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RESEARCH ARTICLE

EVALUATION OF THE ANTI-INFLAMMATORY AND MEMBRANE STABILIZING ACTIVITIES OF SMILAX ANCEPS LEAVES

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ABSTRACT

Smilax anceps leaves are used in the treatment of arthritis and rheumatism in traditional medicine. The anti-inflammatory effects of ethanol leaf extract of *Smilax anceps* were investigated using carrageen an and egg albumin assays with in vitro human red blood cell (HRBC) membrane stabilization method. Fifty albino rats divided into twenty-fivealbino rats for each in-vivo model were then sub-divided into five groups of five rats each and were used. The first three groups received doses of the extract at (250, 500 and 1000mg/kg) respectively, while the control groups were treated with ibuprofen 20mg/kg and distilled water. All administrations were done through the oral route. Inflammation was induced by right hind limb sub-plantar administration of 0.1ml of 1% w/v carrageenan injection and 0.1 ml of fresh egg albumin in the respective in-vivo models. Preliminary phytochemical screening revealed the presence of steroids, tannins and flavonoids while the LD_{50} was found to be greater than 5000mg/kg. There was a statistically significant (P<0.05-0.01) inhibition of inflammation in both carrageen an and egg albumin induced inflammation when the extracts at doses of (250, 500 and 1000mg/kg) were compared against the positive control group at 5 hours, and at 300 minutes respectively in both models after induction of inflammation. The plant extract also showed a good inhibition of erythrocyte membrane lysis. The findings showed that ethanol leaf extract of *Smilax anceps* possesses anti-inflammatory and membrane stabilizing activities.

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INTRODUCTION

Inflammation is considered as a characteristic physiological defense mechanism of body tissues that is evoked in response to tissue injury such as physical trauma like burns, infections by microbiologic agents, allergens, and other harmful stimuli (1). At the onset of an inflammation, the cells in the body undergo a cascade of reactions that lead to the activation and release of inflammatory mediators. These inflammatory mediators include endogenous molecules such as histamine, serotonin, prostaglandins and various plasma enzyme systems such as the complement system, clotting system, fibrinolytic system, etc. Activation of these mediators produces the characteristic signs of inflammation; such as pain, heat, redness, and swelling. Though inflammation is a protective defense mechanism, however if left uncontrolled may serve as an etiologic factor for chronic diseases conditions in the body.

Currently, there are several therapeutic agents designed to facilitate or aid the resolution process of inflammation, and deter or improve the quality of life of persons affected with inflammatory conditions such as rheumatism, arthritis. These drugs are known to exert adverse effects such as gastrointestinal irritations. Therefore, there is necessity for the development of newer and more potent anti-inflammatory drugs with lesser side effects. This has resulted in an increased interest to research into the use of complementary medicine involving the use of medicinal plant extracts.Smilax species are widely distributed in tropical regions and rainforests. They are widespread in Africa in places like Nigeria, South Africa, Madagascar, Mauritius, and Comoros (2). *Smilax anceps* has found a wide range of uses among the locals of southern Nigeria. *Smilax anceps* leaves are used in the treatment of pain, arthritis and rheumatism. (3). Previous studies have reported that plants belonging to *Smilax* genus possess biological activities such as anti-inflammatory, anti-nociceptive, antifungal, estrogenic, anti-estrogenic, diuretic, and anti-hyperuricemic properties. (4).

Osuagwu *et al*, (5), had earlier sthat the ethanolic extracts of the leaves of *S. anceps* had anti-inflammatory and diuretic properties. Previous study by Osuagwu *et al*, (5), reported that the leaves of *S. anceps* had anti-inflammatory property. Hence, this present study is aimed at providing additional information on the anti-inflammatory activity of the leaves of Smilax anceps using both in vivo and in vitro assays.

MATERIALS AND METHODS

Collection and identification of plant materials: Smilax anceps was collected from Ibaa town in Emohua Local Government area of Rivers State and identified by Dr. Oladele at the Department of Forestry and Wildlife of the University of Port Harcourt. The plant material was later authenticated at the herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt by Dr Ekeke Chimezie with voucher specimen number UPH/V/1285.

Preparation of extract: The cleaned plant materials were allowed to air-dry under room temperature for two weeks, then shredded into pieces, and pulverized by means of an electrical grinder. Approximately 2.475kg of the powdered material was soaked in 18.29 L of absolute ethanol for 72 hours in a macerating jar with occasional shaking and stirring. The extract was filtered through whatmann filter paper after which the filtrate was concentrated using rotary evaporator. The concentrated filtrate was carefully evaporated to dryness over a water bath at a temperature of 45˚C for the biological evaluations.

Animals used: Fifty albino rats of average weight of 165kg were obtained from the animal house of the Department of Pharmacology, Faculty of Basic Medical Sciences; University of Port Harcourt. They were housed at the animal house of the Department of Experimental Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Port Harcourt with free access to standard commercial diet and water.

Drugs and Chemicals: Absolute Ethanol (JHD, Guagdong Guanghua Sci-Tech.Co Ltd. Shantou, Guangdong, China), normal saline (Dana Ashmina Pharmaceuticals, Nigeria), diclofenac potassium 50mg tablets (Fenal 50) (Eurolife Healthcare, India), acetyl salicylic acid 75 mg tablet (Vasoprin) (Juhel PLC, Nigeria), ibuprofen 400mg tablet (Ebu - 400) (Me Cure, Nigeria). Carrageenan powder (Sigma Aldrich, USA)

Phytochemical Screening: The phytochemical screening of the leaf extract of *S. anceps* was carried out in accordance with (6).

Experimental Protocol

Acute Toxicity Study: This was done according to the method described by Lorke, (7) . Eighteen albino mice were weighed and allotted into two groups of nine animals each. The first group was subdivided into three groups of three animals each and were treated with extract doses of 10,100 and 1000mg/kgrespectively. They were kept under observation for 24hours for toxicity signs and mortality. No death was recorded, after which the second group of animals were administered with extract doses of 1,600,2,900 and 5,000mg/kg respectively and observed in a similar manner as above. There was no record of death.

Evaluation of Anti-inflammatory Activity

Carrageenin-Induced Inflammation: Rats (5 per group) were administered with the extract (250, 500 and 1000mg/kg, p.o), ibuprofen (20mg/kg, p.o) and normal saline (2ml/kg, p.o) respectively. Half an hour later, each rat was injected with 0.1 ml of 1% carrageenin into the sub-plantar tissue of the right hind paw of the rats. The linear paw circumference was measured after injection of carrageenan at intervals of 0, 1, 2, 3, 4, 5, and 6 hours using vernier caliper. The increase in paw thickness, and percentage inhibition of paw edema in the treatment groups was calculated at the end of the sixth hour using the following formula (8).

Increase in paw thickness in control (PC), or treatment group (PT)

$= Pt - Po$

Percentage inhibition in paw edema in the treatment group

$$= \frac{(PC - PT)}{PC} \times 100$$

Where;
Pt = Paw thickness at time t,
Po = Initial paw thickness,
PC = Increase in paw thickness of the control group, and
PT = Increase in paw thickness of treatment group

Egg Albumin-induced Paw Edema: The animals (5 per group) were treated with the extract (250, 500 and 1000mg/kg, p.o), aspirin (100mg/kg, p.o) and distilled water (2ml/kg, p.o) respectively. Half an hour later, each rat was injected with 0.1 ml of 1% carrageenin into the sub-plantar tissue of the right hind paw of the rats. Thirty minutes later, edema was induced by injection of 0.1 ml of fresh egg albumin into the sub-plantar tissue of the right hind paw of the rats. The linear paw circumference was measured at intervals of 0, 30, 60, 120, 180, 240, and 300 minutes using vernier caliper. The increase in paw thickness, and percentage inhibition of paw edema in the treatment groups was calculated at the end of the sixth hour using the following formula (9).

Increase in paw thickness in control (PC), or treatment group (PT)

$= Pt - Po$

Percentage inhibition in paw edema in the treatment group

$$= \frac{(PC - PT)}{PC} \times 100$$

Where;

Pt = paw thickness at time t,
Po = initial paw thickness,
PC = Increase in paw thickness of the control group, and
PT = Increase in paw thickness of treatment group

Assay of Erythrocyte Membrane Stabilization: Anti-inflammatory activity of *Smilax anceps* was investigated using stabilization of red blood cell membrane lysing technique according to (10).

Preparation of Erythrocyte Suspension: Stabilization of red blood cell membrane lysing technique as outlined by Shinde *et al*, (11),with minor adjustment was used to evaluate the in-vitro anti-inflammatory activity of *Smilax anceps* leaf extract.
A 5ml fresh whole human blood was collected and transferred to an ethylene-di-amine tetra-acetate (EDTA) centrifuge tube, and centrifuged at 2000rpm for 5 minutes, washed three times with equal volume of normal saline. The cellular component was measured and reconstituted into 40%v/v suspension with phosphate buffer saline (10mM, pH 7.4).The erythrocyte suspension obtained was used for the assay.

Heat Induced Hemolysis: Ethanol leaf extract of *S. anceps* at six graded concentrations of 20,40,60,80,100 and 120 µg/ml was placed into centrifuge tubes prepared in triplicates of two sets each. To each of these tubes was added 5ml isotonic buffer solution. The negative control tubes contained 5ml of buffer solution only while the positive control tubes had 5ml buffer solution with diclofenac potassium 100 µg/ml (all in two sets and in triplicates) as well. In addition, to each of all these tubes was added 0.005ml (0.5µg/ml) erythrocyte suspension, and was gently mixed by inversion to produce a mixture. All the tubes from the first set were incubated at 54 ^{0}C for 20minutes in a regulated water bath, and all tubes from the other set were incubated at 0-4 ^{0}C in a freezer for 20 minutes. At the end of the incubation, the tubes were centrifuged at 1000rpm for 3minutes and the absorbance of the supernatant was measured with a spectrophotometer at 540nm. The percent inhibition of hemolysis by the extract was calculated using the following formula:

$$\% \text{ Inhibition of hemolysis} = 100\times [1 - (OD_2\text{-}OD_1/OD_3\text{-}OD_1)$$

Where;
OD_1 = absorbance of test sample unheated
OD_2 = absorbance of test sample heated
OD_3 = absorbance of control sample heated.

Statistical Analysis: The results obtained were expressed as mean ± SEM. Statistical analysis of data was done using Microsoft Excel 2010, Statistical Package for the Social Sciences (SPSS, Version 23.0), followed by one-way ANOVA analysis to determine the difference between control groups and treatment groups. P values were considered statistically significant at values of $P<0.05$.

RESULTS

Preliminary qualitative phytochemical evaluation: Preliminary qualitative phytochemical screening of the ethanol leaf extract of S. anceps revealed the presence of flavonoids, tannins, steroidals/ triterpenoids, carbohydrateand saponins. However,anthraquinones and alkaloids were absent in the extract.

Effect of the ethanol leaf extract of *S. anceps* on carrageenan-induced paw edema: The extract at doses of 250, 500, and 1000 mg/kg produced a decrease in carrageenan-induced paw edema. However, this was not statistically significant when compared to the normal control. When the extracts at same doses were compared to the positive control (ibuprofen 20 mg/kg) after 5 hours of induction of paw edema, statistically significant inhibition of paw edema was observed.

Effect of ethanolic extract of leaves of *S. anceps* on egg albumin-induced paw edema in (mm±S.E.M) with percentage inhibition: The extract at doses of 250, 500, and 1000 mg/kg produced a decrease in egg albumin-induced paw edema. This was also, not statistically significant when compared to the normal control. When the extracts at same doses were compared to the positive control (Aspirin 100 mg/kg) after 300 minutes of induction of paw edema, a statistically significant inhibition of paw edema was observed.

Effect of ethanol Leaf Extract of *Smilax anceps* on Heat Induced Hemolysis of Human Red Blood Cells: The heated sample of *Smilax anceps* exhibited a statiscally significant (0.05) difference at100 and 120 µg/ml when compared to negative control.

DISCUSSION

Medicinal plants used in folk medicine can be new sources of bioactive molecules with pharmacological relevance (12).The results of this study has shown the presence of tannins, saponins, flavonoids, steroidal triterpenoids and carbohydrates in the ethanol leaf extract of *Smilax anceps* as it's phytoconstituents. Several lead compounds have been obtained from plant sources, and now are possible source of drugs for the future (13). Carrageenan is used for induction of edema in experimental animals and is a classic test for evaluating anti-inflammatory agents. Carrageenan injection induces inflammation in two phases. Different mediators are known to mediate inflammation in each phase, and these include; histamine, serotonin, and bradykinin, prostaglandins and nitric oxide. The first phase is characterized by edema and is mediated by release of histamine, serotonin and bradykinin. This is subsequently sustained by release of prostaglandins and nitric oxide in the second phase (14).

There was no significant statistical inhibition of inflammation by the ethanol extract of *Smilax anceps* leaf in the carrageenan group when the extracts were compared against the negative control; however, there was significant statistical inhibition ($P<0.05$, $P<0.01$) of inflammation when the extract were compared to ibuprofen at doses of 500and 1000mg/kg at 5 hours. Reduction of edema in the second phase of inflammation (at 5 hours), may be due to the ability of the extract to inhibit prostaglandin, a known mediator of the second phase of carrageenan-induced inflammation (15). Egg albumin is known to cause the release of two inflammatory mediators; histamine and serotonin-5HT (16). In the egg albumin model, there was no significant inhibition of paw edema when the extracts were compared against the negative control. However, significant inhibition ($P<0.05$-0.01) was observed at 300 minutes when the extracts at 250, 500 and 1000 mg/kg were compared against the reference drug aspirin. This indicates weak inhibition of inflammation mediated by serotonin and histamine (16). Exposure of red blood cells toharmful substances such as hypotonic solutions and heatcan cause lysisof RBC membranes, followed by oxidationof haemoglobin (17).

Table 1. Effect of the ethanol leaf extract of *S. anceps* on carrageenan-induced paw edema Mean paw size (mm)±S.E.M and percentage inhibition of paw edema

Groups	Dose (mg/kg)	Time (hour)						
		0	1	2	3	4	5	6
Control	1ml	4.58±0.62	6.46±0.80	5.87±0.28	5.34±0.80	5.09±0.53	4.09±0.50	4.55±0.51
Ibuprofen	20	3.87±0.23	5.96±0.63	5.41±0.28	5.09±0.43	4.72±0.4	4.46±0.26	4.45±0.51
		(-15.5)	(-11.70)	(-18.46)	(-60.50)	(-68.63)	(-66.67)	(-493.33)
Extract	250	4.42±0.25	6.54±0.59	5.84±0.75	5.67±0.54	5.25±0.1	5.2±0.22*	4.91±0.21
		(-3.49)	(-13.30)	(-9.20)	(-64.50)	(-64.71)	(-116.67)	(-433.33)
Extract	500	4.39±0.24	6.32±0.61	5.81±0.75	5.67±0.58	5.23±0.4	4.92±0.43*	4.58±0.37
		(-3.93)	(-3.19)	(-9.23)	(-68.42)	(-66.67)	(-47.22)	(-233.33)
Extract	1000	4.61±0.39	6.73±0.36	5.93±0.50	5.53±0.42	5±0.28	4.87±0.35**	4.58±0.35
		(-0.66)	(-13.30)	(-1.54)	(-22.37)	(-21.57)	(-27.78)	(-220)

Data are expressed as mean ± S.E.M (n = 5).There was statistical significant inhibition of paw edema when compared to Ibuprofen at *P < 0.001, ** P< 0.05 at 5 hours.Values in parenthesis ( ) are percentage inhibition of edema calculated relative to control.

Table 2. Effect of ethanolic extract of leaves of *S. anceps* on egg albumin-induced paw edema in (mm±S.E.M) with percentage inhibition

Groups	Dose (mg/kg)	Time (minutes)						
		0	30	60	120	180	240	300
Control	0.5ml	3.91±0.03	6.19±0.74	5.84±0.47	5.43±0.37	4.98±0.08	4.45±0.34	4.25±0.16
Aspirin	100	3.74±0.12	6.19±0.67	6.02±0.54	5.25±0.16	4.98±0.06	4.26±0.23	4.28±0.21
		(-4.35)	(-6.80)	(-18.30)	(-0.66)	(-18.70)	0	(-67.65)
Extract	250	3.99±0.33	6.13±0.23	5.91±0.36	5.52±0.24	5.06±0.09	4.82±0.11	4.52±0.25**
		(-2.3)	(-6.14)	(-0.052)	(-6.60)	(0.00)	(-53.70)	(-55.88)
Extract	500	4.08±0.36	6.57±0.20	6.23±0.29	5.73±0.40	5.19±0.14	4.95±0.13	4.28±0.21*
		(-4.35)	(-12.28)	(-11.92)	(-8.55)	(-2.80)	(-61.11)	(-41.18)
Extract	1000	3.5±0.18	6.25±0.38	5.52±0.59	5.37±0.11	4.82±0.23	4.67±0.17	4.38±0.18**
		(-10.49)	(-20.61)	(-3.63)	(-23.02)	(-23.36)	(-116.70)	(-155.88)

Data are expressed as mean ± SEM. There was statistical significant inhibition of paw edema when compared to aspirin at *P < 0.001, ** P< 0.05 at 300 minutes. Values in parenthesis ( ) are percentage inhibition of edema calculated relative to control.

Table 3. Effect of ethanol leaf extract of *S. anceps* on heat induced hemolysis of human red blood cells

CONCENTRATION (µg/ml)	MEAN ABSORBANCE ±SEM		% INHIBITION
	A (heat induced)	B (cold induced)	
20	0.046 ±0.005	0.041 ± 0.002	0
40	0.052 ± 0.006	0.054 ± 0.006	75
60	0.056 ± 0.001	0.050 ± 0.006	250
80	0.051 ± 0.001	0.052 ± 0.006	83
100	0.058 ± 0.002*	0.057 ± 0.014	91
120	0.061 ± 0.002*	0.060 ± 0.017	92.9
Diclofenac (100µg)	0.047 ± 0.007	0.052 ± 0.013	83.3
Negative control	0.046 ± 0.006	0.052 ± 0.005	

Hypotonicity induced hemolysis may arise from shrinkage of the cells due to osmotic loss of intracellular electrolyte andfluid components. During inflammation, breakdown of lysosomal granules cause the release of lysosomal enzymes, which when released could trigger cascades of inflammatory responses and disorders. Non-steroidal anti-inflammatory drugs (NSAIDs) are useful in preventing initiation of these inflammatory cascades by stabilizing lysosomal membranes and inhibit the release of lysosomal enzymes (18). From the results obtained from the inhibition of erythrocyte hemolysis, there was no linear dose-response. However, when the extract at the various doses were compared to the Diclofenac, astatistical significant inhibition of hemolysis was observed at extract doses of 100 and 120 µg/ml. The erythrocyte membrane is similar to the lysosomal membrane and its stabilization is a reflection of the ability of *S. anceps* leaf extract to stabilize lysosomal membranes (19). Stabilization of lysosomal membrane is of key importance in the reduction of inflammatory response through the inhibition of lysosomal constituents release from neutrophils. Hence, preventing tissue inflammation and injury (20). The membrane stabilizing activity of S. anceps leaf extract can be related to that of *M.duseni*istem bark as reported by (21).

The anti-inflammatory activity of the extract may be attributed to its'phytoconstituentssush as flavonoids and tannins. Flavonoids have been reported to effectively inhibit the activity of some enzymes such as proteinkinase C, phosphodiesterases and phospholipase A_2 which are involved in inflammatory processes (22). This study has demonstrated the potential ability of the extract to inhibit the release of inflammatory mediators by stabilizing the membrane of inflammatory cells.

CONCLUSION

From the findings of this study, it can be concluded that the ethanol leaf extract of *Smilax anceps* possesses moderate anti-inflammatory activity which may be attributed to the presence of steroids, tannins and flavonoids.

AUTHORS' CONTRIBUTIONS

OAS designed the research and supervised the study while both authors analyzed the data and drafted the manuscript. MIE carried out the laboratory aspect of the study.

CONFLICT OF INTEREST: The authors declare that no conflict of interest exists.

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