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INVITRO ANTI-INFLAMMATORY ACTIVITY OF METHANOL EXTRACT OF *ENICOSTEMMA AXILLARE*

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Abstract

Methanol extract of whole plant of *Enicostemma axillare* (Family: Gentianaceae) was assessed for its anti-inflammatory activity by invitro methods. Invitro anti-inflammatory activity was evaluated using albumin denaturation assay, proteinase inhibitory activity, membrane stabilization, and anti-lipoxygenase activity at different concentrations. Aspirin, Diclofenac sodium, Indomethacin were used as standard drugs. The results showed that *Enicostemma axillare* Methanol Extract (EAME) at a concentration range of 100-500µg/ml significantly ($p<0.01$) protects the heat induced protein denaturation. At the concentration of 400 and 500 µg/ml, EAME showed significant ($p<0.01$) inhibition of 42 and 53% of proteinase inhibitory action, but at the concentration of 100 and 200 µg/ml did not show significant ($p>0.05$) activity. Heat induced haemolysis of erythrocyte was significantly ($p<0.05$) inhibited at the concentration of 400 and 500µg/ml. Hypotonicity induced haemolysis and lipoxygenase activity were significantly ($p<0.01$) inhibited at the concentration range of 200-500µg/ml and 400, 500µg/ml respectively. The results obtained in the present study indicate that methanol extracts of *Enicostemma axillare* can be a potential source of anti-inflammatory agents.

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Enicostemma axillare, anti-inflammatory, HRBC, anti-lipoxygenase.

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Introduction

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When cells in the body are

damaged by microbes, physical agents or chemical agents, the injury is in the form stress. Inflammation of tissue is due to response to stress. It is a defensive response that is characterized by redness, pain, heat, and swelling and loss of function in the injured area. Loss of function occurs depends on the site and extent of injury. Since inflammation is one of the body's nonspecific internal systems of defense, the response of a tissue to an accidental cut is similar to the response that results from other types of tissue damage, caused by burns due to heat, radiation, bacterial or viral invasion [1].

When tissue cells become injured they release kinins, prostroglandins and histamine**.** These work collectively to cause increased vasodilation (widening of blood capillaries) and permeability of the capillaries. This leads to increased blood flow to the injured site. These substances also act as chemical messengers that attract some of the body's natural defense cells a mechanism known as chemotaxis. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Several experimental protocols of inflammation are used for evaluating the potency of drugs. The management of inflammation related diseases is a real issue in the rural community; the population in these areas uses many alternative drugs such as substances produced from medicinal plants.

Enicostemma axillare is a perennial herb found throughout India and common in coastal areas. It is also called as Vellarugu in Tamil, Chota chirayata in Hindi, Mamejavo in Gujarati and Nagajivha in Bengal. The plant is used in folk medicine to treat diabetes mellitus, rheumatisum, abdominal ulcers, hernia, swelling, itching and insect poisoning [2], anti-inflammatory [3], hypoglycaemic [4], [5] & [6] and anticancer [6] activities have been reported. The whole plant is used in medicine as digestive, anti-inflammatory, liver tonic, antimalarial, antipyretic and as a laxative [7], [8]. According to ayurvedic literature survey, the fresh juice of leaves has been used as a bitter tonic, to control arthritis, in typhoid fever and as cooling agent. The plant is traditionally used in the treatment of hepatic diseases and as a blood purifier. It also acts as ethnomedicine for snakebite [9].The plant paste is applied on boils. The leaves are fed to cattle to increase appetite.

A survey of literature indicated no systemic approach has been made to evaluate the anti-inflammatory potential of *Enicostemma axillare* by invitro method. The present study involves determination of anti-inflammatory activity of *Enicostemma axillare by* Inhibition of albumin denaturation, Antiproteinase action, Membrane stabilization and Anti-lipoxygenase activity.

Materials and methods

Plant material

The whole plants of *Enicostemma axillare* were collected in fresh condition from Coimbatore region of Western Ghats, Tamilnadu. Further identified by botanical survey of India (southern circle), Coimbatore. The plant was dried under shade then ground in to a uniform powder using a blender and stored in polythene bags at room temperature.

Preparation of extracts

The plant powder was loaded in to soxhlet extractor and subjected to extraction with methanol. After

extraction, the solvent was distilled off and the extracts were concentrated on water bath to a dry residue and kept in a desiccator.

Assessment of invitro anti-inflammatory activity

Inhibition of albumin denaturation

The anti-inflammatory activity of *Enicostemma axillare* was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima *et al* [10] and Sakat *et al* [11] followed with minor modifications. The reaction mixture was consists of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount of 1N HCl. The sample extracts were incubated at 37 °C for 20 min and then heated to 51 ° C for 20 min, after cooling the samples the turbidity was measured at 660nm.(UV-Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate.

The Percentage inhibition of protein denaturation was calculated as follows: Percentage inhibition = (Abs Control –Abs Sample) X 100/ Abs control

Antiproteinase action

The test was performed according to the modified method of *Oyedepo et al* [12] and Sakat *et al* [11]. The reaction mixture (2 ml) was containing 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37°C for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated.

Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Membrane stabilization

Preparation of Red Blood cells (RBCs) suspension [11], [13]

The Blood was collected from healthy human volunteer who has not taken any NSAIDs (Non Steroidal Anti-Inflammatory Drugs) for 2 weeks prior to the experiment and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline.

Heat induced haemolysis [11], [14]

The reaction mixture (2ml) consisted of 1 ml test sample of different concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the control test tube. Aspirin was used as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 °C for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples.

The Percentage inhibition of Haemolysis was calculated as follows: Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Hypotonicity-induced haemolysis [15]

Different concentration of extract (100-500µg/ml), reference sample, and control were separately mixed with 1ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37°c for 30minutes and centrifuged at 3000rpm. The supernatant liquid was decanted and the haemoglobin content was estimated by a spectrophotometer at 560nm. The

percentage hemolysis was estimated by assuming the haemolysis produced in the control as 100%.

Percentage protection = 100- (OD sample/OD control) x 100

Anti-lipoxygenase activity [14]

Anti-Lipoxygenase activity was studied using linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and added 0.25ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at 25^{o}C. After which, 1.0ml of lenoleic acid solution (0.6mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin was used as reference standard.

The percent inhibition was calculated from the following equation,

% inhibition= [{Abs control- Abs sample}/Abs control] x 100

A dose response curve was plotted to determine the IC50 values. IC50 is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Statistical analysis

Results are expressed as Mean ± SD. The difference between experimental groups was compared by One-Way Analysis Of Variance (ANOVA) followed by Dunnet Multiple comparison test (control Vs test) using the soft ware Graph Pad Instat.

Results and discussion

Inhibition of albumin denaturation

Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of plant extract to inhibit protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation. Maximum inhibition of 71% was observed at 500 µg/ml. Aspirin, a standard anti-inflammation drug showed the maximum inhibition 68% at the concentration of 100 µg/ml compared with control (Table 1).

Table 1: Effect of EAME on heat induced protein denaturation

Treatment (s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of protein denaturation
Control	-	0.38±0.05	-
EAME	100	0.26±0.02**	32
EAME	200	0.20±0.02**	47
EAME	300	0.16±0.03**	57
EAME	400	0.13±0.01**	65
EAME	500	0.11±0.07**	71
Aspirin	100	0.12± 0.01**	68

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant. EAME: *Enicostemma axillare* Methanol Extract.

Proteinase Inhibitory Action

Neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors [16]. EAME exhibited significant antiproteinase activity at different concentrations as shown in Table 2. It showed maximum inhibition of 53% at 500µg/ml. Aspirin showed the maximum inhibition 55% at 100µg/ml.

Table 2: Effect of EAME on proteinase inhibitory action

Treatment(s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of proteinase action
Control	-	0.38±0.09	-
EAME	100	0.30± 0.05^{ns}	21
EAME	200	0.28± 0.01^{ns}	27
EAME	300	0.24± 0.07*	36
EAME	400	0.22±0.01**	42
EAME	500	0.18± 0.03**	53
Aspirin	100	0.17± 0.01**	55

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant; *p<0.05, considered significant; ns p>0.05, non significant. EAME: *Enicostemma axillare* Methanol Extract.

Membrane stabilization

The HRBC membrane stabilization has been used as a method to study the invitro anti inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane [17], [18] and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce a various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane [19].

Heat Induced Haemolysis

The extract was effective in inhibiting the heat induced haemolysis at different concentrations. The results showed that EAME at concentration 400 and 500µg/ml protect significantly (p<0.05) the erythrocyte membrane against lysis induced by heat (Table 3). Aspirin 100µg/ml offered a significant (p<0.01) protection against damaging effect of heat solution.

Table 3: Effect of EAME on heat induced haemolysis of erythrocyte

Treatment(s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of haemolysis
Control	-	0.30 ± 0.03	-
EAME	100	0.24 ± 0.08^{ns}	21
EAME	200	0.21 ± 0.07^{ns}	30
EAME	300	0.19 ± 0.05^{ns}	36
EAME	400	0.17 ± 0.01*	43
EAME	500	0.15 ± 0.03*	51
Aspirin	100	0.09 ± 0.06**	71

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant; *p<0.05, considered significant; ns p>0.05, non significant. EAME: *Enicostemma axillare* Methanol Extract.

Hypotonicity Induced Haemolysis

The results showed that EAME at concentration range of 200-500µg/ml protect significantly ($p<0.01$) the erythrocyte membrane against lysis induced by hypotonic solution (Table 4). Diclofenac sodium (100µg/ml) offered a significant ($p<0.01$) protection against the damaging effect of hypotonic solution. At the concentration of 500µg/ml, EAME showed maximum of 75% protection, whereas, Diclofenac sodium (100µg/ml) showed 51% inhibition of RBC haemolysis when compared with control.

Table 4: Effect of EAME on hypotonicity induced haemolysis of erythrocyte

Treatment(s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of haemolysis
Control	-	0.31 ± 0.02	-
EAME	100	0.22 ± 0.01^{ns}	30
EAME	200	0.16 ± 0.09**	48
EAME	300	0.14 ± 0.04**	54
EAME	400	0.12 ± 0.07**	61
EAME	500	0.07 ± 0.02**	75
Diclofenac sodium	100	0.15 ± 0.01**	51

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant; ns p>0.05, non significant.
EAME: *Enicostemma axillare* Methanol Extract.

Anti-lipoxygenase activity

The establishment of new *invitro* test systems has stimulated the screening of plants aiming to find leads for the development of new drugs. The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals [20]. For this reason, the in vitro inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential [21]. LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy- radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX.

EAME has been checked at 100,200,300,400, 500µg/ml, it showed 09, 17, 26, 48, 62% anti-lipoxygenase inhibition respectively. From these result, the strongest inhibition was obtained at concentration 500µg/ml. The standard Indomethacin showed an 86% inhibition at a concentration of 100µg/ml. At the concentration of 100 and 200µg/ml, EAME not showed significant difference ($p >0.05$) when compared with control (Table 5).

The results obtained from our studies on EAME have shown a potential anti-inflammatory activity. The EAME extracts inhibited the lipoxygenase enzyme activity. This indicates that plant EAME is more useful in studies of inflammation and in various related physiological studies, aging and diseases such as cancer, neurological disorder etc.

Table 5: Effect of EAME on lipoxygenase inhibitory action

Treatment(s)	Concentration (µg/ml)	Absorbance at 660nm	% inhibition of lipoxygenase action
Control	-	0.40 ± 0.02	-
EAME	100	0.36 ± 0.03^{ns}	09
EAME	200	0.33 ± 0.05^{ns}	17
EAME	300	0.30 ± 0.01*	26
EAME	400	0.21 ± 0.07**	48
EAME	500	0.15 ± 0.02**	62
Indomethacin	100	0.06 ± 0.05**	86

Each value represents the mean ± SD. N=3, Experimental group were compared with control **p<0.01, considered extremely significant; *p<0.05, considered significant; ns p>0.05, non significant. EAME: *Enicostemma axillare* Methanol Extract.

Conclusions

In the present study, results indicate that the methanol extracts of *Enicostemma axillare* possess anti-inflammatory properties. These activities may be due to the strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols, The extract fractions serve as free radical inhibitors or scavenger or acting possibly as primary oxidants and inhibited the heat induced albumin denaturation, proteinase activity and stabilized the Red Blood Cells membrane. EAME also reduced the activity of lipoxygenase. Purification of each bioactive compound is necessary and this purified form of the compound can be used which may show increased activity. This study gives on idea that the compound of the plant *Enicostemma axillare* can be used as lead compound for designing a potent anti-inflammatory drug which can be used for treatment of various diseases such as cancer, neurological disorder, aging and inflammation.

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