ORIGINAL ARTICLE

Evaluation of immunomodulatory activity of ethyl acetate extract of *Leucas aspera* in Swiss albino mice

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Background: *Leucas aspera* is a widely used ethno-medicinal plant for various diseases in India. In our recent research work, this plant showed powerful anti-cancer activity in various cancer cell lines by stimulating macrophage cells which plays a central role in the immune system. **Objective:** The present study was conducted to evaluate the immunomodulatory activity of ethyl acetate extract of aerial parts of *Leucas aspera* (EALA). **Materials and Methods:** In the current study, we have used neutrophil adhesion test, carbon clearance test, haemagglutinating antibody titre test, delayed-type hypersensitivity reaction test and cyclophosphamide-induced immunosuppression test. EALA 200 and 400 mg/kg oral dose were selected for the study after conducting the acute dose toxicity study. All the studies were performed in Swiss albino mice. **Results:** EALA showed a dose dependant increase in the neutrophil adhesion to the nylon fibres, produced a significant increase in the phagocytic index in carbon clearance test and a significant protection against cyclophosphamide-induced immunosuppression. Moreover both doses of EALA produced an increase in the circulating serum immunoglobulins in haemagglutinating antibody titre test along with an increase in the foot pad edema in delayed-type hypersensitivity reaction test. **Conclusion:** From the above findings it is concluded that EALA has the ability to modulate both humoral and cell-mediated immunity.

Key words: Cell-mediated immunity, humoral immunity, immunomodulation, *Leucas aspera*

INTRODUCTION

The immune system is known to be involved in the aetiology as well as pathophysiological mechanisms of many diseases.[1] Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host-defence mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy.[2] System of medicines like Ayurveda gives emphasis on promotion of health through strengthening host defences against different diseases.[3] Many plants used in traditional medicine possess immunomodulatory activities and they generally act by stimulating both specific and non-specific immunity.[4] Although many plants are reported for immunomodulatory activities a lot more are still to be explored.

Leucas aspera Willd belongs to the family Lamiaceae is widely distributed throughout India from the Himalayas down to Ceylon. It is an annual, branched, herb erecting to a height of 15-60 cm with stout and hispid acutely quadrangular stem and branches.[5] Ethno-medicinally this plant is used as antipyretic, stimulant, expectorant, aperient, diaphoretic and insecticide. Phytoconstituents like nicotine, sterols, diterpenes together with other compounds like asperphenamate, maslinic acid, isololiolide, linifolioside, nectandrin B, macelignan, acacetin, apigenin, chrysoeriol, apigenin, myristargenol B, machilin C and chicanine were already reported from this plant.[6] Literature survey reveals that extracts of *Leucas aspera* exhibit analgesic, anti-inflammatory, anti-arthritic and anti-pyretic efficacies.[7]

Recently we had checked the anti-cancer activity of *Leucas aspera* and found that the plant exhibits anti-cancer activity through stimulation of macrophage cells which is a key component of immune system. The present study was, therefore, undertaken to explore the

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preliminary phytochemical screening, acute toxicity studies and immunomodulatory activity of *Leucas aspera* aerial parts on cellular and humoral immune responses.

MATERIALS AND METHODS

Drugs and Chemicals

Alsever's solution, cyclophosphamide and carboxy methyl cellulose (CMC) were purchased from Sigma Aldrich. Indian ink (Himedia), WBC diluting fluid (Nice Chemicals) and all other reagents used were of analytical grade.

Plant Material

Aerial parts of *Leucas aspera* were collected from local areas of Guwahati, Assam, India in the month of January 2012. The plant material was authentified by Dr. G. C Sharma, Curator, Department of Botany, Gauhati University, Guwahati (voucher specimen no. 17701).

Preparation of the Extract

The collected plant parts were washed with water; shade dried in open air and pulverised using electric grinder. About 200 gm of *Leucas aspera* powder was packed into Soxhlet apparatus and subjected to hot continuous percolation using ethyl acetate as the solvent. The extract was filtered through Whatmans filter paper no. 40, evaporated using vacuum rotary evaporator (Buchi) and heated on water bath at 45 ± 5°C and stored in vacuum desiccator. The yield of ethyl acetate extract of aerial parts of *Leucas aspera* (EALA) obtained was 9.3% w/w.

Animals

Swiss albino mice (25-30 gm) and Wistar albino rats (150-200 gm) of either sex were used for present investigation and were obtained from Central Animal Facility, NIPER, Guwahati. Animals were housed under standard environmental conditions of temperature (25 ± 2°C) and light and dark cycle (12:12 h). Animals were fed with standard pellet diet and water *ad libitum*. All experimental studies were done after getting permission from the Institutional Animal Ethics Committee, Gauhati Medical College, Guwahati.

Antigen Preparation

Fresh sheep blood was collected from the local slaughterhouse in Alsever's solution and stored at 4-7°C in a refrigerator.[8] During the experimentation, adequate amount of stock solution of Sheep red blood cells (SRBCs) was taken and washed three times with pyrogen-free normal saline by centrifugation at 3000 × g for 10 min on each occasion. The settled SRBCs were then suspended in normal saline and concentration adjusted to 0.5×10^9 cells/ml.

Preliminary Phytochemical Investigation

Preliminary phytochemical screening of extract was performed using standard procedures and tests with little modifications.[9,10] Test for flavonoids: 1 ml of the extract was mixed with dilute NaOH; golden yellow precipitate confirmed the presence of flavonoids. Test for saponins: 1 ml of the extract was mixed with 10 ml of warm distilled water; formation of persistent foam indicated the presence of saponins. Test for glycosides: 1 ml of extract was mixed with 1 ml of water and 5-6 drops of 10% sodium hydroxide solution; a yellow colour confirms the presence of glycosides.

Acute Dose Toxicity Study

The acute toxicity study was carried out as per the OECD guidelines 425.[11] Initially EALA was administered orally at a limit dose of 2000 mg/kg to a single female rat. The rat was observed closely for the first 4 h and then periodically up to 24 h for any toxic symptoms and mortality. After 24 h same dose was administered to four more female rats.

Immunomodulatory Studies

Experimental Protocol

For all the studies except cyclophosphamide-induced immunosuppression, Swiss albino mice were divided in to three groups and each group contains six animals.

- Group 1: Control (0.5% CMC 10 ml/kg p.o.)
- Group 2: EALA 200 mg/kg p.o.
- Group 3: EALA 400 mg/kg p.o.

For cyclophosphamide-induced immunosuppression, experimental design is follows: Swiss albino mice were divided into four groups and each group contains six animals.

- Group 1: Control (0.5% CMC 10 ml/kg p.o.)
- Group 2: Cyclophosphamide 30 mg/kg i.p
- Group 3: Cyclophosphamide 30 mg/kg i.p. + EALA 200 mg/kg p.o.
- Group 4: Cyclophosphamide 30 mg/kg i.p. + EALA 400 mg/kg p.o.

0.5% CMC and distilled water were used as solvents for EALA and Cyclophosphamide, respectively.

Neutrophil Adhesion Test

The mice were pre-treated orally with vehicle or extracts for 14 days. On 14th day of drug treatment, blood samples were collected by puncturing retro-orbital plexus into heparinised vials and analyzed for total leukocyte cell (TLC) and differential leukocyte cell (DLC) counts. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and percent neutrophil gives neutrophil index of blood sample.[12] The percent neutrophil adhesion was calculated as shown below:

Neutrophil adhesion (%) = [(NIu − NIt)/NIu] ×100

where NIu is the Neutrophil index of untreated blood samples and NIt is the Neutrophil index of fibre-treated blood samples.

Carbon Clearance Test

Swiss albino mice were administered EALA and vehicle orally for 10 days according to the experimental protocol. Forty-eight hours after the last dose of the drug, animals of all the groups received intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) via the tail vein. Blood samples were withdrawn from each animal by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. A 50-µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm using UV Visible spectrophotometer (Thermo scientific).[13] The phagocytic index, K was calculated using the following formula:

$$K = (Log_e OD1 - Log_e OD2)/15$$

where OD1 and OD2 are the optical densities at 0 and 15 min, respectively.

Haemagglutinating Antibody (Ha) Titre

Mice of various groups were pre-treated with the EALA and vehicle for 14 days as described in the experimental protocol. On 14th day, all animals were immunised with 0.5 × 10^9 sheep red blood cells (SRBCs) i.p. The drug treatment was continued for 14 more days and blood samples were collected from each mouse at 21st and 27th days of the drug treatment to estimate the primary and secondary antibody titres respectively. The titre value was determined by titrating serum dilutions (50-100 µl) with SRBC (0.025 × 10^{9}cells) in microtiter plates. Equal volumes of individual serum samples of each group were pooled and two-fold serial dilution of pooled serum samples made in 25 µl of 1% v/v suspension of SRBCs in saline. After mixing thoroughly, microtiter plates were incubated at 37°C for 1 h and examined visually for agglutination. The minimum volume of serum showing haemagglutination was expressed as haemagglutination (HA) titre.[14]

Delayed-type Hypersensitivity Reactions

Swiss albino mice were treated with test drug and vehicle for 27 days as described in the experimental protocol. All the animals were immunised by i.p. administration of 0.5 × 10^9 SRBCs/mice on the 14th day and challenged by s.c. administration of 0.025 × 10^9 SRBCS/mice into right hind foot pad on 28th day. Paw oedema was measured at 24 and 48 h after SRBCs challenge using digital plathysmometer (Ugobasile, Italy).[15]

Cyclophosphamide-Induced Immunosuppression

Swiss albino mice were treated with test drug and vehicle for 10 days as described in the experimental protocol. After the drug treatment, groups 2, 3 and 4 were injected with Cyclophosphamide (30 mg/kg i.p.) on the 11th, 12th and 13th days. On day 14th, blood sample was collected from the retro-orbital plexus of individual animals and analyzed for haematological parameters using automated cell counter (Invitrogen).[16]

Statistical Analysis

Values were expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA (Graph pad prism version 6) followed by Dunnets *post-hoc* test and values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Preliminary Phytochemical Investigation

Preliminary phytochemical investigation of EALA showed the presence of alkaloids, glycosides, tannins and flavonoids.

Acute Dose Toxicity Study

The EALA did not show any toxic reactions and mortality up to a dose of 2000 mg/kg. No changes in food consumption, water intake or behaviour (tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma) were observed in the female rats after dose administration. Hence, EALA 200 mg/kg and 400 mg/kg were taken as treatment dose for the current study.

Immunomodulatory Studies

Neutrophil Adhesion Test

Incubation of blood with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. Pre-treatment with EALA 200 mg/kg and 400 mg/kg evoked a significant increase in the *in vitro* neutrophil adhesion to nylon fibres ($P < 0.01$ and $P < 0.001$, respectively) as compared to the control group [Table 1].

Carbon Clearance Test

The phagocytic activity of the reticulo-endothelium system was measured by the carbon clearance test. Both doses of EALA showed significant increase in phagocytic index as compared to the control animals ($P < 0.01$ and $P < 0.001$) [Table 2].

Table 1: Effects of EALA on neutrophil adhesion test

Treatment groups	Neutrophil index		Neutrophil adhesion (%)
	UB	FTB	
Control (0.5% CMC)	211.23±10.25	173.46±9.14	17.88±1.72
EALA 200 mg/kg	348.91±14.36	257.46±12.90	26.21±1.12^b
EALA 400 mg/kg	392.31±15.07	276.73±13.18	29.4 6±1.34^a

Values are expressed as mean±SEM (n=6). ^aP<0.001, ^bP<0.01 as compared to control group, CMC – Carboxy methyl cellulose; UB – Untreated blood; FTB – Fibre treated blood, EALA – Ethyl acetate extract of *L. aspera*

Haemagglutinating Antibody Titre

Both primary and secondary HA titre value was significantly increased in animals that received vaccination along with the EALA treatment as compared to animals that received vaccination alone ($P < 0.05$ and $P < 0.01$) [Table 3].

Delayed-Type Hypersensitivity Reactions

Paw volume was noted at 24 and 48 h after the antigen treatment. EALA treatment in both doses significantly increased the paw oedema as compared to the control group ($P < 0.01$ and $P < 0.001$) [Table 4].

Cyclophosphamide-Induced Immunosuppression

Cyclophosphamide administration caused a significant reduction in the RBCs, WBCs and platelets count. Pre-treatment with EALA (both doses) significantly prevented the myelosuppression as compared to cyclophosphamide-alone-treated mice ($P < 0.01$ and $P < 0.001$) [Table 5].

DISCUSSION

Immunomodulatory agents enhance the immune responsiveness of an organism against a pathogen by activating the immune system.[17] Many plant products used in traditional medicine have been reported to have immunomodulating activities. While some of these stimulate both humoral and cell-mediated immunity (CMI), others activate only the cellular components of the immune system, i.e. phagocytic function without affecting the humoral immunity.[18] In the current study, we found that EALA modulates both cellular and humoral immunity in experimental mice.

Acute dose toxicity study, in which the animals treated with the EALA at a higher dose of 2000 mg/kg, did not produce any significant toxicity signs, behavioural changes, body weight changes or macroscopic findings during observational period. So the LD_{50} of EALA should be more than 2000 mg/kg.

In the present study five different models, each of which provides information about effect on different components of the immune system was used. Neutrophil adhesion test is widely used to check the effect of various test drugs in cell-mediated immune reactions. The adhesion of neutrophil to nylon fibres indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of inflammation.[19] Both doses of EALA were found to enhance the adhesion of neutrophil in to the fibre. This might be due to the upregulation of the β2 integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres.[20]

Table 2: Effects of EALA on carbon clearance test

Treatment groups	Phagocytic index
Control (0.5% CMC)	0.0180±0.001
EALA 200 mg/kg	0.032±0.0027[b]
EALA 400 mg/kg	0.0481±0.0032[a]

Values are expressed as mean±SEM (n=6). [a]P<0.001, [b]P<0.01 as compared to control group, EALA – Ethyl acetate extract of *L. aspera*; CMC – Carboxy methyl cellulose

Table 3: Effects of EALA on haemagglutinating antibody titre

Treatment groups	Mean haemagglutinating antibody titre	
	1^0 HA titre	2^0 HA titre
Control (0.5% CMC)	4.26±0.27	7.31±0.46
EALA 200 mg/kg	5.14±0.39	9.02±0.49[c]
EALA 400 mg/kg	6.11±0.32[b]	9.86±0.51[b]

Values are expressed as mean±SEM (n=6). [b]P<0.01, [c]P<0.05 as compared to control group, HA – Haemagglutinating antibody; EALA – Ethyl acetate extract of *L. aspera*; CMC – Carboxy methyl cellulose

Table 4: Effects of EALA on delayed-type hypersensitivity in antigenically challenged mice

Treatment groups	Mean % increase in paw volume	
	24 h	48 h
Control (0.5% CMC)	23.14±2.01	19.63±1.67
EALA 200 mg/kg	36.07±2.96[b]	32.38±2.51[b]
EALA 400 mg/kg	42.34±3.12[a]	37.14±3.07[a]

Values are expressed as mean±SEM (n=6). [a]P<0.001, [b]P<0.01 as compared to control group, EALA – Ethyl acetate extract of *L. aspera*; CMC – Carboxy methyl cellulose

Table 5: Effects of EALA on cyclophosphamide-induced immunosuppression

Treatment groups	Count (cells/mm^3)		
	RBC (×10^6)	WBC (×10^3)	Platelet (×10^3)
Control (0.5% CMC)	6.01±0.24	11.24±0.84	550.37±5.67
Cyclophosphamide 30 mg/kg	3.51±0.14	6.85±0.4	347.61±4.78
Cyclophosphamide 30 mg/kg+EALA 200 mg/kg	5.43±0.32[b]	9.07±0.53[b]	475.32±5.49[a]
Cyclophosphamide 30 mg/kg+EALA 400 mg/kg	6.17±0.34[a]	10.49±0.41[a]	539.14±5.86[a]

Values are expressed as mean±SEM (n=6). [a]P<0.001, [b]P<0.01 as compared to cyclophosphamide group, EALA – Ethyl acetate extract of *L. aspera*; CMC – Carboxy methyl cellulose; RBC – Red blood cell; WBC – White blood cell

The effect of EALA on the reticulo endothelial system (RES) was evaluated using the carbon clearance test. RES mainly consists of phagocytic cells (macrophages), which specialised in the removal of foreign substances from the blood stream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation.[13] Since both doses of EALA augmented the phagocytic index, it can conclude that RES was activated by *Leucas aspera* extract.

The HA titre test was performed to study the effect of EALA on the humoral immune system. Antibody molecules, a product of B-lymphocytes and plasma cells, are central to

humoral immune responses; IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonisation, neutralisation of toxins, etc.[21] The results of HA titre test showed that pre-treatment with both doses of EALA significantly increased the circulating antibodies. So from this model it is clear that EALA stimulates humoral immune system also.

CMI responses are critical to defence against infectious organisms, infection of foreign grafts, tumour immunity and DTH reactions.[21] In the current study DTH model was used to evaluate the effect of EALA in CMI reaction. Therefore, increase in paw oedema in mice in response to T-cell-dependent antigen revealed the stimulatory effect of EALA in CMI.

The immunomodulatory effect of EALA was also checked in cyclophosphamide-induced myelosuppression animal model. Cyclophosphamide is a nitrogen mustard subclass alkylating agent and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. The results showed that cyclophosphamide 30 mg/kg lowered the RBCs, platelets and total WBCs counts in the cyclophosphamide-alone-treated group. Interestingly, pre-treatment with EALA prevented the changes in haematological parameters. The prevention of myelosuppression induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin-1.[22,23]

From the phytochemical analysis we can suggest that EALA exerts immunomodulatory activity through the combined action of tannins, alkaloids, glycosides and flavonoids. Tannins are known to possess immunostimulating activities. The well-known Ayurvedic formulation, Triphala contains *Terminalia chebula*, *Terminalia belerica* and *Emblica officinalis*, which are rich in tannins reported for immunostimulating activity.[20]

CONCLUSIONS

The results of the present investigation showed that EALA is a potent immunostimulant, stimulating both the specific and non-specific immune mechanisms. Further studies are required to elucidate the exact mechanism of immunomodulatory activity of *Leucas aspera*.

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