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Stem Bark Extract of *Sterculia setigera* Delile Exhibits Anti-inflammatory Properties through Membrane Stabilization, Inhibition of Protein Denaturation and Prostaglandin E_2 Activity

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ITH, RA, EOA and FT designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors DK, JAM and FJE managed the analyses of the study. Authors GO and BA managed the literature searches and performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The stem bark of *Sterculia setigera* has been used in several African countries for the treatment of many inflammatory conditions. However, there is no scientific report to authenticate this usage. The current study, therefore, aims at assessing the *in vitro* and *in vivo* anti-inflammatory activities of the stem bark extract of *Sterculia setigera* (SSE).

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Place and Duration of Study: The study was conducted at the Laboratories of Department of Biomedical Sciences, University of Cape Coast, Ghana between October, 2016 and April, 2017.
Methodology: *In vitro* anti-inflammatory activities were assessed using heat and hypotonic solution - induced red blood cell haemolysis assays, as well as egg and bovine serum albumin denaturation assays. *In vivo* anti-inflammatory effect of SSE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, *p.o.*) was assessed using carrageenan as well as prostaglandin E_2 -induced paw oedema models in rats.
Results: SSE inhibited heat and hypotonic solution-induced haemolysis by a maximum effect of 62.81 ± 1.18% and 76.05 ± 3.08% respectively as compared to diclofenac which was 76.63 ± 3.36% and 76.49 ± 1.67% respectively. Also, egg and bovine serum albumin denaturation were markedly inhibited by SSE with a maximum inhibitory effect of 74.84 ± 64% and 45.73 ± 8.41% respectively. Diclofenac had a maximum inhibitory effect of 72.23 ± 3.14% and 52.02 ± 0.72% respectively for egg and bovine serum albumin denaturation assays. Also, SSE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, *p.o.*) significantly and dose-dependently reduced rats' paw oedema in the carrageenan and prostaglandin E_2 – induced inflammation tests.
Conclusion: SSE exhibited anti-inflammatory effects in both *in vitro* and *in vivo* models and this gives credence to its folkloric usage.

Keywords: Sterculia setigera; inflammation; prostaglandin E_2; carrageenan; hypotonicity-induced haemolysis; egg albumin denaturation assay.

1. INTRODUCTION

Plant-based traditional medicine usage traces back to ancient times and still plays a significant role in modern healthcare systems as about eighty per cent of the world's population relies mainly on traditional and/or plant-based medicines for their primary healthcare needs [1]. Drug discovery researchers have in recent times developed an interest in research into the discipline of plant medicine. This is to make maximum utility of unexploited natural resources as medicinal plants still present a major source of novel phytocompounds that can serve as leads for the development of new pharmaceuticals which may be useful in disease prevention and treatment [2]. Moreover, phytochemicals have been found to have fewer side effects, better patient tolerance, relatively less expensive and a long history of use and renewability in nature as compared to the available conventional drugs which are expensive and sometimes associated with life-threatening side effects [3]. Research into plant medicine, therefore, has been aimed at making a more scientific discovery to gather chemical and pharmacological evidence to vindicate various folkloric claims by herbal medicine practitioners and to obtain novel medicinal compounds.

Sterculia setigera is a tree widespread in the Savannah area of tropical Africa. In Ghana, it is mainly found in the Northern region of the country. The Kusasis and Gonjas of Ghana call it '*pumping*' and '*kapolo*' respectively. It is a deciduous plant which grows up to 12 m high and 1.5 m in girth. The bark is pale purplish, smooth with thin scales which peel off to expose its yellowish patches, exuding gummy sap. Various parts of the plant have been used in folk medicine in diverse African countries to treat malaria, rickets, cough, hypertension, headache, asthma, bronchitis, wounds, fever, toothache, stomachache, menstrual pains, abscess, diarrhoea, and snakebite [4-10]. Several studies give supportive evidence to the use of this plant as an antimicrobial agent in folk medicine [11, 12]. Hepatoprotective activity of the stem bark extract has been reported [13]. Effects of *Sterculia setigera* stem bark extract on haematological and biochemical parameters of wistar rats have also been reported [14].

Inflammation has been known to be a part of a complex biological response of vascular tissues to harmful stimuli, such as irritants, pathogens or damaged cells. The cardinal signs of acute inflammation are swelling, redness, pain, heat and loss of function. It is a physiological process by which an organism protects itself, remove injury-causing insults, and initiate a healing process [15]. Inflammation has been implicated in the pathogenesis of many diseases including arthritis, stroke, and cancer [16]. Although there has been the widespread traditional use of the stem bark of *Sterculia setigera* in managing many inflammatory conditions, there is no scientific evidence to back this usage. It is, therefore, the purpose of this study to investigate the anti-inflammatory effects of the plant using *in vitro* and *in vivo* techniques.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

The fresh stem bark of *Sterculia Setigera* was collected from Garu Tempane in the Upper East Region of Ghana in October, 2016. It was authenticated by a botanist in the Herbarium unit of the School of Biological Sciences, University of Cape Coast, where a voucher specimen has been kept.

2.2 Preparation of the Extract

Air dried stem bark (350 g) of *Sterculia Setigera* was powdered using a hammer mill. 200 g of the plant powder was extracted with a mixture of 2.5 L each of dichloromethane and methanol for 72 hours on a Mechanical Shaker. The pooled extract was filtered through a glass wool and concentrated using a rotary evaporator (Rotavapor R - 215 model, BÜCHI Labortechnik AG, Flawil, Switzerland) in vacuum at 40°C. The syrupy mass obtained was further dried in a desiccator and kept there until ready for use. The percentage yield obtained was 2.5%. It was subsequently referred to as *Sterculia setigera* extract **(**SSE) or the extract.

2.3 Phytochemical Screening

Phytochemical screening was performed using an earlier described procedure [17,18].

2.4 Drugs and Chemicals

Diclofenac sodium, aspirin, carrageenan, bovine serum albumin and all the other chemicals used were of analytical grade and were purchased from Sigma-Aldrich Inc, St. Louis, MO, USA.

2.5 Animals

Male Sprague - Dawley rats (170 - 250 g) were purchased from Nuguchi Memorial Institute for Medical Research, University of Ghana, Legon-Ghana. They were kept in stainless cages (34 × 47 × 18 cm) in groups of six at the animal house facility of the Department of Biomedical Sciences, University of Cape Coast, Ghana. They were fed with normal commercial diet bought from Agricare, Kumasi, Ghana. Water was given to them *ad libitum.*

2.6 Collection of Blood Sample

Whole blood was collected into a heparinized vacutainer from a healthy volunteer who had not taken any non-steroidal anti-inflammatory drugs (NSAIDS) for 2 weeks prior to the experiment. The blood was washed three times with 0.9% saline and centrifuged intermittently for 10 minutes at 3000 rpm. The packed cells were washed with 0.9% saline and 10% $^{v}/_{v}$ human red blood cells (HRBC) suspension was prepared using 0.9% saline [19].

2.7 Hypotonic Solution-induced Haemolysis

The test was performed as previously described [20]. Test samples consisted of 0.5 ml of stock erythrocyte suspension (HRBC) mixed with 4.5 ml of hypotonic solution (0.45% NaCl) which contained varying concentrations of SSE (100, 300 and 1000 µg/ml). The negative control sample contained 0.5 ml HRBC suspension mixed with 0.45 ml of hypotonic buffered solution alone. The positive control sample was prepared using 0.5 ml of the HRBC suspension, 4.5 ml of the hypotonic solution containing varying concentrations of diclofenac sodium (100, 300 and 1000 µg/ml). The experiment was carried out in triplicates. The mixtures were incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm and haemoglobin content of the supernatant was measured spectrophotometrically at 560 nm. The percentage inhibition of haemolysis was calculated by using the following equation:

$$\%\ Inhibition\ of\ heamolysis = \left(\frac{Optical\ Density\ of\ Control - Optical\ Density\ of\ Test\ Sample}{Optical\ Density\ of\ Control}\right) X\ 100$$

2.8 Heat-induced Haemolysis

The test was carried out as has been previously described [21]. The reaction mixture (2 ml) consisted of 1.0 ml of 10% HRBC and 1.0 ml of various concentrations of SSE (100, 300 and 1000 µg/ml). The negative control sample consisted of 1.0 ml of 10% HRBC and 1.0 ml of normal saline. The positive control samples comprised of 1.0 ml of 10% HRBC and 1.0 ml of different concentrations of diclofenac sodium (100, 300 and 1000 µg/ml). The experiment was carried out in triplicates. The samples were heated at 56°C for 30 minutes then cooled to room temperature followed by centrifugation at 3000 rpm for 10 minutes. Supernatants were collected, and their absorbances were measured at 560 nm. Percentage inhibition of haemolysis was calculated using the formula below:

$$\% \, Inhibition = \left(\frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample}{Absorbance\ of\ Control}\right) X\ 100$$

2.9 Egg Albumin Denaturation Assay

The test was carried out as has been previously described [21]. Test samples (5 ml) consisted of 0.2 ml of egg albumin (from fresh egg), 2.8 ml of phosphate-buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations (100, 300 and 1000 µg/ml) of the extract. The negative and positive control samples contained the same volume of the egg albumin and PBS, but the extract was replaced with either 2 ml of distilled water or diclofenac (100, 300 and 1000 µg/ml) respectively. The mixtures were incubated at 37 ± 2°C for 15 minutes and then heated at 70°C for five minutes. After cooling, their absorbances were measured using a spectrophotometer at 660 nm. The experiment was performed in triplicates and the percentage inhibition of protein denaturation was calculated using the formula below:

$$\% \, Inhibition = \left(\frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample}{Absorbance\ of\ Control}\right) X\ 100$$

2.10 Bovine Serum Albumin (BSA) Model

A method previously described was used [19]. The reaction mixtures consisted of 0.5 ml of 1% BSA fraction and 0.5 ml of either normal saline (negative control), diclofenac (100, 300 and 1000 µg/ml) or SSE (100, 300 and 1000 µg/ml). The samples were incubated at 37°C for 20 minutes and then heated to 51°C for 20 minutes. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. The Percentage inhibition of protein denaturation was calculated as follows:

$$\% \, Inhibition = \left(\frac{Absorbance\ of\ Control - Absorbance\ of\ Test\ Sample}{Absorbance\ of\ Control}\right) X\ 100$$

2.11 Carrageenan-induced Paw Oedema in Rats

The acute anti-inflammatory activity of the extract was evaluated in rats using a method described previously [22]. Five groups of male Sprague-Dawley rats (n = 5) weighing 170 - 250 g were used for the study. Paw oedema was induced by the administration of 0.1 ml of 1% suspension of carrageenan in 0.9% sterile saline solution into the plantar region of the rats' right hind paw. This was preceded by pre-treatment of separate groups with SSE (30 - 300 mg/kg, *p.o.*), diclofenac (10 mg/kg, *p.o.*) or normal saline (10 ml/kg, *p.o.*) 1 hour before paw oedema was induced. Rat's paw oedema was measured using Starrett 798A – 6 / 150 Electronic Digital Callipers before intraplantar injection of carrageenan and at hourly intervals for five hours post oedematous injury. Raw scores of the foot oedema were individually normalized as a percentage of change in their paw diameter at time 0 and then averaged for each treatment group. This was used to plot a time course curve for the 5 - hour period. Total oedema response for each treatment was then calculated as area under the time course curves. The effect of the drugs was evaluated using percentage inhibition of oedema calculated using the formula below:

$$\% \ inhibition = \left(\frac{AUC_{\ control} - AUC_{\ treatment}}{AUC_{\ control}}\right) \times 100$$

2.12 Prostaglandin E_2 (PGE_2) – Induced Paw Oedema

The effect of pre-treatment of rats with SSE on prostaglandin E_2-induced paw oedema was evaluated. Rats (n = 5) were pre-treated with either vehicle (10 mL/kg, *p.o.*), SSE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, *p.o.*) before intraplantar injection of PGE_2 (100 ng/paw; 20 µL) into their right hind paw. Paw oedema was measured before and at 1, 2, 3, 4 and 5 h post PGE_2 injection using Starrett 798A – 6 / 150 Electronic Digital Callipers and analysed as described above under section 2.11.

2.13 Statistical Analysis

A sample size of five rats per group was used in the *in-vivo* experiment (n = 5). Data were presented as mean ± SEM. Time-course curves were subjected to two-way (*treatment x time*) analysis of variance (ANOVA) with Dunnet's *post hoc*. To compare differences between treatment groups (AUCs), one-way ANOVA followed by Bonferroni's *post hoc* test. GraphPad® Prism for Windows Version 7.0 (Graphpad Software, San Diego, CA, USA, 2016) was used for all statistical analysis. $P < 0.05$ was considered statistically significant for all tests.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Preliminary phytochemical screening conducted on the plant revealed the presence of flavonoids,

reducing sugars, triterpenoids, saponins, and tannins.

3.2 Heat-induced Haemolysis

SSE and diclofenac produced a significant ($F_{6, 14}$ = 69.77, P < 0.001) protection of human red blood cells against the damaging effect of heat. The maximum inhibitory effects were 62.81 ± 1.18% and 76.63 ± 3.36% respectively at their highest doses of 1000 µg/ml (Fig. 1).

3.3 Hypotonic Solution-induced Haemolysis

In the hypotonic solution-induced haemolysis, SSE at concentrations of 100, 300 and 1000 µg/ml showed a significant ($F_{6, 14}$ = 134.9, P < 0.0001) protection of human red blood cells in hypotonic solution by 48.77 ± 4.29%, 76.05 ± 3.08% and 66.5 ± 5.14% respectively. Diclofenac sodium on the other hand, at same concentrations, inhibited red blood cells lyses by 51.28 ± 3.17%, 67.56 ± 0.78% and 76.49 ± 1.67% respectively (Fig. 2).

3.4 Egg Albumin Denaturation Assay

SSE significantly ($F_{6, 14}$ = 40.67, P < .001) inhibited heat - induced egg albumin denaturation. Maximum inhibition of 74.84 ± 64% was observed at 1000 µg/ml. Diclofenac, a standard anti-inflammatory drug showed the maximum inhibitory effect of 72.23 ± 3.14% at the concentration of 1000 µg/ml compared with the negative control (Fig. 3).

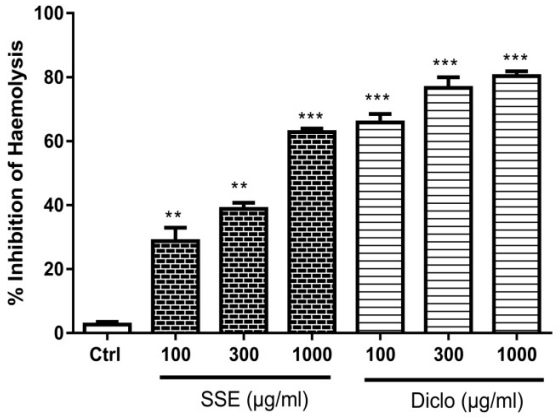

Fig. 1. Effect of SSE and diclofenac on heat-induced haemolysis of human red blood cells
*Data are presented as a mean ± standard error of the mean (n = 3). ** P <.01 and *** P < .001*

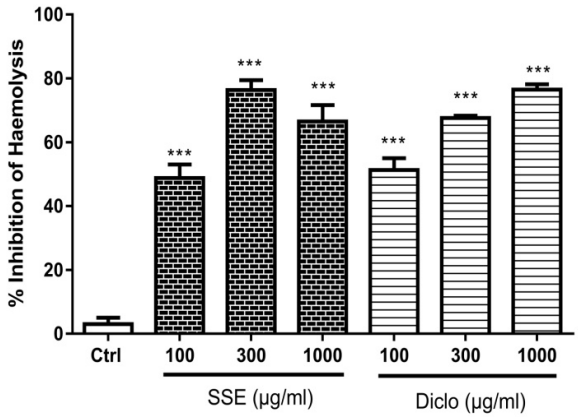

Fig. 2. Effect of SSE and diclofenac on hypotonic solution-induced haemolysis of human red blood cells
*Data are presented as a mean ± standard error of the mean (n = 3). *** P < .001*

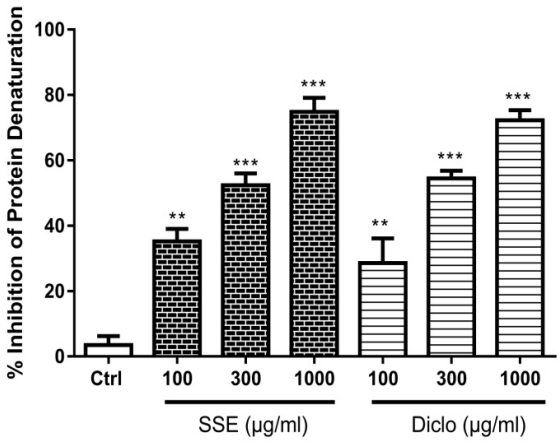

Fig. 3. Effect of SSE and diclofenac on inhibition of protein denaturation in egg albumin assay
*Data are presented as a mean ± standard error of the mean (n = 3). ** P < .01 and *** P < .001*

3.5 Bovine Serum Albumin (BSA) Denaturation Model

SSE significantly ($F_{6, 14}$ = 7.326, P = .001) protected proteins in the bovine serum albumin against denaturation with a maximum inhibitory effect of 45.73 ± 8.41% at a dose of 1000 µg/ml. Also, diclofenac similarly inhibited BSA protein denaturation by 52.02 ± 0.72% at its maximum dose of 1000 µg/ml (Fig. 4).

3.6 Carrageenan-induced Paw Oedema

Time course curves are shown in Fig. 5 (insert), two-way ANOVA (*treatment* x *time*), revealed a significant effect of drug treatments on the change in paw oedema in rats calculated as the percentage change in paw oedema ($F_{4, 120}$ =21.95, *P* < .001). Also, SSE (100 and 300 mg/kg, *p.o.*) dose-dependently and significantly reduced paw oedema similar to diclofenac 10 mg as shown in Fig. 5.

3.7 Prostaglandin E_2 (PGE_2) – induced Paw Oedema

Results presented in Fig. 6 show that intraplantar administration of prostaglandin E_2 irritant (100 ng/paw, 20 µL) resulted in a significant increase in paw oedema over time. Pre-treatment of the rats with SSE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac (10 mg/kg, *p.o.*) resulted in a significant increase in paw oedema ($F_{4, 90}$ = 13.02, P < .001) and total anti-nociceptive scores ($F_{4, 15}$ = 4.62, P = .01).

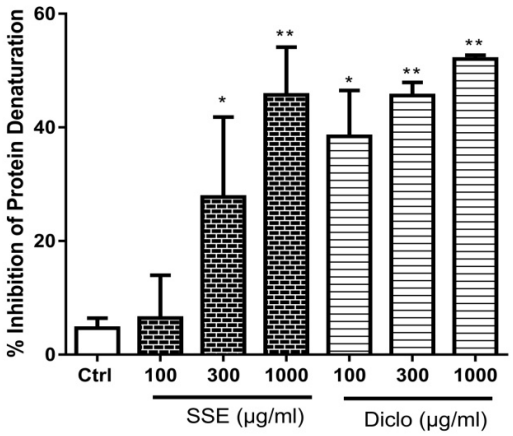

Fig. 4. Effect of SSE and diclofenac on inhibition of protein denaturation in bovine serum albumin assay
*Data are presented as a mean ± standard error of the mean (n = 3). * P < .05, ** P < .01 and *** P < .001.*

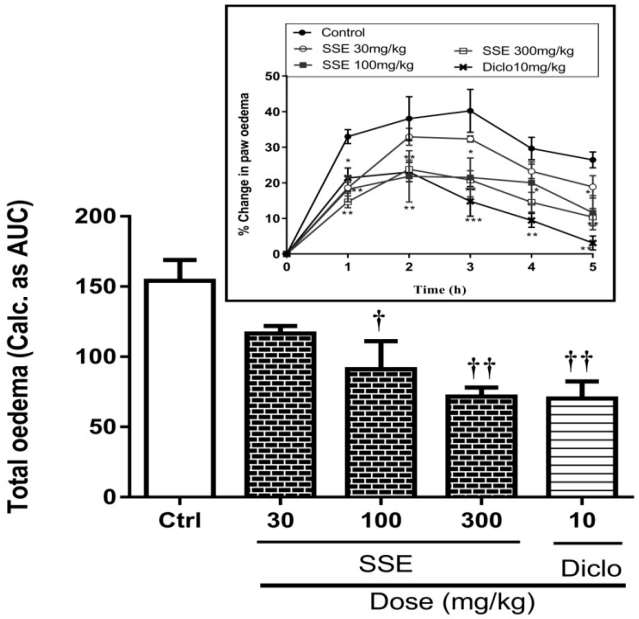

Fig. 5. The effects of SSE (30, 100 and 300 mg/kg, *p.o.*) and diclofenac (Diclo 10 mg/kg, *p.o.*) on total change in paw oedema (calculated as AUCs) in carrageenan-induced paw oedema test in rats

*† P < .05, †† P < .01 Insert: Percentage change in paw oedema over 5 h period. *P < .05, **P < .01 and ***P < .001. Each data represents mean ± standard error of mean, n = 5*

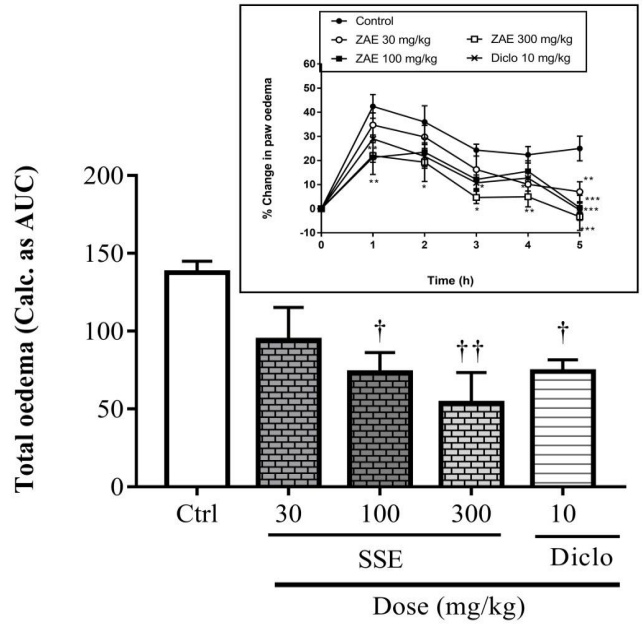

Fig. 6. The effects of SSE (30, 100 9and 300 mg/kg, *p.o.*) and diclofenac (Diclo 10 mg/kg, *p.o.*) on total change in paw oedema (calculated as AUCs) in prostaglandin E_2-induced paw oedema test in rats

*† P < .05, †† P < .01. Insert: Percentage change in paw oedema over 5 h period. *P < .05, **P < .01 and ***P < .001. Each data represents mean ± standard error of mean, n = 5.*

4. DISCUSSION

The vitality of red blood cells depends upon the integrity of their membranes particularly upon exposure to the injurious environment such as hypotonic medium which results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin [23,24]. Such an injury to a red blood cell membrane leads to secondary damage through free radical-induced lipid peroxidation [25]. Compounds with membrane stabilizing effects are known to confer significant protection to cells against injuries possibly through the inhibition of phospholipases that trigger the formation of inflammatory mediators [26]. From the results obtained (Figs. 1 and 2), SSE markedly inhibited heat, as well as hypotonicity-induced haemolysis and this is an indication of a possible anti-inflammatory activity.

Also, proteins are said to be denatured when they lose their secondary or tertiary structures upon application of external stresses or substances such as strong acids or bases, concentrated inorganic salts, organic solvents or heat [19]. Protein denaturation has been well correlated with the occurrence of the inflammatory response and has been implicated in several inflammatory diseases such as arthritis [27-29]. Many tissue injuries occur because of denaturation of protein constituents of cells or intercellular substances. As such, the main drugs used for the management of inflammatory conditions in clinical settings (non-steroidal anti-inflammatory drugs) have been reported to have the ability to inhibit protein denaturation [30,31]. From the results obtained, Figs. 3 and 4, SSE inhibited heat-induced protein denaturation in both egg and bovine serum albumin assays. This ability of a substance to inhibit protein denaturation is an indication of its potential anti-inflammatory activity.

The observed *in vitro* anti-inflammatory effect was further confirmed in *in vivo* studies using the carrageenan-induced paw oedema test in rats. It is a suitable and most widely used method for evaluating anti-inflammatory effects of plant extracts [32,33]. Carrageenan-induced inflammatory model is characterized by a biphasic event which involves various inflammatory mediators such as bradykinins, prostaglandins, serotonin, histamine etc. Histamine and serotonin play a key role in the first phase (first 2 hours following carrageenan injection) whiles bradykinins, prostaglandins and other slow reacting substances of inflammation are involved in the second phase (3 - 4 hours following carrageenan injection) [34]. From the results of the study, Fig. 5a and b, SSE (30 - 300 mg/kg, *p.o.*) showed a significant anti-inflammatory activity within the first two hours after drug administration and the effect lasted over the 5-hour duration of the experiment. This suggests a possible activity of the extract on both phase-1 and phase-2 inflammatory mediators.

It is well documented that intraplantar injection of inflammatory insults such as histamine, bradykinin, serotonin and prostaglandin E_2 into the paws of rodents induces inflammatory response and hyperalgesia in experimental models [35,36]. In this present research, the inflammatory response induced by PE_2 was significantly reversed by the plant extract and the effect was comparable to that of diclofenac, a non-steroidal anti-inflammatory drug (NSAID). This agrees with an earlier report which suggest that plant extracts have the ability to inhibit prostaglandin-induced inflammatory response possibly through the inhibition of further synthesis of PGE_2 via the cyclooxygenase pathway [37].

The anti-inflammatory activity exhibited by SSE can be attributed to the presence of phytochemicals such as flavonoids, reducing sugars, triterpenoids, saponins, and tannins that were found to be present in the extract following the phytochemical screening. The presence of these biologically active phytochemicals in plants has been documented to be responsible for the pharmacological activities of medicinal plants [38-40]. For instance, flavonoids are reported to have anti-inflammatory activities and the mechanism by which they exert this effect involves inhibition of cyclo - oxygenase (COX) effects, lipoxygenase (LOX) effects, neutrophil degranulation and eicosanoid biosynthesis [41, 42]. Triterpenoids and saponins have also been reported to inhibit inflammation by suppressing the actions of tumour necrosis factor (TNF)-alpha, interferon gamma, prostaglandin E_2, inducible nitric oxide synthase *(*iNOS*),* and nuclear factor -kappa B (NF - κB) [42,43]. The phytochemical results obtained also confirm an earlier report on the plant [14].

5. CONCLUSION

The study demonstrated SSE possesses anti-inflammatory activities and this may be attributed to the presence of secondary metabolites such as flavonoids, triterpenes, saponins, alkaloids

and reducing sugars. However, toxicity studies on the extract are recommended to ensure that it is safe for human use.

CONSENT

Declaration of consent was not applicable to the studies.

ETHICAL APPROVAL

All authors hereby declare that "Principles of Laboratory Animal Care" (NIH Publication No. 85-23, Revised 1985) were followed. All protocols used in the study were approved by the Department of Biomedical Sciences' ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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