

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF ALCOHOLIC EXTRACT OF LEAVES OF *LEUCAS ASPERA* IN ALBINO RATS

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ABSTRACT

Objective: Alcoholic extract of leaves of *Leucas Aspera* was tested for anti-inflammatory activity in animal models.

Methods: Three doses, 100 mg/kg, 200 mg/kg and 400 mg/kg of the plant extract prepared as a suspension in 1% tween 80 in normal saline. The control group received only Tween 80 (2%, v/v) orally. Acute and sub-acute inflammatory activities were studied in rats by carrageenan induced paw edema and cotton pellet induced granuloma models respectively. In both models, the standard drug used was diclofenac 150 mg/kg.

Results: All the three test doses (100mg/kg, 200mg/kg, 400mg/kg) at different time intervals (0-4 hours) did exhibit anti-inflammatory activity in both models ie. Carrageenan model and cotton pellet granuloma model in comparison to control. The test dose of 400mg/kg of *leucas aspera* produced a percentage inhibition of 60.64% which was comparable to standard drug Diclofenac which produced a percentage inhibition of 60.70% in carrageenan model, whilst the percentage inhibition by cotton pellet method for standard drug was 60.27% and for three different doses of test (100, 200, 400mg/kg) was 50.85%, 57.63%, and 58.42% respectively.

Conclusion: *Leucas Aspera* possesses significant anti-inflammatory activity with p value (0.0001).

Keywords: Anti-inflammatory activity, *Leucas Aspera*, carrageenan induced paw edema, cotton wool pellet granuloma, NSAIDs (Non-steroidal antiinflammatory drugs).

INTRODUCTION

Inflammation is a pathophysiological process by which a living tissue reacts to injury and particularly concerns vascular compartment and the connective tissue [1]. The term inflammatory reaction refers to the events, which occurs in the tissue in response to an invading pathogen or the presence of a noxious substance. However, if the inflammatory stimulus persists or if the defence system of the body is inappropriately mobilized, inflammation becomes chronic. Chronic inflammatory diseases like rheumatoid arthritis, gout and connective tissue disorders result in a great deal of morbidity which affects the quality of life. NSAIDs, DMARDs, Corticosteroids are widely used in the treatment of a variety of inflammatory disorders. However, the prolonged use of any of the above mentioned drugs can lead to a variety of adverse drug reaction like headache, gastrointestinal bleeding, liver disorders, dermatological reactions, bone marrow depression and the list goes on and on.

Drug research and development (R & D) is comprehensive, expensive, time-consuming and full of risk. It is estimated that a drug from concept to market would take approximately 12 years and capitalizing out-of-pocket costs to the point of marketing approval at a real discount rate of 11% yields a total pre-approval cost estimate of US\$ 802 million [2].

The corollary is, a physician would want such a drug which achieves high therapeutic plasma concentration with least adverse effects. Hence, the search for a better drug has been a perennial pursuit and the use of medicinal plants could offer a better and effective treatment for inflammatory disorders with least or no adverse effects and moreover is cost effective.

In the present study, *Leucas aspera* commonly known as Thumbai [3] or dronapushpi in Sanskrit is distributed throughout India from Himalayas down to Ceylon [4].

The plant is used traditionally as an antipyretic and insecticide. Flowers are valued as stimulant, aperient, Diaphoretic, insecticide and emmenagogue. Leaves are considered useful in chronic rheumatism, psoriasis and other chronic skin eruptions. Bruised leaves are applied for snake bites [5]. The presence of various

phytochemicals like triterpenoids [6], sitosterols [7], nicotine [8], glycoside [9], diterpenes [10], isoamyl propionate [11], ceryl alcohol [12], phenolic compounds [13] and alkaloids play a vital role of their application as anti-inflammatory [14], antioxidant, antifungal [15], antimicrobial [16], diabetes [17], analgesic, asthma [18].

Leucas aspera has the property to counteract the effect of nitric oxide formation due to the presence of tannins and flavonoids and return be of considerable interest in preventing the ill effects of excessive nitric oxide generation. The possibility of *leucas aspera* extract of having anti-inflammatory response could not have been overruled. Hence, by keeping the above concept in mind, the present study "Anti-inflammatory activity of alcoholic extract of leaves of *Leucas aspera* in albino rats" was undertaken. The ethanolic extract was used in this investigation because ethanol being nonpolar, the major active chemical constituents of *leucas aspera* including volatile oils, would be expected to be more soluble in ethanol fraction of the extract.

MATERIALS AND METHODS

Description of plant material

The whole plant of *Leucas aspera* was collected in and around fields of Davangere during September 2011 and was authenticated at the department of botany DRM College Davangere. After authentication, the plants were cleaned and shade dried and milled into coarse powder by a mechanical grinder.

The dried powder (total 2kg) was subjected to Soxhlet extraction successfully with alcoholic extract (ethanol 95%) at a boiling point of 78 °C exhaustively for 32 hours.

The extract was then kept in a desiccator and stored in refrigerator for further pharmacological evaluation. The test doses were prepared in Tween 80 (1%) suspension in normal saline of each extract to get the desired content of the extract. After extraction the solvent was distilled off and the extract was concentrated under reduced pressure on a water bath at a temperature below 50°C to give a semisolid syrupy consistency residue of 56.6 g (yield approximately 3%, w/w) which was stored in a closed bottle and

kept in a refrigerator at temperature below 4°C until tested. Phytochemical screening of the extract was done which shows the presence of tannins, flavonoids, triterpenoids, sterols, glycosides and saponins. A dose of 100mg/kg, 200mg/kg and 400mg/kg of ethanolic extract of *Leucas aspera* was used after concluding toxicity studies.

Selection of animals, caring and handling

Animals: Albino rats of either sex of average weight 150-200g used for the study were bred in the central animal house of JJM Medical College, Davangere, under suitable conditions of housing, temperature, ventilation and nutrition were used to study the anti-inflammatory effect of *Leucas aspera* on carrageenan induced hind paw edema and inflammatory effect of cotton wool granuloma. They were housed under controlled conditions of temperature of 23±20°C, relative humidity of 30-70% and 12 h light-12 h dark cycle. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment. All animals were fed with sterile commercial pelleted rat chow supplied by Hindustan Lever Ltd. (Mumbai, India) and had free access to water *ad libitum*. Animals were kept under fasting for overnight and weighed before the experiment. The study was undertaken after obtaining approval of Institutional Animal Ethics Committee. The experiment was conducted according to CPCSEA. (Committee for the Purpose of Control and Supervision on Experiments on Animals) recommended OECD guideline 425[19].

Study design

A total of 60 animals (N=60) are taken for the study, in 2 groups, each group consisting of 30 animals subdivided in 5 subgroups of 6 animals each.

Group I: Carrageenan induced hind paw edema

The animals were divided into following groups:

Group A: Received Tween 80 (2%, v/v) orally as control.

Group B: Received Diclofenac sodium 150mg/kg orally as standard.

Group C: Received Ethanolic extract of *Leucas aspera* 100mg/kg orally.

Group D: Received Ethanolic extract of *Leucas aspera* 200mg/kg orally.

Group E: Received Ethanolic extract of *Leucas aspera* 400mg/kg orally.

Group 2: Cotton wool pellet Granuloma

Group F: Received Tween 80 (2%, v/v) orally as control

Group G: Received Diclofenac sodium 150mg/kg orally as standard.

Group H: Received Ethanolic extract of *Leucas aspera* 100mg/kg orally.

Group I: Received Ethanolic extract of *Leucas aspera* 200mg/kg orally.

Group J: Received Ethanolic extract of *Leucas aspera* 400mg/kg orally.

MATERIALS AND METHODS

Drugs

Diclofenac (Genu Pharmaceuticals, Goa), Ether (Sigma chemical Co. St Louis, USA).

Inflammatory agents used

Inj carrageenan (0.1 ml of 1% w/v suspension) - Sigma Aldrich Switzerland, Cotton pellets.

Instruments

Digital plethysmometer (INCO), Soxhlet Extractor.

Determination of the drug dosage and dosing schedule

Doses were selected and determined according to the previous acute toxicity studies of ethanolic extract of *Leucas aspera*. Different doses of ethanolic extract of *Leucas aspera* (100,200,400) mg/kg were administered orally. The animals were observed during 1 hour for signs and symptoms of toxicity and the number of deaths each 24 hour period for 7 days was recorded. Two methods were used for evaluation of anti-inflammatory effect

1. Carrageenan induced Hind paw edema — For Acute Inflammation.

2. Cotton wool pellet granuloma — For Chronic Inflammation

Inclusion criteria

- Albino rats of either sex weighing 150-200 grams.
- Healthy animals with normal behaviour.

Exclusion criteria

- Albino rats weighing more than 200grams or less than 150grams.
- Pregnant female rats.
- Animals previously used for any other experimental procedures.

A total of 60 animals (N=60) are taken for the study, in 2 groups, each group consisting of 30 animals subdivided in 5 subgroups of 6 animals each.

Acute inflammatory model

Group I: Carrageenan induced hind paw edema

Acute inflammation was produced by injecting 0.1ml of 1% carrageenan suspension in normal saline to the right hind paw of the rats. In control group only vehicle (Normal saline 3ml/kg) is injected. Test drug (Ethanolic extract of *Leucas aspera* at doses 100,200,400mg/kg) as well as standard (Diclofenac 150mg/kg) was administered orally one hour before carrageenan challenge. A mark is made at the ankle joint of each rat. Paw volume up to the ankle joint is measured at 0,1, 2,3,4 hours after carrageenan challenge using digital plethysmograph. The percentage inhibition in various treated groups was then calculated using the formula:

$$\text{Percentage edema inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

V_t - Is the mean edema volume in a drug treated group.

V_c - Is the mean edema volume in control group.

The difference in the initial 0hr and volume at +1h indicate paw edema at 1hr following carrageenan administration. Accordingly paw edema at +2, +3 and +4 was calculated. Then percentage inhibition of paw edema was calculated.

Cotton pellet induced granuloma model

Cotton pellet granuloma model is a model for chronic inflammation in rats. In this model cotton pellet granulomas are induced by subcutaneous implantation of cotton pellets[20]. Removing and weighing pellets after 7 days can measure the amount of granulation tissue formed. More intensive granulation formation has been observed if the cotton pellets have been impregnated with carrageenan, formalin, capsicum oleoresin[21]. In this model, cotton pellets are implanted subcutaneously, which leads to the infiltration of fluid, histological giant cells and proliferation of the connective tissue which are the hallmarks of chronic inflammation.

Procedure

Under light ether anaesthesia, ventral aspect of abdomen is shaved and an incision of 3-4cms is taken. Then the path towards the axilla is cleared with a finger, so that subcutaneous pocket is formed. Sterile cotton pellet weighing (10mg) impregnated with foreign material carrageenan is implanted subcutaneously bilaterally in the axilla. Drug treatment is started 2 hours after cotton pellet implantation and continued for 7 consecutive days. After 7 days rat

is sacrificed and cotton pellets are removed by dissection. While dissecting, granulation tissue is identified as firm vascular tissue, which is generally well defined from the surrounding normal subcutaneous tissue. Pellets are dried at 60 °C for 24 hours to their dry weight. The net granuloma formation was calculated by subtracting the initial weight noted (10mg)[22].

Efficacy variable

Change in weight of cotton pellet expressed as percentage change. Percentage inhibition can be calculated as

$$\text{Percent inhibition} = \frac{WC - Wt}{WC} \times 100$$

WC – Mean dry weight of pellet granuloma in control group

Wt – Mean dry weight of pellet granuloma for test group

Statistical analysis

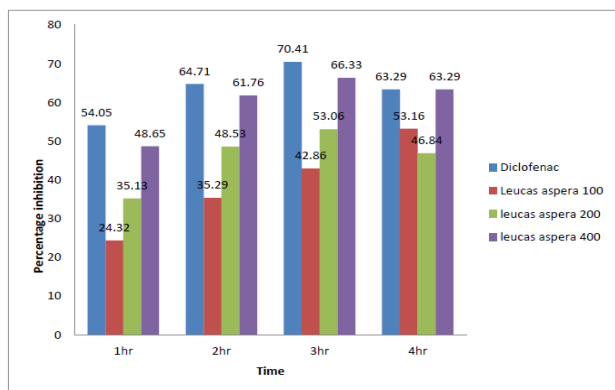
Descriptive data that include Mean, Standard Deviation and Range were found for each group and used for analysis. Tukey’s post hoc test was used for comparison between two groups. One way ANOVA was used for multiple group comparison. P value of 0.05 or less was considered for statistical significance.

RESULTS

The results obtained from carrageenan induced paw edema showed the mean values for control, Diclofenac, and the test compound *Leucas aspera* with 3 different doses to be, 0.62, 0.2436, 0.402, 0.336, 0.2442 respectively, while the percentage inhibition by carrageenan induced paw edema method from 0-4 hours for the standard (Diclofenac) was 60.70%, and for the 3 different test doses of *Leucas aspera* (100mg, 200mg, 400mg/kg) was 35.16%, 45.80% and 60.64% respectively. Moreover the test dose of 400mg/kg of *Leucas aspera* produced a percentage inhibition of 60.64% which is almost equal to that of standard drug which produced a percentage inhibition of 60.70%. The above results showed that the ethanolic extracts of leaves of *Leucas aspera* with three different doses (100, 200, 400mg/kg) at different time intervals (0-4 hours) had high significant anti-inflammatory activity with p value less than 0.001 by carrageenan induced paw edema. in acute inflammation. The results obtained from cotton wool pellet granuloma method showed the mean values for control, Diclofenac, and the test compound *Leucas aspera* with 3 different doses to be, 17.7, 7.033, 8.7, 7.5, 7.36 respectively, while the percentage inhibition by cotton wool pellet granuloma method for the standard (Diclofenac) was 60.27%, and for the 3 different test doses of *Leucas aspera* (100mg, 200mg, 400mg/kg) was 50.85%, 57.63% and 58.42% respectively. These results by cotton wool pellet method i.e. for chronic inflammation showed that the ethanolic extracts of leaves of *Leucas aspera* with three different doses (100, 200, 400mg/kg) at different time intervals (0-4 hours) had high significant anti-inflammatory activity with p value less than 0.001.

Method 2: carrageenan induced paw edema

RESULTS



Graph 1: depicting percentage inhibition of paw edema by standard drug (Diclofenac) and test drug (*Leucas aspera*) at different time intervals by CARRAGEENAN method

Table 1: ANOVA of the results of carrageenan induced paw edema method

Time	F value	P value
1 hour	59.22	<0.001
2 hours	161.08	<0.001
3 hours	475.46	<0.001
4 hours	389.38	<0.001
P value (<0.001) being significant at time intervals 1, 2, 3 and 4 hours		

Table 2: Post Hoc (Tukey’s) test of the results of carrageenan induced paw edema method - Comparison values between different groups for 0 hour, 1, 2, 3 and 4 hours

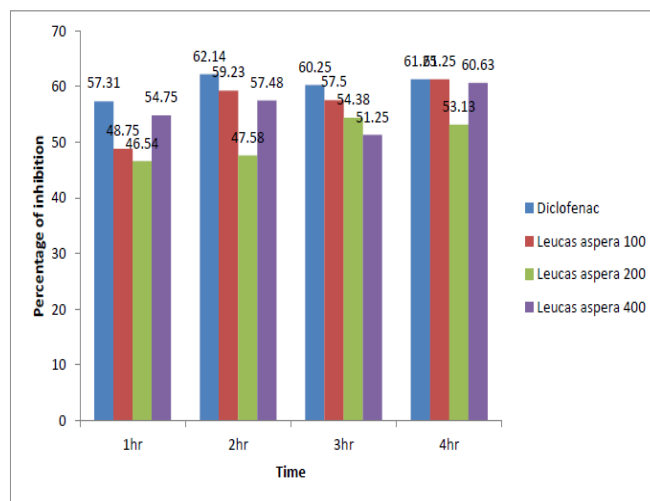
Comparison	P value
Control* vsDiclofenac**	<0.01
Control* vs <i>Leucas aspera</i> (100mg/kg)	<0.01
Control* vs <i>Leucas aspera</i> (200mg/kg)	<0.01
Control* vs <i>Leucas aspera</i> (400mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (100mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (200mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (400mg/kg)	<0.01
<i>Leucas aspera</i> (100mg/kg) vs <i>Leucas aspera</i> (200mg/kg)	<0.01
<i>Leucas aspera</i> (100mg/kg) vs <i>Leucas aspera</i> (400mg/kg)	<0.01

** Normal saline 3ml/kg) ** Diclofenac 150mg/kg)

Comparison between groups (control, standard and the 3 different test doses 100,200 and 400mg/kg of *Leucas aspera*) is highly significant with a p value < 0.01

Method 2: cotton wool pellet granuloma method

RESULTS



Graph 2: Depicting the comparison of the percentage inhibition of paw edema by standard drug (Diclofenac) and test drug (*Leucas aspera*)

Table 3: ANOVA of the results by cotton pellet granuloma method

Time	F value	P value
1 hour	100.90	<0.001
2 hours	71.78	<0.001
3 hours	71.56	<0.001
4 hours	102.29	<0.001

* p value (<0.001) was highly significant at all the time intervals (1 to 4 hours).

Table 4: Post Hoc (Tukey's) test of the results of Cotton wool pellet granuloma method - Comparison values between different groups for 0 hour, 1, 2, 3 and 4 hours

Comparison	P value
Control* vsDiclofenac**	<0.01
Control* vs <i>Leucas aspera</i> (100mg/kg)	<0.01
Control* vs <i>Leucas aspera</i> (200mg/kg)	<0.01
Control* vs <i>Leucas aspera</i> (400mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (100mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (200mg/kg)	<0.01
Diclofenac** vs <i>Leucas aspera</i> (400mg/kg)	<0.01
<i>Leucas aspera</i> (100mg/kg) vs <i>Leucas aspera</i> (200mg/kg)	<0.01
<i>Leucas aspera</i> (100mg/kg) vs <i>Leucas aspera</i> (400mg/kg)	<0.01

Normal saline (Saline3ml/kg) ** 150mg/kg *

Comparison between the groups (control, standard and the 3 different test doses 100, 200 and 400mg/kg of *Leucas aspera*) is highly significant with p value <0.01.

DISCUSSION

The current study of ethanolic extract of leaves of *Leucas aspera* was done to evaluate the anti-inflammatory effect both for acute inflammation as well as chronic inflammation.

Two widely used screening methods were employed:

1. Carrageenan induced hind paw edema – for acute inflammation
2. Cotton pellet granuloma – for chronic inflammation.

The results obtained from carrageenan induced paw edema showed the mean values for control, Diclofenac, and the test compound *Leucas aspera* with 3 different doses to be, 0.62, 0.2436, 0.402, 0.336, 0.2442 respectively, while the percentage inhibition by carrageenan induced paw edema method from 0-4 hours for the standard (Diclofenac) was 60.70%, and for the 3 different test doses of *Leucas aspera* (100mg, 200mg, 400mg/kg) was 35.16%, 45.80% and 60.64% respectively. Moreover the test dose of 400mg/kg of *Leucas aspera* produced a percentage inhibition of 60.64% which is almost equal to that of standard drug which produced a percentage inhibition of 60.70%. The above results showed that the ethanolic extracts of leaves of *Leucas aspera* with three different doses (100, 200, 400mg/kg) at different time intervals (0-4 hours) had high significant anti-inflammatory activity with p value less than 0.001 by carrageenan induced paw edema. In acute inflammation.

The results obtained from cotton wool pellet granuloma method showed the mean values for control, Diclofenac, and the test compound *Leucas aspera* with 3 different doses to be, 17.7, 7.033, 8.7, 7.5, 7.36 respectively, while the percentage inhibition by cotton wool pellet granuloma method for the standard (Diclofenac) was 60.27%, and for the 3 different test doses of *Leucas aspera* (100mg, 200mg, 400mg/kg) was 50.85%, 57.63% and 58.42% respectively. These results by cotton wool pellet method i.e. for chronic inflammation showed that the ethanolic extracts of leaves of *Leucas aspera* with three different doses (100, 200, 400mg/kg) at different time intervals (0-4 hours) had high significant anti-inflammatory activity with p value less than 0.001.

Saundane AR et al revealed that ethanol and distilled water extracts exhibited significant anti-inflammatory activity when compared to respective controls and were comparable with those standard drugs Diclofenac sodium and Analgin.

N M Goudgoan et al evaluated the presence of anti-inflammatory activity of *Leucas aspera* with different extracts (crude, alkaloid fraction, non-alkaloid fraction) with control as phenylbutazone and his experiments revealed in which phenylbutazone showed the highest anti-inflammatory effect followed by alkaloid fraction and

crude extracts. K Srinivas et al performed a study using 4 different crude extracts (petroleum ether, chloroform, ethanol and water) of *Leucas aspera* , respectively at a dose of 400mg/kg body weight orally, in albino rats, when compared with respective controls and in comparison with the standard drugs Diclofenac sodium and Analgin, ethanol and distilled water extracts exhibited significant anti-inflammatory effects.

Saha MR et al studied the extracts of four medicinal plants (*Leucas aspera* , *Hibiscus mutabilis*, *Ixoracoccinea* and *Polyalthialongifolia*) of which *Leucas aspera* showed the greatest nitric oxide scavenging effect which in turn is known for its anti-inflammatory effect.

The results obtained in our study also exhibits that the ethanolic extract of leaves of *Leucas aspera* also exhibits anti-inflammatory effect with both the methods i.e. Carrageenan induced paw edema and cotton wool pellet which suggests its use in both acute and chronic anti-inflammatory activity.

Carrageenan-induced hind paw edema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effects.

The development of edema in carrageenan induced rat paw edema model follows a biphasic response. The first phase that occurs between 0-2.5 hours after injection has been attributed to histamine, serotonin or kinin release[23]. The second phase occurs at 3 hours and is caused by the release of bradykinin, protease, prostaglandin and lysosome[24, 25, 26]. Hence results were considered from 0 to 4 hours.

In response to infection or tissue damage, an array of soluble and lipid mediators as well as cytokines and growth factors cause both immune and non-immune cells to produce large amounts of nitric oxide. Nitric oxide and its oxidation products are toxic and can cause tissue injury[27]. Macrophages when activated via T-cell responses secreting interferon gamma, elicit a TNF-dependent nitrite response. Inhibition of nitric oxide activity by either suppressing nitric oxide synthase (NOS2) or via inhibition of TNF activity results in marked suppression of macrophage activation. Macrophages regulate T cell responses, in part via nitric oxide production[28].

Leucas aspera has the property to counteract the effect of nitric oxide formation due to the presence of tannins and flavonoids and in turn may be of considerable interest in preventing the ill effects of excessive nitric oxide generation. Ethanolic extract of *Leucas aspera* has showed the greatest nitric oxide scavenging effect which in turn is known for its anti-inflammatory effect[29]. Chronic inflammation includes proliferation of fibroblasts and the infiltration of neutrophils and exudation[30, 31]. Chronic inflammation occurs by means of the development of proliferative cells. These cells can either be spread from granuloma form. Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation[32].

Three major families of compounds present in EELA may explain these activities: catechins (epicatechin, beta epicatechin), flavonoids (procyanidin), phytosterols (beta-sitosterol) apart from glycosides, phenolic compounds and tannins.

CONCLUSION

The ethanolic extract of leaves of *Leucas aspera* at different doses showed significant (p< 0.001) anti-inflammatory activity in both acute as well as chronic inflammation. Further studies might help in the establishment of its role in the treatment of inflammatory disorders.

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