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SCREENING OF AERIAL PARTS OF *LEUCAENA LEUCOCEPHALA* FOR ITS ANTI-INFLAMMATORY ACTIVITY (*IN VITRO*)

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ABSTRACT
Leucaena leucocephala belongs to the family Fabaceae and is naturalized throughout the tropical areas. The aim of this study is to evaluate the anti-inflammatory activity of 50% hydroethanolic extracts of aerial parts (leaves, seeds, stems) of *Leucaena leucocephala* through *in vitro* assays such as albumin denaturation, membrane stabilization and proteinase inhibiton assay. The present study showed that among the aerial parts tested, the leaves found to possess notable anti-inflammatory property when compared to seeds and stem.

KEY WORDS: *Leucaena leucocephala*, Anti-inflammatory, Albumin denaturation, Proteinase inhibition, Membrane stabilization.

INTRODUCTION
Leucaena leucocephala is a flowering plant, commonly known as lead tree (Hughes, 1998a). The plant is native to America (Hughes, 1998) but they are now distributed throughout the tropics (Brewbaker and Sorensson, 1990). It is the fastest growing species of woody plants and referred to as the "miracle tree" due to its broad spectrum applications in various fields (Yeung *et al.*, 2004; Brewbaker and Sorensson, 1990). The aerial parts of *Leucaena leucocephala* has reported to have different medicinal uses which include anthelmintic, antioxidant, contraceptive, abortive and nutritive activities (Mohammed *et al.*, 2015; Syamsudin and Partomuan, 2010; McMahon,2008).

Inflammation is a complex process, accompanied with pain, increase of vascular permeability, increase of protein denaturation and membrane alteration. Denaturation of protein is the major cause of inflammation (Ingle and Patel, 2011; Leelaprakash and Mohan Dass, 2010). Inflammation is also referred to as the reaction of living tissues to injury, infection or irritation (Vane and Botting, 1995).

Inflammation is caused in response to stress and it is a defensive response produced in the injured area which is characterized by redness, pain, heat, swelling and loss of function (Gerard and Sandra, 1993). Though it serves a physiological defense mechanism, the complex events and mediators involved in the inflammatory reaction can induce many diseases (Persoone, 1980). An uncontrolled inflammation may serve as a major factor for other chronic illnesses.

Currently used synthetic anti-inflammatory drugs have several side effects. Thus the development of anti-inflammatory drugs with fewer side effects is essential from medicinal plants (Persoone, 1980). In this study, *Leucaena leucocephala* is used to screen the anti-inflammatory activity using various assays which include inhibition of protein denaturation, membrane stabilization and inhibition of proteinase enzyme.

MATERIALS AND METHODS
Plant material: The aerial parts (leaves, seeds and stem) of *Leucaena leucocephala* were collected in fresh condition without any physical defect from Coimbatore, Tamil Nadu. They were cleaned, air dried in shade, powdered using a blender and stored in polythene bags at room temperature for further use.

Preparation of plant extract
10 g of dried powder (leaves, seeds and stem) were subjected to cold extraction using 50% hydroethanol (100 ml) with occasional stirring for 3 days. After 3 days, the extracts were filtered through Whatmann No. 1 filter paper and were evaporated to dryness at low temperature (<40ºC) under reduced pressure in a rotary vacuum evaporator. The powders obtained were stored in a refrigerator and were used for the further analyses.

Assessment of *in Vitro* Anti-Inflammatory Activity
Inhibition of albumin denaturation: The anti-inflammatory activity of aerial parts of *Leucaena*

leucocephala was evaluated by inhibition of albumin denaturation assay which was studied according to Mizushima and Kobayashi (1968); Sakat *et al.*, (2010) followed with minor modifications. The reaction mixture consisted of 1 ml of plant sample at various concentrations (62.5, 125, 250, 500, 1000 µg/ml) and 1% aqueous solution of bovine serum fraction, the pH of the reaction mixture was adjusted to 6.5 by adding small amount of 1N HCl. The reaction mixture was incubated at 37ºC for 20 minutes and further heated at 57ºC for another 20 minutes. The samples were cooled and the turbidity was measured spectrophotometrically at 660 nm. The experiment was carried out in triplicates. The percentage inhibition of albumin denaturation was calculated as follows.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

Proteinase inhibition assay

The test was carried out according to the modified method of Oyedepo and Femurewa (1995). The reaction mixture 2 ml contained 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml plant sample at different concentrations (62.5, 125, 250, 500, 1000 µg/ml) were incubated at room temperature for 20 minutes. The reaction was stopped by the addition of 70% perchloric acid. The cloudy suspension was centrifuged and the absorbance of the supernatant was recorded at 210 nm against a blank. The experiment was performed in triplicates. The percentage inhibition of proteinase activity was calculated as follows.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

Membrane stabilization test

Preparation of Red Blood cells (RBCs) suspension

Human blood was collected from healthy volunteers and transferred to the heparanized centrifuge tubes. It was centrifuged at 3000 rpm for 20 minutes. The RBC pellet was collected and it was washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

Heat induced haemolysis

The test was done according to the modified method of Shinde *et al*., (1999). The reaction mixture 2 ml consisted of 1ml plant sample at various concentrations (62.5, 125, 250, 500, 1000 µg/ml) and 1 ml of Red Blood cells suspension. For the control tube, instead of plant sample 1 ml of saline was added. Aspirin was used as a standard drug. All the tubes were incubated in a water bath at 56ºC for 30 minutes. After pre-incubation, the tubes were cooled under running tap water and centrifuged at 2500 rpm for 5 minutes. The absorbance of the supernatants was taken at 560 nm. The experiment was carried out in triplicates. The percentage inhibition of haemolysis was calculated as follows.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of Control} - \text{Absorbance of Sample}) \times 100}{\text{Absorbance of Control}}$$

RESULTS

The results of anti-inflammatory activity of aerial parts of *Leucaena leucocephala* on inhibition of albumin denaturation and proteinase action are shown in table 1. As the concentration of plant extract increases, there is an increase in inhibition of albumin denaturation and proteinase action. Maximum inhibition of albumin denaturation for hydroethanolic extract of leaves, seeds and stem was found to be 65.7%, 50.83% and 35.42% at a concentration of 1000µg/ml respectively. A standard anti-inflammatory drug, aspirin showed the maximum inhibition as 86.08% at a concentration of 1000µg/ml. Leaves showed maximum inhibitory activity followed by seeds and stem. The highest inhibitory action on proteinase enzyme was found to be 78.24%, 28.62% and 69.07% respectively for leaves, seeds and stem at a concentration of 1000µg/ml. Aspirin, a standard anti-inflammatory drug showed the maximum inhibition of 89.34% at a concentration of 1000µg/ml. The results revealed that maximum inhibitory potential on albumin denaturation and proteinase action was observed with leaves followed by stem and seeds.

Table. 1. Effect of 50% hydroethanolic extract of aerial parts of *Leucaena leucocephala* on albumin denaturation and proteinase inhibitory activity.

Concentration (µg/ml)	Albumin denaturation (%)				Proteinase inhibitory activity (%)			
	Leaves	Seeds	Stem	Aspirin	Leaves	Seeds	Stem	Aspirin
62.5	11.33±0.6	10.67±0.3	12.65±0.8	12.73±0.5	23.62±0.7	11.32±0.6	30.27±0.56	15.04±0.94
125	16.58±0.70	13.09±0.57	13.11±0.50	15.03±0.63	30.67±1.19	12.65±0.49	36.30±1.41	18.60±0.80
250	18.72±0.75	17.42±0.75	17.45±0.71	30.90±1.25	57.13±1.89	24.47±1.08	49.41±1.83	38.61±1.41
500	21.78±0.98	19.20±0.80	21.8±0.81	38.36±1.35	70.37±2.23	25.27±1.09	57.08±2.10	47.81±2.10
1000	65.70±2.65	50.83±1.63	35.42±1.21	86.08±3.68	78.24±3.35	28.62±1.22	69.07±3.10	89.34±3.14

The values are expressed as Mean ± SD. (n=3)

The results of membrane stabilization test are shown in table 2. The membrane stabilization action for extracts of leaves, seeds and stem was found to be 84.26%, 81.52% and 80.5% respectively. The maximum stabilizing

capacity was shown by the leaves followed by seeds and stem with relevance to the standard drug aspirin (86.46% at 1000µg/ml).

Table. 2. Effect of 50% hydroethanolic extract of aerial parts *Leucaena leucocephala* on membrane stabilization activity.

Concentration (µg/ml)	Membrane stabilization (%)			
	Leaves	Seeds	Stem	Aspirin
62.5	27.62±0.90	47.32±0.62	32.67±0.47	43.92±0.55
125	49.82±1.92	58.34±2.21	40.33±1.52	55.94±2.23
250	65.57±2.78	63.52±2.57	60.47±1.84	60.50±1.90
500	75.17±3.21	68.71±2.97	65.51±2.88	65.38±2.71
1000	84.26±3.03	81.52±3.27	80.50±1.80	86.46±3.74

The values are expressed as Mean ± SD. (n=3)

DISCUSSION

Denaturation of proteins is a well reported cause of inflammation. Most inflammatory drugs show dose dependent ability to protein denaturation (Mizushima and Kobayashi, 1968). During denaturation, most of the biological proteins lose their function (Megha *et al.*, 2013). Mechanism of denaturation involves the alteration of electrostatic force, hydrogen, hydrophobic and disulphide bonds. Auto antigens are produced in certain inflammatory diseases which may be due to denaturation of proteins (Grant *et al.*, 1970).

It was reported that leukocytes proteinase have an important role in the development of tissue damage during an inflammatory reaction. Neutrophils are the rich source of proteinase which carries many serine proteinases in their lysosomal granules (Das and Chatterjee, 1995). The aerial parts of *Leucaena leucocephala* extract inhibit the release of lysosomal content of neutrophils at the site of inflammation (Chou, 1997). Proteinases are mainly implicated in arthritic reactions (Das and Chatterjee, 1995).

Membrane stabilization method was done for in vitro assessment of anti-inflammatory activity since the erythrocyte membrane is analogous to the lysosomal membrane. A variety of disorders are produced during inflammation by the release of lysosomal enzyme. The extracellular activity of the lysosomal enzymes is related to acute or chronic inflammation (Rajendran Vadivu). The compounds which prevent the lysis of hydrolytic enzymes present in the lysosomes may relieve some symptoms of inflammation (Hes and Milonig, 1972). Stabilization of lysosomal membrane is important in limiting the inflammatory response (Chippada and Neena, 2011; Azeem *et al.*, 2010). Hence the results of our findings confirmed the use of Leucaeana leucocephala as a potent anti-inflammatory agent.

CONCLUSION

The present study revealed the anti-inflammatory activity of 50% hydroethanolic extract of aerial parts of *Leucaena leucocephala*. Further investigations are required to find the *in vivo* anti-inflammatory activity of *Leucaena leucocephala*.

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CONFLICT OF INTEREST

There is no conflict of interests.

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