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ANTI-INFLAMMATORY ACTIVITY OF *SAPINDUS EMARGINATUS* LEAF AQUEOUS EXTRACT BY USING IN-VITRO MEMBRANE STABILIZATION OF HUMAN RBC AND IN-VIVO FORMALIN INDUCED PAW EDEMA MODELS

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ABSTRACT

To appraise the Anti-inflammatory activity of *Sapindus emarginatus* (S. emarginatus), leaf aqueous extract by using *in-vitro* membrane stabilization of human RBC and *in-vivo* formalin induced paw edema models.The acute toxicity study was performed by administering 2g/kg aqueous extract of *S. emarginatus* leaves in rats by oral gavage. The anti-inflammatory activity was evaluated by using both *in-vitro* membrane stabilization of human RBC and *in-vivo* formalin induced paw edema model in rats. The dose of aqueous extract of *S. emarginatus* leaves was given at a dose of 200 and 400 mg/kg *p.o.* In acute toxicity study of the plant extract, at a dose of 2g/kg *p.o.* was not shown any toxic events in rats. It was observed that SEAE have demonstrated dose dependent increase in the % membrane stabilization property significantly. The indomethacin had shown significant ($p<0.001$) reduction in paw edema at 1^{st} , 3^{rd} and 6^{th} hr when compared with normal control. SEAE-200 had shown significant ($p<0.05$) action at 6^{th} hr but not 1^{st} and 3^{rd} hr. SEAE- 400 had shown significant ($p<0.05$, $p<0.001$) actions at 3^{rd} and 6^{th} hr but not 1^{st} hr. In the present study the aqueous extract of *S. emarginatus* leaves poses anti- inflammatory activity.

Key words: Formalin, *Sapindus emarginatus*, Anti-inflammatory activity

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INTRODUCTION

At present situation herbal medicine is gaining importance and it has become a topic of world. It is showing impact on both world health as international trade. In the developing countries large proportion of the population are using herbal medicine due to high cost of western pharmaceuticals in the health care. Since times immemorial plants have been using as stores of potential biochemical factories, man is able to extract many of the potential bio chemicals form the medicinal plants. These useful chemicals can be extracted from any part of the plant like bark, leaves, flowers, fruits, seeds, roots etc. Inflammation is a complex biological response of vascular tissue due to different harmful stimuli, which results in pain and discomfort along with actual or potential injury to the body. Inflammation with pain is associated with

different diseases like rheumatism, encephalitis, pneumonia, oesophagitis, cancer, heart problems, fibrosis etc. Non steroidal anti-inflammatory drugs and opioid analgesic are normally used in the treatment of inflammation and pain but can cause a lot of adverse effect. Several herbal medicines constitute a potentially important avenue leading to novel therapeutic agents for inflammation that may not only prevent structural damage of arthritic joints caused by tissue and bone breakdown, but also be safe, relatively inexpensive, highly tolerated and convenient for many patients. Every year a lot of plants from traditional medicinal system have been screened for their potential anti-inflammatory and analgesic activity but only few of them are included in health care system after clinical research. Thus there is a need to emphasise on research for discovery of anti-inflammatory agents from natural sources. Inflammation is one of the body unique mechanisms that help body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. It is a body defence reaction in order to eliminate or limit the spread of injurious agent. The process is created by immune cells invading the tissue like an army in full battle mode. There are various components of inflammatory reaction that can contribute to the associated symptoms and tissue injury. During inflammation, innate cells and molecules are usually stimulated to isolate, destroy infectious agents and repair tissue, or sometimes the adaptive immune system is also stimulated. Consequently, the mechanism works in a cascade, where the inflammation is often triggered by circulating immune complexes that enter tissue. Although the inflammatory process is the body's mechanism to overcome the stress, it involves a complex array of enzyme activation, mediator release, Fluid extravasations, cell migration, tissue breakdown and repair which are aimed at host defence and usually activated in most disease conditions. The several phenomena may alter the antigenicity of endogenous proteins, including protein denaturation and glycosylation: protein denaturation may occur during chronic inflammatory phenomena *in-vivo* and albumin denaturation was observed in patients with rheumatic diseases and in rats with inflammatory lesions. The NSAIDs are the best studied. Their property of protecting albumin against heat-induced denaturation *in-vitro* was first considered strictly related to their anti-inflammatory activity *in-vivo*.

Sapindus emarginatus Vahl family Sapindaceae is a medium-sized deciduous tree found in south india. It is commonly called as soap nut tree. Native to South India *S. emarginatus* is found wild or introduced in tropical and sub-tropical regions, particularly the Indo-Malayan region. Traditionally, *S. emarginatus* is used as anti-inflammatory and antiprurutic. It is used to purify the blood. The seed is in intoxicant and the fruit rind has oxytropic action. Its powder is used as nasal insufflations. *S. emarginatus* also showed strong antibacterial activity against the tested bacterial strains[9]. Antifertility and antiandrogenic activities of *S. emarginatus* extract have been reported[10]. High content of saponins has been reported in the pericarp[11].Two Pisicidal triterpenoid saponins[12], acetylated triterpene saponins, hederagenin, sweet acyclic sesquiterpene glycoside, Mukurozioside IIb15 have been isolated from the Pericarps of *S. emarginatus*. Flavonoids have been isolated from the pericarp of *S. emarginatus*. As we know many plants contain phytochemicals constituents that possess medicinal properties, among them majority of plants shows antioxidant activity. Hence, the present study was conducted to evaluate the anti-inflammatory activity of *S. emarginatus.* aqueous extract. Aim of the present study is to detect the anti-inflammatory activity of aqueous extract of *S. emarginatus*. The anti-inflammatory activity was screened against *in-vitro* anti-inflammatory activity by membrane stabilization activity by using Human RBC and *In-vivo* anti-inflammatory activity by formalin induced paw edema model (1-4).

MATERIALS AND METHODS

Collection and Authentication of Plant Material

The plant leaves of *S. emarginatus*. used for the present study was collected from A.N.U campus, Guntur district of Andhra Pradesh. The plant was identified, confirmed and authenticated by comparing with voucher specimen available at survey of Medicinal plants and collection unit, Department of Botany, by field Botanist Dr. SK.kasim, Assistant Professor, Acharaya Nagarjuna University, Guntur. A copy of certificate is attached. The plant leaves

material were cut into small pieces and shade dried. The dried material was then pulverized separately into coarse powder by a mechanical grinder. The resulting powder was then used for extraction.

Preparation of Aqueous Plant Extract

The powder was extracted directly with water, which was used for *in-vitro* and *in-vivo* anti-inflammatory investigations and, after subjecting it to preliminary qualitative phytochemical studies[40]. The extract was concentrated under reduced pressure and stored in vacuum desiccators.

Preliminary Phytochemical Screening of SEAE Extract

Phytochemical analysis was carried out by using the standard procedures. Alkaloids, carbohydrates, flavonoids, glycosides, phytosterols/terpenes, proteins Tannins and saponins were qualitatively analysed.

In -Vitro **Human RBC Membrane Stabilization Bioassay (5, 6)**

Inflammation is caused by inflammatory mediators released due to rupture of lysosomes. Lysosomal membrane is structurally similar to Human erythrocyte membrane. Protective effect of an agent on heat / hypotonic saline induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of the agent. This is determined by measuring the intensity of red colour of the hemoglobin released due to rupture of RBC at 560 nm.

Standardization of *In-vitro* Human RBC Membrane Stabilization Bioassay

In-vitro Human RBC membrane stabilization inhibition bioassay was standardized using diclofenac sodium as standard.

Procedure

The method described by Chatterjee S et al was used to study the membrane stabilization. The bioassay was standardized by using Diclofenac sodium as standard. The reaction mixture 4.5ml consisted of 2ml hyposaline (0.25% NaCl), 1ml of 0.15M phosphate buffer (pH 7.4) and 4, 8 & 20µl of Diclofenac sodium 25 µg/ml injection, 0.5ml of 10% human RBC in normal saline was added. The mixtures were incubated at 56^0C for 30 minutes. The tubes were cooled under tap water for 20 minutes. The mixtures were centrifuged and the absorbance of the supernatants read at 560 nm. A control was also maintained where drug was replaced by buffer solution.

In-Vitro **Human RBC Membrane Stabilization Activity Bioassay of *Sapindus emarginatus* Extract (7, 8)**

Preparation of Sample Solution

Stocks solutions of SEAE, 10,000 µg/ml were prepared by using normal saline as a solvent. From this stock solution 3 different concentrations of 100, 200, 400 µg/ml were prepared by using normal saline as a solvent.

Procedure

The method described by Chatterjee S et al was used to study the membrane stabilization assay of the drug extract. The reaction mixture 4.5ml consisted of 2ml hypo saline (0.25% NaCl), 1ml of 0.15M phosphate buffer (pH 7.4) and 1ml of test solution (100,200,400µg/ml of SEAE) in normal saline, 0.5ml of 10% human RBC in normal saline was added. For control tests, 1ml of Isosaline was used instead of test solution while product control tests lacked red blood cells. The mixtures were incubated at 56^0C for 30 minutes. The tubes were cooled under tap water for 20 minutes. The mixtures were centrifuged and the absorbance of the supernatants read at 560 nm. Percentage membrane stabilization was calculated. Graphical representation of *In-vitro* HBRC membrane stabilization of standard Diclofenac sodium and *S. emarginatus* extract was shown.

Experimental Animals

Albino wistar rats weighing 150-250g was procured from Biogen, Bangalore. They were maintained in the animal house of Acharya Nagarjuna University College of Pharmacy. Animals were maintained under controlled condition of temperature at $27^o \pm 2^o$ C and 12-h light-dark cycles. They were housed in polypropylene cages and had a free access to standard pellets (Amruth) and water *ad libitum*.

Evaluation of *In-vivo* Anti-inflammatory Activity by Formalin Induced Rat Paw Edema Model

In the dose response experiment, albino rats were randomly assigned into 4 groups of 6 animals each.**Group I**-Normal saline (*p.o*) was administered daily once throughout the experiment; **Group II**-This group of animals received indomethacin (10 mg/kg, *p.o*.) on the second day, one hour before the induction of paw edema by using 0.2 ml of formalin (1% w/v);

Group III-The animals of this group received 200 mg/kg, *p.o.* of SEAE for two days; **Group IV-** The animals of this group received 400 mg/kg, *p.o.* of SEAE for two days. On the second day, all the groups of animals were given with 0.2 ml of formalin (1% w/v) injected into the rat hind paw, one hour after the treatment of normal saline, Indomethacin, SEAE low dose and SEAE high dose for groups I,II,III and IV respectively. Before formalin injection, the paw volumes for each rat were measured separately by means of plethysmometer. Edema caused by formalin was measured at 0, 3, 6 and 24 hours. The anti-inflammatory potency of the extract was determined by comparing it with a group in which a 10 mg/kg dose of indomethacin was administered orally. Then percentage of inhibition of edema was calculated for each group with respect to the control group as follows.

Percentage of inhibition of paw edema = $(1-V_t/V_c) \times 100$

RESULTS AND DISCUSSION

Extraction of *Sapindus emerginatus* was carried out by using distilled water as a solvent, the percentage yield was found to be 1.6% The extract of *S. emarginatus* was prepared by aqueous extraction procedure and was subjected to preliminary phytochemical screening. It was observed that alkaloids, carbohydrates, flavanoids, glycosides, terpenoids, proteins, tannins and saponins present in the SEAE. Bioactivity of any plant drug is attributed to its phytochemical components. Anti- inflammatory activity reported for different plant drugs reveal that, diverse classes of phytoconstituents are responsible for its activity. In order to co-relate the anti- inflammatory activities with the phytoconstituents of *S. emarginatus*, we have identified its phytoconstituents like alkaloids, carbohydrates, flavanoids, glycosides, terpenoids, proteins, tannins and saponins from the SEAE. Reported methods, were used for the purpose.

Acute Toxicity Studies (LD_{50})

In both phase I and II procedures, none of the animals did not show any toxicity upon the single administration of SEAE (2000mg/kg, *p.o*). Thus, a low dose 200 mg/kg, *p.o* and a high dose 400 mg/kg, *p.o* were selected for the present study. In order to evaluate anti-inflammatory activity of *S. emarginatus* extract*; in-vitro* bioassay involving HRBC membrane stabilization was performed. This bioassay is widely reported for investigations on anti-inflammatory activity of plant drugs

In-vitro Anti-inflammatory Activity

In-Vitro Human RBC Membrane Stabilization Activity Bioassay

It was observed that SEAE have demonstrated dose dependent increase in the % membrane stabilization property (Table-1).

Table-1 *In-Vitro* Human RBC Membrane Stabilization Activity Bioassay of SEAE

Groups	Absorbance Mean ± SEM	%Membrane Stabilization
Control	0.258 ± 0.0006***	-
Diclofenac 10µg	0.181 ±0.001***	29.84
Diclofenac 25µg	0.174 ±0.002***	32.55
SEAE 100 µg	0.236 ±0.001***	8.52
SEAE 200 µg	0.205 ±0.001***	20.54
SEAE 400 µg	0.152 ± 0.001***	41.08

Values are the mean ± S.E.M., n=3, ***Significant at $p<0.001$ compared to control.

SEAE: *Sapindus emarginatus* aqueous extract

In the *in-vitro* membrane stabilization activity, diclofenac was used as standard anti-inflammatory drug and it had showed significant membrane stabilization by preventing haemolysis. SEAE had shown significant dose dependent % membrane stabilization activity in HRBC. Thus *S. emarginatus* extract may have anti-inflammatory activity by preventing release of inflammatory mediators from lysosomal granules whose membrane is structurally similar to erythrocyte membrane. Formalin induced rat paw edema model was used to evaluate the *in-vivo* anti- inflammatory activity. Formalin induced paw edema model, this model based upon the ability of test drug to inhibit the edema produced in the hind paw of the rat after injection of formalin. The nociceptive effect of formalin is biphasic, an early neurogenic component followed by a later tissue mediated response. In the first phase there is release of histamine, 5-HT and kinin, while the second phase is related to the release of prostaglandins. The enzyme, Phospholipase A2, is known to be responsible for the formation of mediators of inflammation such as prostaglandins and leukotrienes. Phospholipids in the cell membrane are converted into arachidonic acid with the help of Phospholipase A2, which is highly reactive and is rapidly metabolized by cyclo-oxygenase (prostaglandin synthesis) to prostaglandins, which are responsible for the induction of pain and inflammation

***In-vivo* Anti-inflammatory Activity**

Effect of SEAE on Formalin Induced Rat Paw Edema Model: Effect on Change in paw volume

The indomethacine had shown significant ($p<0.001$) reduction in paw edema at 1 st , 3 rd and 6 th hr when compared with normal control. SEAE-200 had shown significant ($p<0.05$)action at 6 th hr but not 1 st and 3 rd hr. SEAE- 400 had shown significant ($p<0.05$, $p<0.001$) actions at 3rd and 6 th hr but not 1 st hr (Table-2 and fig-1).

Effect on Change in paw volume

Table-2 Effect of SEAE on Formalin Induced Rat Paw Edema

Group	Treatment	Change in paw volume (ml) and % Inhibition		
		1hr	3hr	6hr
Control	saline	0.607 ± 0.03	0.663 ± 0.04	0.703 ± 0.02
Standard	Indomethacin(10mg/kg)	0.280 ± 0.02***(53.8)	0.330 ± 0.02***(50)	0.333 ± 0.02***(52.6)
SEAE-200	Extract-200 mg/kg	0.490 ± 0.03^{ns}(19.2)	0.527 ± 0.03^{ns}(20.5)	0.580 ± 0.03*(17.4)
SEAE-400	Extract-400 mg/kg	0.483 ± 0.03^{ns}(20.4)	0.487 ± 0.04*(26.5)	0.490 ±0.017***(30.2)

Values are Mean ± SEM (n=6) one way ANOVA followed by Dunnett's test. Where, ***P<0.001, ** P<0.01, *p<0.05 and ns . All values are compared with control.

SEAE: *Sapindus emarginatus* aqueous extract

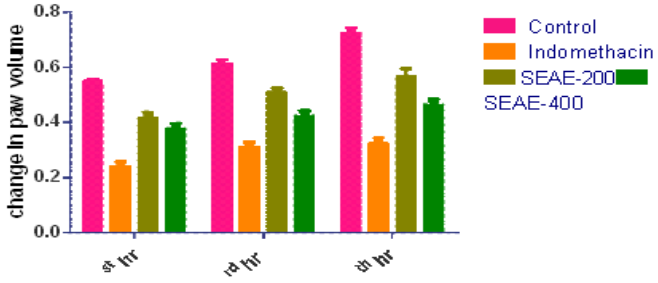

Fig-1 Effect of SEAE on Formalin Induced Rat Paw Edema

CONCLUSION

The SEAE demonstrated anti inflammatory activity by dose dependent increase in %membrane stabilization of HRBC. The acute toxicity study indicated that the SEAE is devoid of major toxic effects. The SEAE-200 mg/kg had shown significant reduction in formalin induced paw edema at 6^{th} hr but not 1^{st} and 3^{rd} hr. The SEAE-400 mg/kg had shown significant reduction in formalin induced paw edema at 3^{rd} and 6^{th} hr but not at 1^{st} hr, whereas maximum significant effect was shown at 6^{th} hr. Further studies will be necessary to establish the probable mechanism of action of plant extracts of *Sapindus emarginatus.* The present investigation has also opened avenues for further research especially with reference to the development of potent phytomedicine for treatment of inflammation from the title plant.

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