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Rajesh A
Department of Medicinal Botany, Govt. Siddha Medical College, Palayamkottai, Tamil Nadu, India

Doss A
PG & Research Department of Botany, V.O. Chidambaram College, Tuticorin, Tamil Nadu, India

Tresina PS
PG & Research Department of Botany, V.O. Chidambaram College, Tuticorin, Tamil Nadu, India

Mohan VR
Department of Biomedical Science and Technology, Noorul Islam Center for Higher Education, Kumaracoil, Tamil Nadu, India

Correspondence
Mohan VR
Department of Biomedical Science and Technology, Noorul Islam Center for Higher Education, Kumaracoil, Tamil Nadu, India

In-vitro anti-inflammatory activity of ethanol extract of *Crotalaria longipes*

Rajesh A, Doss A, Tresina PS and Mohan VR

Abstract
Ethanol extract of *Crotalaric longipes* aerial part (Fabaceae) was judged for anti-inflammatory activity by *In-vitro*. Qualitative phytochemical analysis revealed the presence of alkaloid, flavonoid, phenol, glycosides, saponins, steroids etc. *In-vitro* anti-inflammatory activity was calculated using, proteinase inhibitory activity, albumin denaturation assay, membrane stabilization and antilipoxygenase activity at different concentrations. Aspirin was employed as standard drug. The results showed that *Crotalaric longipes* ethanol extract at a concentration range of 100 -500 µg/ml significantly (p<0.01) protects the heat induced haemolysis. The results obtained the present study indicate the ethanol extract of *Crotalaric longipes* can be a potential source of anti-inflammatory agents.

Keywords: *Crotalaric longipes* protein denaturation, HRBC, hypotonicity

Introduction
Inflammation is a basic mechanism in which the body responds to infection, irritation or injury of the body cells and tissues, and the key feature being redness, warmth, swelling and pain. Inflammation is frequently associated with pain and involved occurrences such as; the increase of vascular permeability, increase of protein denaturation and membrane alternation. Denaturation of protein is a well documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability to plant extract to inhibit protein denaturation was studied [1, 2]. Most of the present anti-inflamamtory drugs inhibit the production of cyclooxygenase (COX) enzymes, COX1 and COX2 which synthesis prostaglandins and thromboxane, inflammatory mediators. The commonly used drug which pose several adverse effects especially skin irritation and gastric irritation leading to formation of rashes and ulcers respectively [3].
The genus *Crotalaria longipes* has 300 species worldwide and about 18 species are reported in India. The genus produces mainly pyrrolizindine alkaloids but some flavonoid, glycosides have also been reported [4]. *Crotalaria longipes* are woody shrub growing upto 4 m tall with bright yellow flowers endemic to The Nilgiris and Kollihills. However, perusal of literature survey reveals that *In-vitro* antiinflamamtory activity of *Crotalaria longipes* is totally lacking and hence the present investigation was undertaken.

Material and methods
The aerial parts of *Crotalaria longipes* was collected in fresh from Kothagiri, Nilgiri Biosphere Reserve, Tamil Nadu, India. With the help of local flora, specimen were identified and authenticated by Botanical Survey of India (Southern Circle), Coimbatore, Tamil Nadu, India.

Solvent extraction
Ethanol was used as solvent to prepare the plant extracts. The whole plant was directly soaked for 12 hrs in 500 ml ethanol and then subjected to extraction by reflexing for 6 to 8 hrs below the boiling point of the solvent. The ethanol extracts were concentrated by evaporating at a reduced pressure using rotary evaporator. The concentrated extracts were further dried at 37 ^{0}C for 3 to 4 days in order to facilitate complete evaporation of the solvents. The concentrated extracts were used to qualitative analysis of phytochemicals with standard protocols.

Assessment of *In-vitro* anti-inflammatory activity
Inhibition of albumin denaturation
The anti-inflammatory activity of *Crotalaria longipes* was premeditated by using inhibition of albumin denaturation technique. This was calculated according to Mizushima *et al.* [5] and Sakat *et al.* [6] followed with minor modifications.

The reaction mixture consists of test extracts and 1% aqueous solution of bovine albumin fraction. The pH of the reaction mixture was altered using small amount of 1N HCl. The sample extracts were protected at 37 ºC for 20 min. Then it is heated to 51 ºC for 20 min. After cooling the samples the turbidity was calculated at 660nm. (UV Visible Spectrophotometer Model 371, Elico India Ltd) The experiment was performed in triplicate.

The Percentage inhibition of protein denaturation was computed 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 and Femurewa, [7] and Sakat *et al*. [6]. The reaction mixture (2 ml) was containing 1 ml 20 mM Tris HCl buffer (pH 7.4), 0.06 mg trypsin, and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was kept warm at 37 ºC for 5 min. To this 1 ml of 0.8% (w/v) casein was added. The mixture was kept warm for an extra 20 min. 2 ml of 70% perchloric acid was added to it inorder to arrest the reaction. Followed by this the cloudy suspension was centrifuged. Then the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was repeated thrice. The percentage inhibition of proteinase inhibitory activity was computed. Percentage inhibition = (Abs control –Abs sample) X 100/ Abs control

Membrane stabilization

Preparation of Red Blood cells (RBCs) suspension [6, 8]

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. At 3000 rpm for 10min the tubes were centrifuged and were washed three times with equal volume of normal saline. The volume of blood was determined and re constituted as 10% v/v suspension with normal saline.

Heat induced haemolysis [6, 9]

The reaction mixture (2ml) consisted of 1 ml test sample of dissimilar concentrations (100 - 500 µg/ml) and 1 ml of 10% RBCs suspension, in its place of test sample, only saline was adjoined to the control test tube. As a standard drug Aspirin was used. All the centrifuge tubes enclosing reaction mixture were incubated in water bath at 56 ºC for 30min. At the end of the incubation the tubes were cooled using running tap water. At 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm the reaction mixture was centrifuged. The experiment was completed in triplicates for all the test samples.

The Percentage inhibition of Haemolysis was computed as follows:

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

Hypotonicity-induced haemolysis [10]

Different reference sample, different concentration of extract (100-500µg/ml), and control were individually mixed with 2ml of hyposaline, 1ml of phosphate buffer, and 0.5ml of HRBC suspension. Diclofenac sodium (100µg/ml) was utilized as a standard drug. All the assay mixtures were kept warm at 37 ^{0}C for 30minutes. Then centrifuged at 3000rpm. The supernatant liquid was poured and the haemoglobin content was approximated by a spectrophotometer at 560nm. The percentage hemolysis was calculated approximately by assuming the haemolysis produced in the control as 100%. Percentage protection = 100- (OD sample/OD control) x 100

Anti-lipoxygenase activity [9]

Anti-Lipoxygenase activity was considered using linoleic acid as substrate and lipoxidase as enzyme. Test samples were dissolved in 0.25ml of 2M borate buffer pH 9.0 and additional 0.25ml of lipoxidase enzyme solution (20,000U/ml) is added and kept warm for 5 min at 25 ^{0}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 computed from the following equation,

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

A dose response curve was plotted to establish the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were repeated thrice and averaged.

Statistical analysis

Results are articulated as Mean ± SD. The difference between experimental groups be compared by One Way Analysis Of Variance (ANOVA). This is followed by Dunnet Multiple comparison test (control Vs test) making use of the soft ware Graph Pad Instat.

Results and Discussion

Qualitative Phytochemical screening

The qualitative phytochemical analysis of ethanol extract of *Crotalaria longipes* aerial part showed the presence of alkaloids, flavonoids, saponins, steroids, glycosides, terpenoids, phenol, tannin and sugar

***In-vitro* anti-inflammatory activity**

Inhibition of albumin denaturation

Protein denaturation is a procedure in which protein lose their tertiary structure and secondary structure by using external stress or compound such as strong acid or base a concentration inorganic salt, an organic solvent or heat induced biological protein lose their biological function when denatured. Denaturation of protein is a well documented reason for inflammation. As a part of the study on the mechanism of the anti-inflammatory activity, ability of plant extract to inhibit protein denaturation was examined. It was effective in inhibitory heat induced albumin denaturation at different concentrations as shown in Table 1. Maximum inhibition, 81% was observed at 500 µg/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition 67% at the concentration of 100 µg/ml.

Proteinase inhibitory action

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinase. 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 [11]. *Crotalaria longipes* aerial part ethanol extract exhibited significant antiproteinase activity at different concentrations as shown in table 2. It showed maximum inhibition 76% at 500 µg/ml concentration. Aspirin showed the maximum inhibition 62% at 100 µg/ml.

Heat induced haemolysis

Stabilization of the RBCs membrane was studied to further establish the mechanism of anti-inflammatory activity of *Crotalaria longipes*. The extract was effective in inhibiting the heat induced haemolysis at different concentrations. These provide confirmation for membrane stabilization as an additional mechanism of their anti-inflammatory effect. This extract may perhaps inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutrophil lysosomal constituents include bactericidal enzymes and protect, which upon extracellular release cause further tissue inflammation and damage [12]. Ethanol extract of *Crotalaria longipes* aerial part (100 -500 mg/ml) inhibited the heat induced haemolysis of RBCs to varying degree as shown in table 3. It showed the maximum inhibition 85% at 500 µg/ml concentration. Aspirin, standard drug the maximum inhibition 78% at 100 mg/ml concentration.

Hypotonicity induced haemolysis

The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury of RBCs membrane will further vender the cell more susceptible to secondary damage through free radical induced lipid peroxidation [13, 14]. This notion is constituent with the observation that the breakdown of biomembranes leads to the formation of free radicals which is enhanced cellular damage [15]. It is therefore expected that compounds with membrane stabilizing properties, should offer significant protection of cell membrane against injurious substances [15, 16, 17]. The results showed that ethanol extract of *Crotalaric longipes* aerial part at concentration range of 100 – 500 mg/ml protect significantly (p<0.01) protection against the damaging effect of hypotonic solution. At the concentration 500 mg/ml, ethanol extract of *Crotalaria longipes* showed maximum 88% production whereas, aspirin showed 75% inhibition of RBC haemolysis when compared with control (Table 4).

Antilipoxygenase activity

Lipoxygenase (LOXs) catalyzes the addition of molecules oxygen to fatty acids containing a cis, cis-1,4, pentadiene system. This reaction originates unsaturated fatty acid hydroperoxides. These products are further converted into others that play a key role in inflammatory process [18]. Hence, the compounds which are able to inhibit that enzyme can be considered as antioxidant and possessing anti-inflamamtory properties [19]. Ethanol extract of *Crotalaria longipes* at different concentration (100 -500 mg/ml) has been checked antilipoxygenase activity. From these result, the strongest inhibition was obtained at concentration 500 mg/ml. The standard aspirin showed an 82% inhibition at a concentration of 100 mg/ml (Table 5).

The results obtained from the studies on ethanol extract of *Crotalaria longipes* have shown a potential anti-inflammatory activity. The results of anti-inflammatory studies showed that the inhibition of albumin denaturation at the concentration 500 mg/ml and membrane stabilization at the concentration 500 mg/ml confirmed the aerial parts of *Crotalaria longipes* a potential inhibitor of inflammation. The presence of alkaloids, flavonoids and related phytocompounds may be responsible for the activity.

Table 1: Effect of CL Extract on heat induced protein denaturation

Treatment	Concentration Ug/ml	Absorbance at 660 nm	% of inhibition of heat induced protein denaturation
Control	-	0.36±0.04	-
CL Extract	100	0.21±0.01**	42
CL Extract	200	0.16±0.07**	56
CL Extract	300	0.11±0.03**	69
CL Extract	400	0.09±0.01**	75
CL Extract	500	0.07±0.007**	81
Aspirin	100	0.12±0.04**	67

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

Table 2: Effect of CL Extract on proteinase inhibitory action

Treatment	Concentration Ug/ml	Absorbance at 210 nm	% of inhibition of proteinase action
Control	-	0.37±0.06	-
CL Extract	100	0.28±0.03	24
CL Extract	200	0.22±0.01	41
CL Extract	300	0.16±0.03**	57
CL Extract	400	0.12±0.04**	68
CL Extract	500	0.09±0.002**	76
Aspirin	100	0.14±0.01**	62

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

Table 3: Effect of CL Extract on heat induced haemolysis of erythrocyte

Treatment	Concentration Ug/ml	Absorbance at 560 nm	% of inhibition of heat induced haemolysis of erythrocyte
Control	-	0.33±0.02	-
CL Extract	100	0.21±0.07	36
CL Extract	200	0.16±0.03**	51
CL Extract	300	0.11±0.07**	67
CL Extract	400	0.07±0.007**	78
CL Extract	500	0.05±0.003**	85
Aspirin	100	0.07±0.01**	78

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

Table 4: Effect of CL Extract on hypotonicity induced haemolysis of erythrocyte

Treatment	Concentration Ug/ml	Absorbance at 560 nm	% of inhibition of hypotonicity induced haemolysis of erythrocyte
Control	-	0.32±0.04	-
Extract	100	0.27±0.05	16
CL Extract	200	0.21±0.04	34
CL Extract	300	0.16±0.06**	50
CL Extract	400	0.11±0.03**	66
CL Extract	500	0.07±0.002**	88
Aspirin	100	0.10±0.03**	75

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

Table 5: Effect of CL Extract on lipoxygenase inhibitory action

Treatment	Concentration Ug/ml	Absorbance at 234 nm	% of inhibition of lipoxygenase action
Control	-	0.40±0.06	-
CL Extract	100	0.27±0.02	32
CL Extract	200	0.21±0.04**	47
CL Extract	300	0.16±0.06**	60
CL Extract	400	0.13±0.03**	67
CL Extract	500	0.11±0.05**	72
Aspirin	100	0.07±0.01**	82

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

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