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Phytochemical and Pharmacological investigation of an indigenous medicinal plant *Leucas aspera*

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Abstract : *Leucas aspera* commonly known as 'Thumbai' has a wide variety of medical applications. The aqueous extract of *Leucas aspera* (leaf extract) was assessed for its various *in vitro* activities such as anti-inflammatory, anti-helminthic, anti-arthritic, anti-oxidant and anti-bacterial activity. The *in vitro* Anti-inflammatory activity was studied by human red blood cell (HRBC) membrane stabilization method by using various concentration of *L. aspera*. The results showed that the HRBC Membrane stabilization activity of the aqueous leaf extract of *L. aspera* at concentration of 100µg/ml showed 73.25% inhibition of denaturation in hypotonic solution while the standard Diclofenac 100µg/ml showed 79.25% inhibition of denaturation. The crude extracts of the leaves of *Leucas aspera* showed statistically significant anti-inflammatory activity in *in vitro* assay.The crude aqueous leaves extract of *Leucas aspera* also showed that significant anti-helmintic effect causing death of the worm at all the concentrations but the time of death was different in each case. *Invitro* anti-arthritic activity was carried out by bovine serum protein denaturation method and egg albumin denaturation method. The results suggested that the aqueous extract of *L. aspera* showed a very good anti-arthritic activity. Antioxidant and Antibacterial activity was also evaluated. The present study concluded that the plant can be formulated in broad spectrum antibiotics and also confirms the traditional uses in pathogenic disease.
Key words: Anti-inflammatory; Albendazole; Diclofenac; Hydrogen per oxide; Anti-arthritic, *Leucas aspera*.

Introduction

The medicinal value of plants has been documented in almost all ancient civilizations. As plants are known as the store houses of drugs majority of the drugs are derived directly or indirectly from these plants. Each and every part of the plant such as roots, stems, leaves, flowers, fruits and seeds are known to have various medicinal properties[1,2]. The medicinal properties of plants have been investigated due to their economic viability, less toxicity and various pharmacological activities[3,4]. The revival of interests in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than expensive synthetic drugs which have adverse side effects[5]. Medicinal plants contain some organic compounds which provide definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids. Based on the above context, the present investigation is carried out to evaluate the various *invitro* activities in *Leucas aspera.*

Leucas aspera commonly known as Thumbai is distributed throughout India[6]. Traditionally, the whole plant is taken orally for analgesic, antipyretic, anti-rheumatic treatment and its paste is applied topically to

inflamed areas[7]. The entire plant is also used as an insecticide and indicated in traditional medicine for cough, cold, painful swelling and chronic skin eruption[8].

This study is an attempt to investigate about the activities against helminths, inflammatory, arthritic and bacterial activity.

Materials and Methods

Preparation of *L. aspera* aqueous plant extract

The whole plant was collected from VIT University, Vellore, Tamil Nadu in the month of October 2015. The Plant was thoroughly washed with water. All the plants were sliced into small pieces and spread on tray allowing shade drying. The dried leaves were ground to coarse powder with a mechanical grinder (Grinding mill). Then, an aqueous extract was made with 10g of dried powder boiled with 100 ml of double distilled water at 60°C for 1 hour. Then, the extract was filtered through Whatmann No.1 filter paper and used for further experiments[9, 10]

Qualitative Phytochemical Screening

The aqueous extracts of*Leucas aspera* were screened for different phytochemical constituents' viz., carbohydrates, phenol, alkaloid, tannin, flavonoid and saponin. Phytochemical screening of the extracts was carried out by the standard methods[11,12]

In-Vitro Anti-Inflammatory Activity

The human red blood cell (HRBC) membrane Stabilization method

The blood was collected from healthy human volunteer. It was mixed with equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3,000 rpm[13]. The packed cells were washed with iso-saline and a 10% suspension was made. Various concentrations of extracts were prepared (25, 50, 75, 100 µg/ml) using distilled water and to each concentration 1 ml of phosphate buffer, 2 ml hypo-saline and 0.5 ml of HRBC suspension were added[14,15,16]. Percent inhibition of protein denaturation was calculated as follows:

%Inhibition= (Abs control - Abs sample)/Abscontrol×100

Antihelminthic activity

The anthelmintic activity was performed according to the method of[1]on adult Indian earth worm *Pheretima pothuma* as it has anatomical and physiological resemblance with the intestinal round worm parasites of human beings. *Pheretima pothuma* was placed in petridish containing four different concentrations (5, 25, 50, 100 mg/ml) of aqueous leaf extract of *Leucas aspera*. Each petri dish was placed with 3 worms and observed for paralysis or death. The mean time for paralysis was noted when no movement of any sort could be observed, except when the worm was shaken vigorously; the time death of worm (min) was recorded after ascertaining that worms neither moved when shaken nor when given external stimuli. In the same manner albendazole was included as reference compound[17,18]. The Test results were compared with Reference compound Albendazole (15mg/ml) treated samples.

Group 1: Normal control - Treated with Normal saline
Group 2: Negative control - Tween 20 with Normal Saline
Group 3: Positive Control - Standard Drug Albendazole
Group 4: Test - Different Concentration of Extract

Anti-arthritic activity

The *Invitro* anti-arthritic activity was studied using bovine serum protein denaturation method and Egg Albumin denaturation method.

Bovine Serum Protein Denaturation Method

Preparation of Reagents

0.5% Bovine Serum Albumin (BSA): Dissolved 500mg of BSA in 100 ml of water

Phosphate Buffer Saline PH 6.3: Dissolved 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) in 800 ml distilled water. The pH was adjusted to 6.3 using 1N HCl and make up the volume to 1000 ml with distilled water.

Method

Test solution (0.5ml) consists of 0.45ml of Bovine serum albumin (0.5%W/V aqueous solution) and 0.05ml of test solution of various concentrations (100, 250, 500 µg/ml). Test control solution (0.5ml) consists of 0.45ml of bovine serum albumin (0.5%W/V aqueous solution) and 0.05ml of distilled water. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution. Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (0.5%w/v aqueous solution) and 0.05ml of Diclofenac sodium of various concentrations (100, 250, 100 µg/ml).

Procedure

0.05 ml various concentrations (100, 250,500 µg/ml) of test dugs and standard drug diclofenac sodium

(100, 250, 500 µg/ml) were taken respectively and 0.45 ml (0.5% w/V BSA) mixed. The samples were incubated at 37°c for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 255 nm. The control represents 100% protein denaturation. The results were compared with Diclofenac sodium. The percentage inhibition of with Diclofenac sodium[10,15].

The percentage inhibition of protein denaturation can be calculated as
% inhibition = 100 X (Vt / Vc - 1)
Where,
Vt = absorbance of test sample; Vc = absorbance of control

Egg Albumin Denaturation Method:

The reaction mixture (5 mL) consisted of 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate-buffered saline (PBS, pH 6.4) and 2 mL of varying concentrations (100, 250, 500 µg/mL) of drug. A similar volume of double-distilled water served as the control. Next, the mixtures were incubated at 37 ± 2°C in a BOD incubator for 15minutes and then heated at 70°C for five minutes. After cooling, their absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac sodium in the concentrations of 100, 250, 500 µg/mL was used as the reference drug and treated similarly for the determination of absorbance[19,20].

The percentage inhibition of protein denaturation was calculated by using the following formula:

% inhibition = 100 × [V t / V C - 1]
Where,
V t = absorbance of the test sample
V c = absorbance of control.

Antioxidant activity

The ability of the *Leucas aspera* extract to scavenge hydrogen peroxide was determined according to the method of [21,22]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (100 µg/mL) in distilled water were added to a hydrogen peroxide solution (0.6 mL, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide [23]. The percentage of hydrogen peroxide scavenging of both *Leucas aspera* extracts and standard compounds were calculated:

% Scavenged [H_2O_2] = [(AC – AS)/AC] x 100

Where
AC is the absorbance of the control
AS is the absorbance in the presence of the sample of *Leucas aspera* extracts or standards.

Antibacterial activity

Antibacterial activity of aqueous extract of *Leucas aspera* has been evaluated *in vitro* against *E. coli*, *Listeria* sp, *Staphylococcus aureus*, *Salmonella* sp, *Klebsiella* sp by the standard disc diffusion method. Sterilized disc were dipped in aqueous plant extract and placed in the Prepared Muller-Hinton agar plates which were seeded with the culture. Then, the plates were incubated for 18-24 hours at 37˚C. The assay was carried out in triplicate[24,25, 26]. The zone of inhibition was measured from the centre of disc to the clear zone in millimeter and the results were recorded.

Results and Discussions

Qualitative Phytochemical screening

The results of phytochemical screening were representated in Table (1)

Table (1): Results of Phytochemical analysis

S. No	Phytochemical Test	Reagents used	Interference	Result
1.	Alkaloid	Mayer' Reagent	Appearance of yellow cream ppt.	Positive
		Hager's Reagent	Formation of yellowish white ppt	Positive
2.	Carbohydrate Test	Molish's Reagent	Formation of Violet ring	Positive
		Benedict's Reagent	Formation of orange red ppt	Positive
		Fehling's solution	Formation of red ppt.	Positive
3.	Saponin test	Foam Test	Produce foam that lasts for more than 10 minutes	Positive
4.	Glycoside test	Modified Brontrager's Reagent	No formation of pink colour	Negative
5.	Phytosterol test	Salkowski's Test	Golden brown colour obtained	Positive
6.	Fats and Fixed oil test	Filter Paper press Test	No oily stain was obtained	Negative
7.	Resin test	Acetone Water Test	No Appearance of Turbidity	Negative
8.	Phenol test	Ferric Chloride Test	Appearance of bluish black ppt	Positive
9.	Tannin test	Gelatin Test	No formation of white ppt.	Negative
10.	Diterpenes test	Copper Acetate Test	No formation of bright green colour	Negative
11.	Flavonoids test	Alkaline Reagent Test	No intense yellow colour obtained	Positive
		Lead Acetate Test	Yellow ppt. obtained	Positive
12.	Proteins and amino acids test	Xanthoproteic Test	Formation of yellow colour	Positive

In-vitro Anti-inflammatory activity

The human red blood cell (HRBC) **membrane Stabilization method**

The investigation is based on the need for newer anti-inflammatory agents from natural source with potent activity and lesser side effects as substitutes for chemical therapeutics. *Leucas aspera* has significant anti-inflammatory activity which may be due to presence of chemical profile such as Flavones, Tri-Terpenoids, Flavonones and Phenols. The HRBC Membrane stabilization activity of the aqueous leaf extract of *Leucas aspera* at concentration 100µg/ml showed 73.25% inhibition of denaturation in hypotonic solution while the standard Diclofenac 100µg/ml showed 79.25% inhibition of denaturation. The crude extracts of the leaves of

Leucas aspera showed statistically significant anti-inflammatory activity in *in vitro* assay which were represented in Table (2). In a study[27], researchers revealed the significant *in vitro* membrane stabilizing effect of two Bangladeshi medicinal plants namely *Mesua nagassarium*, *Kigelia pinnata*, which indicates the anti-inflammatory activity of the medicinal plants. From *Persicariastagnina*[28], *Scoparia dulcis*[29], *Polygonum viscosum*[30] and *Sida cordifolia*[31]researchers isolated potent anti inflammarory compounds and tested using standard methods. The compounds were of sesquiterpene, diterpene, flavonoid glycoside and alkaloid types. In case of the rest of the medicinal plants the researchers conducted the anti-inflammatory study using the crude extracts and found significant activity.

Table (2): Anti-inflammatory Effect – HRBC Membrane Stabilization method.

S.no	Drug	Concentration (µg/ml)	% inhibition
1.	Control	-	-
2.	Plant Extract	25 50 75 100	30.25 51.75 64.05 73.25
3.	Diclofenac sodium	25 50 75 100	44.05 57.85 69.83 79.25

Anti helminthic activity

The aqueous extract of leaves of *Leucas aspera* samples, which were used to evaluate anthelmintic activity, showed variable times at different concentrations and the mean time values were calculated for each parameter. The crude aqueous leaves extract of *Leucas aspera* showed the significant anthelmintic effect causing death of the worm at all the concentrations but the time of death was different in each case (Fig.1). However, when observed the response of worms in case of paralysis, there was significant variation among the results produced by the extracts at different concentrations like 5, 25, 50 and 100 mg/ml. The results were tabulated below (Table (3)). Similar observations were made in the anthelmintic activity as well. The data presented in the table and observations made thereof, lead to the conclusion that the different degree of helminthiasis of the different extracts are due to the level of tannins present in compounds. Tannins, the secondary metabolite, occur in several plants have been reported to show anthelmintic property by several investigators[31, 32]. Tannins, the polyphenolic compounds, are shown to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation or, binds to the glycoprotein on the cuticle of parasite and cause death.

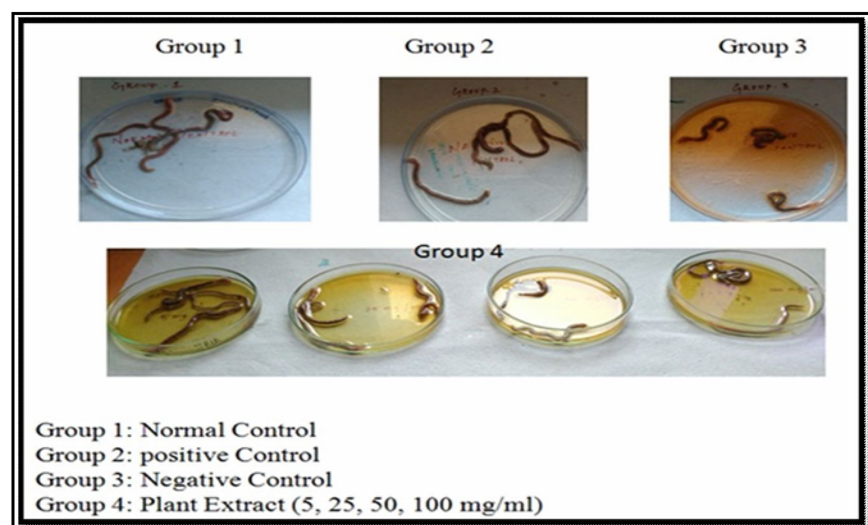

Fig. 1 Anti helminthic activity

Table (3) Antihelminthic activity

S.No	Treatment	Concentration (mg/ml)	Time taken for paralysis	Time taken for death
1.	Normal Control	-	-	-
2.	Positive Control	5	27	35
3.	Negative Control	-	-	-
4.	Plant Extract	5 25 50 100	64 59 43 34	70 63 54 42

Antiarthritic activity

Bovine Serum Denaturation Method

In *Invitro anti-arthritic activity by* Bovine Serum denaturation method at concentration of 100, 250 and 500 mcg/ml showed 62, 74 and 89 % inhibition of denaturation of bovine serum whereas, standard diclofenac at 100, 250 and 500 mcg/ml which showed 93.20, 95.41 and 96.91% inhibition of denaturation of bovine serum (Table 4).

Table (4): Anti-arthritic activity – BSA Denaturation

S. No	Drug	Concentration (µg/ml)	% inhibition
1.	Control	-	-
2.	Plant Extract	100 250 500	62 74 89
3.	Diclofenac sodium	100 250 500	93.20 95.41 96.91

Egg Albumin Denaturation Method

In *Invitro* anti-arthritic activity by Egg Albumin denaturation method at concentration of 100, 250 and 500 mcg/ml showed 75.00, 80.31 and 84.15% inhibition of Egg Albumin denaturation whereas, standard diclofenac at 100, 250 and 500 mcg/ml which showed 27.78, 45.84 and 69.77% inhibition of Egg Albumin denaturation.(Table (5)).

Table (5): Anti-arthritic activity – Egg Albumin Denaturation Method

S. No	Drug	Concentration (µg/ml)	% inhibition
1.	Control	-	-
2.	Plant Extract	100 250 500	75 80.31 84.15
3.	Diclofenac sodium	100 250 500	27.78 45.54 69.77

Some literature reported that denaturation of protein is one of the cause of rheumatoid arthritis is one of the cause of rheumatoid arthritis[33, 34]. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins. Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. Several anti-inflammatory drugs have shown dose dependent ability to inhibit thermally induced protein denaturation[35]

Antioxidant activity

Hydrogen Peroxide Scavenging Capacity

The scavenging ability of aqueous extracts of *Leucas aspera* on hydrogen peroxide is shown Table and compared with α-tocopherol as standards. The *Leucas aspera* extracts were capable of scavenging hydrogen peroxide in an amount dependent manner. 100 µg of water extracts of *Leucas aspera* exhibited 22% scavenging activity on hydrogen peroxide. On the other hand, using the same amount α- tocopherol exhibited 44.58% hydrogen peroxide scavenging activity. Results show that the scavenging activity values on hydrogen peroxide of 100 µg of the extracts of *Leucas aspera* decreases than that of α-tocopherol (Table (6)). Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells [36,37]. Thus, the removing of H_2O_2 is very important for antioxidant defense in cell or food systems.

Table (6): Hydrogen Per Oxide Scavenging activity

Samples	**% H_2O_2 Scavenging Activity (100 µg/mL)**
Control	0
α-tocopherol	44.58
Leucas aspera	22

Antibacterial activity

Plant extract (*Leucas aspera*) had tested for its ability to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp, *Listeria* sp, *Klebsiella* sp. Among the tested bacterial isolates, the plant extract showed maximum inhibition against *E. coli* followed by *Listeria* sp, *S.aureus*, *Salmonella* sp, *Klebsiella* sp. The zones are presented in Table (7). This result suggests that the Plant extract (*Leucas aspera*) have the potential against several bacteria species.

Table (7): Antibacterial activity – Disc Diffusion method

Microorganism	**Zone of inhibition (mm)**
E. coli	16
Listeria	11
Staphylococcus aureus	8
Salmonella	7
Klebsiella	7

Conclusion

The results of this study showed that the aqueous leaf extract exhibited anti-inflammatory, anti-arthritic, anti-bacterial, anti-helmintic activity and anti oxidant property. The presence of some bioactive phytochemical constituents in the leaves may be responsible for the observed effect. However, further studies are necessary in order to isolate and characterize the active compounds of *Leucas aspera* responsible for the above properties.

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