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Anti-inflammatory and antioxidant activities of ethylacetate fraction of *Sida linifolia* L. (Malvaceae)

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ABSTRACT

Sida linifolia L., a known weed found in West Africa and other parts of the world, is being used in African traditional medicine for many purposes, including the relief of uncomfortable teething, and the prevention of malaria. This study aimed to fractionate the crude extract of *S. linifolia* and determine the anti-inflammatory, and antioxidant properties of the most potent fraction. In the examination of anti-inflammatory compounds, in vitro tests for platelet aggregation, albumin denaturation, protease, and phospholipase A_2 were utilized. To assess the in vivo anti-inflammatory effects, rat paw edema was induced with carrageenan and egg albumin. The total antioxidant capacity (TAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing power (FRAP), and nitric oxide (NO) assays were used in the in vitro antioxidant assessment. The result of the phytochemical screening revealed that there were varying concentrations of terpenoids, saponins, steroids, alkaloids, flavonoids, tannins, and other phenols. The ethylacetate leaf fraction of *S. linifolia* EALFSL displayed robust, concentration-dependent anti-inflammatory effects by significantly inhibiting hypotonicity- and heat-induced hemolysis, platelet aggregation, protein denaturation, protease activity, and phospholipase A_2 activity, which were comparable to those of the reference drugs (aspirin/prednisolone). In vivo studies also revealed that EALFSL was able, at different doses, to inhibit the progress of carrageenan-induced rat paw edema and egg albumin models. Though it was less active than the butylated hydroxytoluene BHT (0.30 mg/ml), ascorbic acid (0.32-0.50 mg/ml), and gallic acid (0.47 mg/ml), the EALFSL fraction's IC_{50} values ranged from 0.93 to 1.20 mg/ml. The results demonstrated that EALFSL has significant concentration-dependent antioxidant activity. These suggest that the ethylacetate leaf fraction of *S. linifolia* possesses anti-inflammatory and antioxidant effects.

1. Introduction

Inflammation is one of the body's defensive mechanisms in response to harmful physico-chemical and microbiological irritants (Ammendolia et al., 2021). It typically shows up as painful episodes, heat, swelling, and redness of the infected area due to increased vascular permeability of the affected area. Inflammation is a beneficial process that can be used to alter a problem or illness situation inside the body system, but it can also occasionally cause pain and tissue damage (Chen et al., 2018). Most times, the outcome includes fluid exudation and tissue function loss. Neutrophils and other leucocytes are attracted consequently, and they begin to behave aggressively in the inflammatory milieu, leading to lysosomal leakage, protein denaturation, and cell death. Tissue damage and organ failure can result from ongoing and increased inflammation (Sarveswaran et al., 2017). The goal of the inflammatory response is to eliminate or inactivate invasive organisms, remove toxins, and start the process of tissue repair.

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According to Laveti et al. (2013), inflammation has been associated with several illness states, including rheumatoid arthritis, osteoporosis, cancer, obesity, inflammatory bowel disease, cardiovascular disease, diabetes, asthma, depression, and other neurological disorders.

Vasodilatation-increased vascular permeability and the recruitment of plasma proteins and leukocytes to the infected or wounded tissue are some of the events that define inflammation. Several potent vasoactive compounds, including serotonin, tumor necrosis factor-α (TNF-α), prostaglandins, lymphokines, prostacyclins, leukotrienes, and interleukin (IL)-1, IL-8, interferon-γ (IFN- γ), and IFN-α, are also commonly involved in its mediating effects (Abdulkhaleq et al., 2018; Ricciotti & FitzGerald, 2011). Numerous glucocorticoids and non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used to treat pain brought on by acute or chronic inflammation; however, they are not known to be completely effective in treating chronic inflammatory diseases and frequently cause other side effects like liver damage, ulcers, strokes, and heart attacks (Marcum & Hanlon, 2010). These are what appear to have caused the shift in research priority for the treatment of inflammatory illnesses from chemical synthetic formulations to natural formulations. The question of "How to produce potent anti-inflammatory chemicals from natural sources?" has long been a topic of discussion. Because of the richness of their phytochemical makeup, plant products are ideal starting points for the development of novel pharmaceuticals, and they have significantly improved basic healthcare in many impoverished countries (Dias et al., 2012; Sofowora et al., 2013).

A range of bioactive secondary metabolites found in aromatic plants makes them useful as both food and medicine (Altemimi et al., 2017; Samarth et al., 2017). Plants create these phytocompounds, which include phenolic chemicals (tannins, flavonoids, and other phenols), terpenoids, carotenoids, coumarins, alkaloids, and glucosinolates as defense mechanisms against adversaries, infections, predators, and environmental stress (Tungmunnithum et al., 2018). Some of these phytochemicals, such as phenolics and terpenoids, are well known for their potential advantages to human health. African communities use herbs as food and as a cure-all for illnesses in folk remedies since they are gifted with a variety of medicinal plants (Lin et al., 2016; Sofowora et al., 2013). Many weeds and plants are utilized by traditional healers in African traditional medicine to treat pain and inflammatory illnesses (Mintah et al., 2019).

Sida linifolia L. is a widespread weed that can be found in West Africa and other countries. It is a member of the Malvaceae family and the genus *Sida*, which comprises over 180 species and a wide range of ethnomedical uses (Dinda et al., 2015; Saensouka et al., 2016). West tropical Africa, notably Nigeria and Sierra Leone, is home to the highly sought *S. linifolia*. *S. linifolia* leaves are used in combination by traditional healers to treat a variety of diseases, such as cutaneous infections, malaria, and whitlow and as anti-depressants, stimulants for the genitals, and anti-abortifacients effects (Akubue et al., 1983; Burkill, 1994; Kokwaro, 2009; Neuwinger, 2000; Saensouka et al., 2016). The anti-inflammatory, and antioxidant properties of *S. linifolia*'s crude leaf extracts have been investigated (Nwankwo et al., 2023a; Nwankwo et al., 2023b). This study aimed to fractionate the crude leaf extract of *S. linifolia* using different solvents based on polarity and to determine the anti-inflammatory and antioxidant potential of the most potent fraction for possible isolation of the bioactive constituents.

2. Materials and methods

2.1. Collection and identification of the research plant

Fresh *S. linifolia* leaves were harvested in May 2021 in Nsukka, Nsukka L.G.A., Enugu State, Nigeria. A botanist, Mr. Alfred Ozioko, at the Bioresources Development and Conservation Programme (BDCP) research center in Nsukka identified the plant (Voucher no: BDCP20210724).

2.2. Techniques for fractionation and extraction

Following the guidelines provided by Hwang et al. (2009) and Parvin et al. (2015), the extraction process was carried out. *S. linifolia* leaves were collected, cleaned, and dried in the shade to maintain a steady bulk and to avoid denaturation of some bioactive components. A high-speed electric grinder was used to crush the dried plant material. Two thousand five hundred grams (2500 g) of the ground material were weighed out and put in an 8.0 l flask with a flat bottom to soak in 98% ethanol. To enable thorough extraction, the content was left in place for 24 hours while being shaken periodically. The resulting mixture was separated using Whatman No. 1 filter paper, and the crude ethanolic leaf extract of *S. linifolia* (CELESL) was obtained by concentrating it at 45 °C under decreased pressure.

2.3. Fractionation of the crude extract

The CELESL obtained from the extraction of the leaves of *S. linifolia* was partitioned (3 times each) in a fractionating column using *n*-hexane, ethyl acetate, and ethanol. The *n*-hexane (n-HLFSL), ethyl acetate (EALFSL), and ethanolic (ELFSL) leaf fractions were obtained by individually concentrating the eluents at 45 °C using a rotary evaporator. The concentrated fractions were divided among sterile, clearly labeled, screw-capped vials and kept in the refrigerator until they were required for research.

2.4. Preliminary phytochemical testing of EALESL and ELESL

Following traditional procedures as described by Harborne (1998) and Trease and Evans (1985), phytochemical screening of EALESL was carried out to identify the classes and amounts of secondary metabolites contained in the leaf fractions. Different kinds of phytochemicals present were assessed using the formulaes given below:

$$Concentrations\ \left(\frac{mg}{100}g\right) = \frac{Sample\ absorbance}{Standard\ absorbance} x\ Dilution\ factor$$

$$Dilution\ factor = \frac{Total\ volume}{Weight\ of\ extract}$$

2.5. Assessment of in vitro inflammatory actions

2.5.1. Assays for membrane stabilization

With a few minor adjustments, the method of Gandhidasan et al. (1991) was used to create the erythrocyte suspension. Whole blood was supplied by several healthy volunteers who had not used NSAIDs in the previous three weeks. A server solution (0.42% NaCl, 2% dextrose, 0.5% citric acid, and 0.8% sodium citrate) was added to test tubes holding equivalent volumes of the blood sample that had been drawn from sodium oxalate-containing tubes. The test tubes were then refrigerated at 4 °C for 24 hours. The packed cell volume was calculated after centrifuging the resultant mixtures at 3000 g

for 10 min. Following that, this volume was reconstituted to a 40% (v/v) suspension in 10 mM phosphate-buffered saline (pH = 7.4).

2.5.1.1. Assay for hypotonicity-induced hemolysis

For this study, the methodology of Umapathy et al. (2010) was applied with a few minor alterations. A quantity, of 950 µl of phosphate-buffered saline and 1 ml of the reaction mixture (RM) containing 37.5 g/ml of the reference drug (aspirin) at different concentrations were added to 20 µl of a 40% (v/v) erythrocyte suspension. The mixtures were incubated for 1 hour at 30 °C before being centrifuged at 5000 g for 5 minutes, and then 200 µl of the supernatants were then poured into microtitre plates. Using a microplate reader (Molecular Devices, Inc., U.S.) and aspirin as the reference medication, the amount of freed hemoglobin was calculated at 540 nm. Cells were incubated with distilled water and phosphate-buffered saline 0.1% (w/v), respectively, to produce the positive and negative controls of 100% and 0% hemolysis. Triplicates of the test were run for each concentration. The proportion of hemolysis inhibited was calculated by using the formulae given below:

$$\%\ Inhibition\ of\ hemolysis\ = 1 - \frac{Abs2 - Abs1}{Abs3 - Abs1} x\ 100$$

where, Abs1 is the absorbance of control I (isotonic solution), Abs2 is the absorbance of test or reference drug sample, and Abs3 is the absorbance of control II (hypotonic solution).

2.5.1.2. Heat-induced hemolysis

Minor alterations were made to the test protocol from that described by Okoli et al. (2008). An acceptable incubation temperature and time for the hemolysis induction were established during preliminary research. Red cell suspension of a known volume (20 ml) was added to a micro-centrifuge tube (1.7 ml) having 980 ml of buffer that had been incubated and left to warm in a water bath (with a thermostat) kept at 55 ± 0.1 °C by controlling with a calibrated mercury thermometer. After 45 minutes at 5-minute intervals, tubes were removed from the water bath and centrifuged for 5 minutes at 5000 g. Using a microplate reader (made by Molecular Devices, Inc., California, USA), the absorbance of the supernatant was measured at 540 nm. To evaluate the impact of EALFSL on heat-induced hemolysis, a 20-minute incubation period at 54 °C was developed from the observation. Briefly, 20 µl of red cell suspension (40%) and 30 µl of different EALFSL or aspirin concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) were combined and dispensed into a 1.5 ml micro-centrifuge tube with 950 µl of pre-incubated buffer in duplicates to test the effectiveness of the EALFSL on heat-induced hemolysis in comparison to standard medications. The negative control was put into a different tube with an equivalent volume (50 µl) of the vehicle (pre-incubated buffer). One tube from each pair was warmed in a water bath for 20 minutes at 54 °C, while the other tubes were freeze-dried in an ice bath for 5 minutes at 0 °C. Following a 5-minute at 5000 g centrifugation of the final mixes, the supernatant absorbance at 540 nm was measured. The percentage of hemolysis inhibited was determined by using the formulae given below:

$$\%\ Inhibition\ of\ hemolysis\ = 1 - \frac{Abs2 - Abs1}{Abs3 - Abs1} x\ 100$$

where Abs1 is the absorbance of the test group, standard drug, and control that are non-heated, Abs2 is the absorbance of the test group, standard drug, and vehicle samples heated, and Abs3 is the absorbance of the heated negative control.

2.5.2. Phospholipase A_2 inhibition assay

The method outlined by Vane (1971), with a few minor adjustments, was used to assess the plant's inhibitory property on phospholipase A_2 activity. At 418 nm, phospholipase A_2 (PLA_2) activity, which is directly proportional to the volume of oxidized hemoglobin in the medium, was calculated. Red blood cells, which served as the PLA_2 substrate, were extracted from a volume of whole blood (5 ml) samples taken from healthy human participants. On the other hand, a pure growth of the *Aspergillus niger* strain yielded PLA_2. Each pair of test tubes with the labels test and blank received graduated amounts of phosphate-buffered saline (1 ml), red cell suspension (0.2 ml), and $CaCl_2$ (0.22 ml), and the test and blank tubes received 1 ml each of free and boiled enzymes. Then, 1 ml of the EALFSL or prednisolone was diluted into separate test tubes at various concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml), with phosphate-buffered saline serving as the vehicle. The test tube used as the control contained RBC suspension, free enzyme, and $CaCl_2$. All of the test tubes underwent a 1-hour incubation at 30 °C followed by a 10-minute centrifugation at 3000 g. The absorbance of the produced supernatants was measured spectrophotometrically at 418 nm. The highest enzyme activity and the percentage of inhibition were assessed by using the formulaes given below:

$$\%\ Maximum\ enzyme\ activity = \frac{Absorbance\ of\ test}{Absorbance\ of\ control} x\ 100$$

$$\%\ Inhibition = 100 - maximum\ enzyme\ activity$$

2.5.3. Test for inhibition of platelet aggregation

With a few minor changes, the test protocol used in this study was largely the same as that described by Born and Cross (1963). The test works as follows: when $CaCl_2$ is added, platelet aggregation increases in the solution medium, which correlates favorably with transmittance and negatively with absorbance. Thus, at 520 nm, the level of $CaCl_2$-induced platelet aggregation in the reaction milieu is calculated spectrophotometrically. In practice, the absorbance of the RM following the addition of $CaCl_2$ might be measured to evaluate the anti-platelet aggregatory potentials of pharmaceutical drugs. Briefly stated whole blood from healthy human volunteers was put into tubes containing 1% EDTA and centrifuged at 3000 g for 10 minutes. Platelet-rich plasma (PRP) was created by centrifuging the resultant supernatant and diluting it twice with normal saline. Three duplicates of each test were run. A graded volume (0.2 ml) of PRP was divided among sets of three tubes, each containing 1 ml of different concentrations of EALFSL or the control medication (aspirin) in normal saline (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml). Using normal saline as the vehicle, the RM was diluted up to 2.2 ml. PRP (0.2 ml) and regular saline (2 ml) were the contents of the control test tube. The RM was then given 0.4 ml of 1.47% $CaCl_2$ after all the test tubes had been preheated at 30 °C for 5 minutes. A spectrophotometer gave measurement for the change in the solutions' absorbance at 520 nm every two minutes for eight minutes. For each test, the reference medications without PRP were used as the blanks.

2.5.4. Test for protease inhibition

With a few minor modifications, the test was carried out using the method of Sakat et al. (2010). A volume of the RM (2 ml) contains 0.06 mg of trypsin dissolved in a 20 mM *tris*-HCl buffer (pH 7.4) together with 1 ml of EALFSL or the reference drug (aspirin) at various doses (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml). The mixtures were treated with 2 ml of 68% perchloric acid to stop the reaction after

20 minutes of incubation at 37 °C. The resulting cloudy suspension was then centrifuged once more for 10 minutes at 3000 g. After that, the *tris*-buffer was used to blank out the supernatant absorbance (Abs), which was then measured spectrophotometrically at 210 nm. Three duplicates of each test were run. The fraction's % protease inhibition at various concentrations was assessed in comparison to the control and reference medication.

2.5.5. Test for albumin denaturation

The test procedure was the same as that used in the study of Mizushima and Kobayashi (1968). The RM contained aspirin or EALFSL in varying amounts (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml), bovine albumin fraction dissolved in water (1%), and bovine albumin fraction. A few drops of 1N HCl were intermittently added to the reaction liquid to change the pH. The different RM was heated for 30 minutes in a water bath at 57 °C after being incubated at room temperature for 18 minutes. The RM was cooled to room temperature while its absorbance at 660 nm was quantified spectrophotometrically. The percentage inhibition of albumin denaturation was assessed after the complete test had been run in triplicate by using the formulae given below:

$$\%\ Protease\ inhibition\ = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} x\ 100$$

2.6. In vitro study of antioxidants

2.6.1. Assay for free radicals using DPPH

In this testing system, the method implemented by Liyana-Pathirana and Shahidi (2005) was followed. 100 ml of each of five different concentrations of the fraction or reference medication [butylated hydroxytoluene (BHT)] were added to sets of tubes holding 200 ml of DPPH solution (0.1 mM) produced in methanol. The resulting mixtures were given a 15-minute incubation period at 37 °C. The mixes' absorbance was measured spectrophotometrically at 517 nm. Three duplicates of each test were run. The amount in gram-equivalents of butylated hydroxytoluene was used to represent the assay results. EALFSL was compared to a reference medication to determine how well it inhibited DPPH and was calculated by using the formulae given below:

$$\%\ Inhibition\ = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} x\ 100$$

The quantity of the sample that scavenged 50% of the starting amount of DPPH radical in the RM, or the IC_{50} value, was also determined using a graph.

2.6.2. Test for ferric-reducing antioxidant power

The testing was done following the methodology of Sahreen et al. (2014). In a nutshell, 2.0 ml of EALFSL or the reference medication (ascorbic acid) at various concentrations were dispersed into sets of test tubes containing 2.0 ml of potassium ferricyanide solution [0.1% (w/v)] dissolved in phosphate-buffered solution (2.0 ml, 0.2 M, pH 6.6) with a potassium ferricyanide concentration of 10 mg/l. For 20 minutes at 50 °C, the RM was heated in a water bath. The mixtures were then given 2.0 ml of trichloroacetic acid solution [100 mg/l, 10% (w/v)] to add. Then, 2.0 ml of the mixture was poured into 0.4 ml of distilled water that had been combined with 0.1% (w/v) ferric chloride ($FeCl_3.6H_20$) solution. After 10 minutes of reaction, the RM absorbance was measured spectrophotometrically at 680 nm. The gram-equivalents of ascorbic acid were used to represent the assay results. The same formula as DPPH was used to evaluate the fraction's % reduction power in comparison to the standard medication.

2.6.3. Total antioxidant capacity (TAC)

The total antioxidant capacity test was performed according to the method of Prieto et al. (1999) based on the phosphomolybdenum technique. The test is based on antioxidants' propensity to convert Mo^{6+} to Mo^{5+}, which leads to the development of a green phosphate/Mo^{5+} combination in an acidic media. The reaction mixes include 0.1 ml of reference medication (gallic acid) or EALFSL in different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml). A specific concentration (0.6 M) of sulfuric acid was added to test tubes containing ammonium molybdate (4 mM) and sodium phosphate (28 mM) to create the reagent solution, which was then combined with 1 ml of the reagent solution (containing 1.0 mg/ml and 1.0 mg/ml). At 95 °C, the RM was incubated for 90 minutes. When the different mixes were cooled to room temperature, the absorbance was measured spectrophotometrically at 695 nm blanking with an identical quantity of the reagents devoid of the test samples. 1 ml of the reagent solution and 0.1 ml of methanol were combined to create the blanking solution. The number of gram-equivalents of ascorbic acid was used to express the test sample's antioxidant potential. By adding ascorbic acid in different quantities to methanol-filled tubes, the calibration curve was drawn. The gram-equivalents of gallic acid were used to measure the test samples' antioxidant activity. Using the same formula as DPPH, the fraction's percent total antioxidant capacity in comparison to the reference medication was assessed.

2.6.4. Test for nitric oxide activity

This test's methodology was comparable to that described by Marcocci et al. (1994). Sodium nitroprusside was used to produce nitric oxide free radicals, and the Griess reaction was used to assess them. The RM (5.0 ml) contained 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml of EALFSL or the reference medication (ascorbic acid) and 5 mM sodium nitroprusside in phosphate-buffered saline (pH 7.3). The RM was allowed to sit at 30 °C for 18 minutes of incubation. The control was handled similarly and had identical amounts of the buffer present but no test sample. The Griess solution, which contains 2% *O*-phosphoric acid, 1% sulfanilamide, and 0.1% naphthyl ethylene diamine dihydrochloride, was added to the RM after 5 hours. The absorbance was then measured spectrophotometrically at 546 nm. The gram-equivalents of ascorbic acid were used to represent the assay results. Using the same formula as DPPH, the fraction's percent total antioxidant capacity in comparison to the reference medication was assessed.

2.7. Animals

Swiss albino mice, both male and female, weighing 28-32 g, were used in the investigation. The animals were bought from the animal holding facility at the University of Nigeria, Nsukka's Department of Zoology and Environmental Biology. Before the experiment began, the animals were kept in standard cages with good quality rat food (Vital feed) in an extremely hygienic environment (relative humidity of 60%, temperature of 23 ± 2 °C, and a 12/12-hour light/dark period). They were also given time to acclimatize to the environment for 14 days.

2.8. Ethical authorization

The ethics commission section of the Department of Biochemistry at the University of Nigeria, Nsukka granted permission and provided ethical clearance documents (approval number: UNN/BCH/9014) for the safe conduct of the experiment and the covert use of animal models. According to the updated National Institutes of Health (NIH) handbook on the use of experimental animals, all studies agreed with worldwide ethics and regulations in research.

2.9. Anti-inflammatory in vivo studies

Two different techniques were used to evaluate the fraction's anti-inflammatory properties. Mice were divided into five groups of four each using a random allocation process for each procedure (n = 4). The vehicle (10 mg/kg bw per oral distilled water), EALFSL in varying concentrations (200, 400, and 600 mg/kg bw p.o.), and the reference drug (100 mg/kg bw p.o. aspirin), respectively, were given to 24-hour-fasted mice assigned to the various groups, 1 hour before the induction of inflammation or pain. Using intraperitoneal injections of egg albumin and carrageenan, the fraction's anti-inflammatory properties were assessed.

2.9.1. Carrageenan-induced paw edema model

The test was carried out using a modified version of the approach of Winter et al. (1962). After the treatment, the mice's left hind paws were intraperitoneally injected with 0.1 ml of fresh carrageenan (0.01 g/ml) solution made in ordinary saline. A digital plethysmometer (model: I-520) was used to measure the paw volumes of the animals' left hands. In actuality, the animal paw is submerged in water that is in a specific water cell whose resistance is altered by the immersion of the animal paw. On the plethysmometer's electronic monitor, this change in resistance, which equates to the change in volume displacement, is calibrated in milliliters. After injecting carrageenan into a mouse's hind paw, the change in volume displacement at time zero was measured every 1 hour for 5 hours. The average edema for each interval was assessed using the difference between the volume displacement after the phlogistic agent was administered and the zero-time volume displacement of the induced paw (Vt - Vo) (Anosike et al., 2012). At each instant, an estimation of the paw edema inhibition percentage was derived.

$$\%\ Inhibition\ of\ edema\ = \frac{(V_t - V_o)control - (V_t - V_o)test}{(V_t - V_o)control} x\ 100$$

where V_o is the time zero mouse paw edema volume and V_t is the various time intervals of mouse paw edema volume.

2.9.2. Egg albumin-induced paw edema model

The test was conducted using the technique described by Okokon and Nwafor (2010). 0.1 cc of undiluted fresh egg albumin was injected intraperitoneally (i.p.) into the subplantar region of the right hind paw of mice one hour after treatment. Using a digital plethysmometer, the right hind paw volumes of the rats were measured immediately, at the start of the experiment, and then every hour after receiving an injection of egg albumin for 5 hours (Anosike et al., 2012). Each interval included an evaluation of the average edema and the percentage of paw edema inhibition.

2.10. Statistical evaluation

The Statistical Product and Service Solutions (SPSS) version 23 was used to perform one-way and two-way ANOVA on the data. Standard error of the mean (SEM) was used to present the analysis's findings. Using Duncan post hoc tables, significant differences between the several groups were examined. The predetermined level of significance was set at $p < 0.05$ for all outcomes.

3. Results and discussion

3.1. Phytochemical constituents of EALFSL

The phytochemicals that make up EALFSL are displayed in Table 1. In EALFSL, phenolic components such as tannins, flavonoids, and other phenols were present in appreciable high concentrations, whereas cyanogenic substances, terpenoids, steroids, glycosides, alkaloids, and other saponins were present in moderate concentrations. A previous studies on crude extracts of *S. linifolia* by Nwankwo et al. (2023a) and Nwankwo et al. (2023b) revealed that it contained a good amount of the above-mentioned phytochemicals. According to research by Barbosa-Filho et al. (2006) and Farooq et al. (2022), medicinal plants' anti-inflammatory activities are attributed to their high concentrations of flavonoids, tannins, terpenoids, phenols, and steroids. The study was conducted using the ethylacetate fraction because among the fractions (*n*-hexane, ethylacetate, and ethanol fractions), it gave better results at the pilot test level and equally provided the highest yield; data not shown.

Table 1. Phytochemical screening of EALFSL

Phytochemical constituents	Concentration (mg/g)
Flavonoids	1.377 ± 0.011
Phenols	0.938 ± 0.010
Tannins	0.526 ± 0.002
Cyanogenic compounds	0.351 ± 0.009
Glycosides	0.255 ± 0.003
Terpenoids	0.253 ± 0.007
Saponins	0.231 ± 0.014
Steroids	0.176 ± 0.005
Alkaloids	0.127 ± 0.007

3.2. Anti-inflammatory activities of EALFSL

The EALFSL's in vitro anti-inflammatory activities are shown in Table 2. According to the findings, EALFSL displayed the majority of its anti-inflammatory activities in a concentration-dependent manner. The EALFSL at different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml) significantly ($p < 0.05$) inhibited hemolysis induced by hypotonicity and heat thereby stabilizing the membrane of the erythrocyte. However, the EALFSL had better activity against heat-induced hemolysis. A similar study by Mendez-Encinas et al. (2023) reported that an extract of *Caborca propolis* stabilized the red blood cell membrane by inhibiting heat-induced and hypotonicity-induced hemolysis.

The different concentrations of EALFSL and the standard drug (aspirin) at the concentration of 0.2 mg/ml exhibited significant ($p < 0.05$) inhibitory activity against protease activity and protein denaturation with a better activity on protease inhibition. In line with a study by Enechi et al. (2020), a flavonoid-rich extract of *Peltophorum pterocarpum* inhibited protease activity and protein denaturation. Studies have revealed that proteases play crucial roles in a variety of pathogenic and inflammatory diseases when leucocytes produce them in excess or when there is lysosomal leakage (Bermúdez-Humarán et al., 2015; Enechi et al., 2019).

Similar to the standard drug (aspirin), the different concentrations of EALFSL successfully reduced the aggregation of platelets. The activity of phospholipase A_2 was greatly inhibited both by the different concentrations of EALFSL and the standard drug (prednisolone). In consonance with the result of this study, both platelet aggregation and phospholipase A_2 activity were reported to have been inhibited by the extract of *S. linifolia* (Nwankwo et al., 2023a). Perhaps the fraction's anti-inflammatory properties were brought about by inhibiting the release of arachidonic acid, which is necessary for the production of pro-inflammatory mediators (Coutinho & Chapman, 2011).

Table 2. In vitro anti-inflammatory activities of EALFSL

Treatments	Concentration (mg/ml)	Hypotonicity-induced hemolysis inhibition (%)	Heat-induced hemolysis inhibition (%)	Platelet aggregation inhibition (%)	Protease inhibition (%)	Phospholipase A_2 activity inhibition (%)	Protein denaturation inhibition (%)
EALFSL	0.2	39.03 ± 0.31^{a}	40.05 ± 0.43^{a}	37.19 ± 0.17^{a}	40.55 ± 1.00^{a}	32.99 ± 0.34^{a}	31.91±0.31^{a}
EALFSL	0.4	40.58 ± 0.23^{b}	53.04 ± 0.23^{c}	40.97 ± 0.40^{b}	44.16 ± 0.99^{b}	41.38 ± 0.99^{b}	36.79±0.23^{b}
EALFSL	0.6	46.13 ± 0.27^{c}	57.30 ± 0.26^{d}	46.89 ± 0.28^{d}	53.03 ± 0.33^{d}	46.94 ± 0.59^{c}	42.14±0.41^{c}
EALFSL	0.8	50.81 ± 0.14^{d}	67.76 ± 0.14^{e}	51.40 ± 0.17^{e}	57.72 ± 0.26^{e}	52.61 ± 1.67^{d}	51.63±0.35^{d}
EALFSL	1.0	57.49 ± 0.25^{e}	74.97 ± 0.30^{f}	55.94 ± 0.21^{f}	62.45 ± 0.19^{f}	54.20 ± 0.50^{d}	56.10±1.02^{e}
Aspirin	0.2	39.26 ± 0.65^{a}	45.27 ± 0.13^{b}	45.52 ± 0.84^{c}	48.72 ± 0.23^{c}	41.61 ± 0.51^{b}	41.12±0.28$^{c\ þ}$

Data are represented as mean ± SEM (n = 3), Down the column, values with different letters superscripts are significantly different ($p < 0.05$), þ signifies that prednisolone served as a reference drug

3.3. In vivo anti-inflammatory activities of EALFSL

3.3.1. Effect of EALFSL on carrageenan-induced edema in mice

The impact of EALFSL on mice with edema induced by carrageenan is presented in **Table 3**. Edema development peaked (5.27-0.05 ml) 4 hours after injection of carrageenan in mice who were not later given any treatment (control). EALFSL's ability to partially block the edema was time- and concentration-dependent. While the largest percentage inhibition (75.46%) was achieved at 5 h after treatment with 600 mg/kg of EALFSL, this percentage is the one of lower concentration after 1 h. Aspirin, the conventional medication, showed 77.82% inhibition 5 hours after treatment with a dose of 100 mg/kg. The first phase of edemogenesis in the carrageenan model is mediated by the release of inflammation mediators, and the observed excellent inhibition of edema formation exhibited by EALFSL at this first phase (1-2 h) suggests that the fraction probably inhibited this release (Georgewill & Georgewill, 2010; Georgewill et al., 2010). In tandem with the result of this study, da Silva et al. (2022) conducted a study on the anti-inflammatory properties of *Pediastrum boryanum*, which revealed that the extract reduced edema progression in carrageenan-induced experimental animals.

Table 3. Effect of EALFSL on carrageenan-induced paw edema in mice

Group	Mean edema volume (ml) (% edema inhibition)				
	1h	2h	3h	4h	5h
Control	6.06 ± 0.10^{aA}	6.16 ± 0.07^{aA}	6.26 ± 0.03^{aA}	6.34 ± 0.19^{aA}	6.05 ± 0.06^{aA}
	-	-	-	-	-
EALFSL (200 mg/kg)	5.32 ± 0.15^{cA}	4.90 ± 0.16^{cB}	3.94 ± 0.23^{cC}	3.19±0.30^{cD}	2.83 ± 0.14^{cE}
	(38.72 %)	(42.03 %)	(52.60 %)	(66.58 %)	(72.73 %)
EALFSL (400 mg/kg)	5.47 ± 0.08^{bcA}	5.22 ± 0.16^{bB}	4.51 ± 0.07^{bC}	3.90 ± 0.12^{bcD}	3.32 ± 0.08^{bE}
	(44.74 %)	(46.94 %)	(54.23 %)	(62.93 %)	(73.66 %)
EALFSL (600 mg/kg)	5.64 ± 0.15^{bA}	5.22 ± 0.16^{bB}	4.85 ± 0.11^{bC}	4.37 ± 0.16^{bD}	3.09 ± 0.12^{bE}
	(41.28 %)	(44.63 %)	(48.10 %)	(53.44 %)	(75.46 %)
Aspirin (100 mg/kg)	5.59 ± 0.07^{bA}	5.01 ± 0.10^{bcB}	4.52 ± 0.39^{bC}	3.99 ± 0.12^{bcD}	3.29 ± 0.07^{bE}
	(45.73 %)	(51.09 %)	(56.64 %)	(64.78 %)	(77.82 %)

Results are presented as mean ± SEM (n = 4). Figures in parentheses indicate inhibition (%) of edema progression. Subsets of a column with different low-case alphabets and rows with different upper-case alphabets as superscripts differ significantly ($p < 0.05$), Control= 10 mg/kg of distilled water.

Table 4. Effect of EALFSL on egg albumin-induced paw edema in mice

Group	Mean edema volume (ml) (% edema inhibition)				
	1h	2h	3h	4h	5h
Control	6.85 ± 0.14^{abA}	6.37 ± 0.07^{aAB}	6.51 ± 0.05^{aA}	6.63 ± 0.04^{aA}	6.48 ± 0.06^{aAB}
	-	-	-	-	-
EALFSL (200 mg/kg)	5.73 ± 0.06^{bA}	5.17 ± 0.11^{cB}	3.79 ± 0.08^{dC}	2.89 ± 0.06^{cD}	2.66 ± 0.04^{cD}
	(34.74 %)	(38.59 %)	(52.61 %)	(69.02 %)	(74.94 %)
EALFSL (400 mg/kg)	6.11 ± 0.07^{aA}	5.60 ± 0.13^{bB}	4.82 ± 0.11^{cC}	3.60 ± 0.10^{bD}	3.14 ± 0.05^{bE}
	(38.92 %)	(42.52 %)	(49.27 %)	(66.24 %)	(75.66 %)
EALFSL (600 mg/kg)	6.39 ± 0.04^{aA}	5.83 ± 0.08^{bB}	4.50 ± 0.17^{cC}	3.41 ± 0.10^{bD}	3.05 ± 0.32^{bE}
	(33.50 %)	(36.75 %)	(47.39 %)	(62.71 %)	(70.61 %)
Aspirin (100 mg/kg)	6.43 ± 0.17^{aA}	6.12 ± 0.24^{aB}	5.55 ± 0.09^{bC}	3.69 ± 0.09^{bD}	3.02 ± 0.10^{bE}
	(32.90 %)	(40.25 %)	(44.25 %)	(66.68 %)	(81.36 %)

Results are presented as mean ± SEM (n = 4). Figures in parentheses indicate inhibition (%) of edema progression. Subsets of a column with different low-case alphabets and rows with different upper-case alphabets as superscripts differ significantly ($p < 0.05$), Control= 10 mg/kg of distilled water.

3.3.2. Effect of EALFSL on egg albumin-induced paw edema in mice

The impact of EALFSL on mice with edema induced by egg albumin is shown in **Table 4**. Edema development peaked (6.85-0.14 ml) 1 hour after injection of egg albumin in mice. After 5 hours of treatment, 400 mg/kg of EALFSL caused the greatest percentage of edema inhibition; after 1 hour, 600 mg/kg of EALFSL caused the least amount of edema inhibition. It could be a result of an antagonizing effect due to the presence of more compounds in the mixture that at 1-hour post-treatment 600 mg/kg caused the least

amount of edema inhibition. The usual medication (aspirin) gave 81.36% inhibition 5 hours after therapy at a dose of 100 mg/kg. The EALFSL also inhibited egg albumin-induced edemogenesis, suggesting that it might lessen inflammation (Akindele et al., 2015). Additionally, as expected from a strong cyclooxygenase inhibitor, aspirin greatly decreased the edema brought on by the injection of egg albumin and was comparable to the EALFSL fraction. Comparing the result of the anti-inflammatory effect of EALFSL on egg albumin-induced rat paw edema, the leaf extract of *Detarium microcarpum* also inhibited edema progression in rats induced with egg albumin (Odoh & Ene, 2020).

3.4. In vitro antioxidant activities of EALFSL

The outcomes of this study demonstrated that the EALFSL was able to successfully scavenge DPPH, nitric oxide radicals, TAC, and FRAP in a concentration-dependent manner. The EALFSL demonstrated a good inhibitory effect on DPPH-generated free radicals **(Figure 1)** with an IC_{50} value of 1.20 mg/ml while the standard gave an IC_{50} value of 0.30 mg/ml. It equally exhibited a strong ferric-reducing antioxidant power (FRAP) **(Figure 2)** with an IC_{50} value of 0.88 mg/ml, which was close to that of the standard (ascorbic acid) which had an IC_{50} value of 0.50 mg/ml. The total antioxidant capacity (TAC) of EALFSL **(Figure 3)** was very promising with an IC_{50} value of 0.93 mg/ml, which was comparable to that of the standard (gallic acid) having an IC_{50} value of 0.47 mg/ml. In the same vein, it exhibited a good nitric oxide scavenging property **(Figure 4)** with an IC_{50} of 1.04 mg/ml while that of ascorbic acid for nitric oxide scavenging was 0.32 mg/mL. A similar study by Chukwuma et al. (2023) proved that the extract of *Cola acuminata* was able to scavenge free radicals generated by DPPH and nitric oxide, and also showed a good FRAP and TAC.

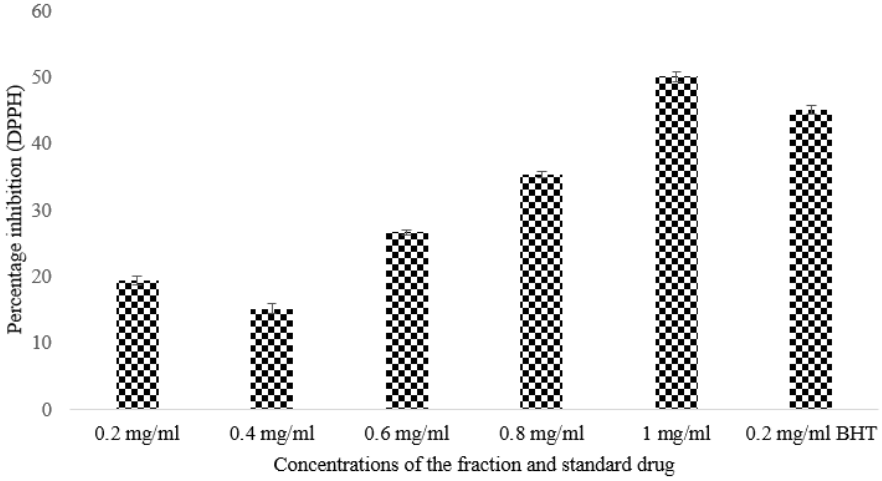

Figure 1. Antioxidant capacity of EALFSL against DPPH

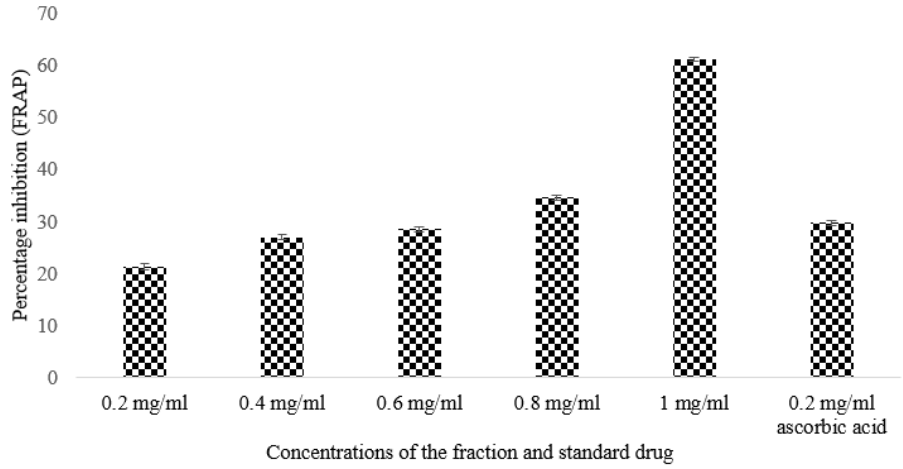

Figure 2. Antioxidant capacity of EALFSL against FRAP

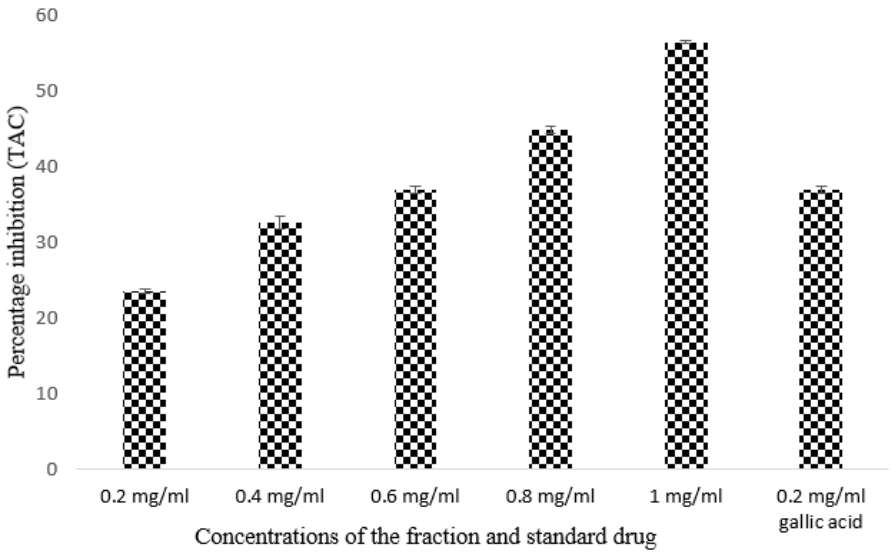

Figure 3. Antioxidant capacity of EALFSL against TAC

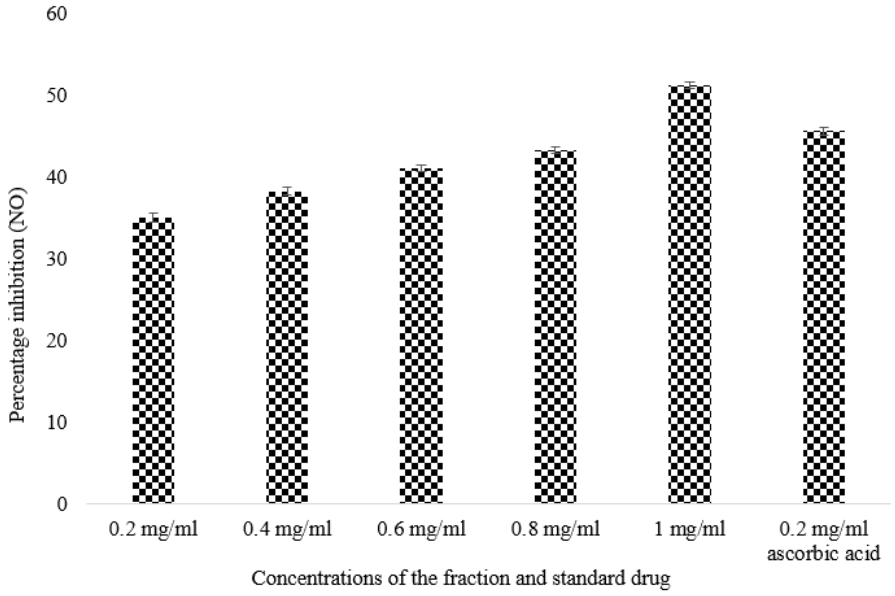

Figure 4. Antioxidant capacity of EALFSL against NO

4. Conclusions

The current study established that the ethylacetate leaf fraction of *S. linifolia* has anti-inflammatory and antioxidant potentials both in vivo and in vitro. These properties of the fraction could be attributed to the abundance of necessary phytochemical constituents contained therein. Now that it has been established that the ethylacetate fraction of the crude ethanol extract holds good potential against inflammation and oxidative stress: the next action should be to possibly isolate the bioactive compounds responsible for the activities.

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None.

Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

The ethics commission section of the Department of Biochemistry at the University of Nigeria, Nsukka granted permission and provided ethical clearance documents (approval number: UNN/BCH/9014) for the safe conduct of the experiment and the covert use of animal models.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

None.

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