartilage and the arrangement of the chondrocytes are maintained, indicating a protective effect of cartilage degradation in the femoral head of the knee joint. According to the study by Singh et al. (2012), the hydroalcoholic extract of *S. alata* at a dose of 200 mg/kg inhibits mast cell degranulation. Meanwhile, the compounds rhein and kaempferol present potent inhibition (>76%) of mast cell degranulation at 5 mg/kg. The extract and rhein inhibit lipoxygenase (LOX) at concentrations of 90.2 and 3.9 µg/mL, respectively. While Ongchai et al. (2019) reported that *S. alata* extract contains rhein (3.65 µg/mL) and aloe-emodin (1.78 µg/mL), and at the concentration of 25 µg/mL, they decrease the release of sulfated glycosaminoglycans (S-GAG) and hyaluronic acid (HA), therefore, preventing the degradation of cartilage proteoglycan. In a review study conducted by Alshehri et al. (2022) the antioxidant and anti-inflammatory activity of the *Senna* genus is verified, whose mechanism of action may be by inhibiting free radicals through the exchange of one or more protons by generating chelation complexes between polyphenolic compounds and free radicals and including metals, and by reducing the formation of reactive oxygen species (ROS).

The results of this research must be considered in the context of several limitations. The main one is that the hydroalcoholic extract of the leaves of *S. alata* has only been studied, not quantified, isolated, and elucidated, and the chemical structure of the phenolic compounds responsible for the anti-inflammatory activity has not been quantified. Other biases that can lead to confusion are having studied only the leaves and not having included other parts of the plant. Likewise, the therapeutic index of the total extract was not determined. For this reason, our research group is considering continuing to study this plant species in a second stage to isolate the bioactive components, carry out preclinical studies in other animal models, and with a greater number to determine the therapeutic index. However, this preliminary research should be considered as a contribution to science that allows studying other experimental models to validate the anti-inflammatory activity. Then, formulate a pharmaceutical form to begin clinical trials in humans, whose ultimate goal is to treat inflammatory diseases with fewer adverse effects.

CONCLUSION

The total content of flavonoids and polyphenols of the hydroalcoholic extract of the leaves of *Senna alata* (L.) Roxb. has potential anti-inflammatory activity in an experimental model of acute inflammation induced by carrageenan in *Rattus norvegicus* (Albino rats) of the Holtzman strain, thus validating the ethnopharmacological claims. At the same time, the results indicate the potential antioxidant activity of HELSA, as has been established in studies of the relationship between total flavonoid content TFC/DPPH and specifically with the TFC/ABTS correlation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Concepts or ideas	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Design	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Definition of intellectual content	x	x	x	x	x	x	x									
Literature search									x	x	x	x	x	x	x	x
Experimental studies	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Data acquisition	x	x	x		x		x	x								
Data analysis									x	x	x	x	x	x	x	x
Statistical analysis						x	x	x	x	x						
Manuscript preparation	x	x	x	x	x	x										
Manuscript editing	x	x			x											
Manuscript review	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

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