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A STUDY ON THE IMMUNOMODULATORY ACTIVITY OF THE LEAF EXTRACTS OF *CASSIA AURICULATA* LINN (*CEASALPINIACEA*) IN RATS

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ABSTRACT

The present study was undertaken to evaluate immunomodulatory activity of aqueous and alcoholic extracts of leaves of *C. auriculata*. Albino rats of either sex were divided into 14 groups (6 animals each). Aqueous and alcoholic extracts of *C. auriculata* leaves were administered orally at a dose of 100, 200, 400 mg/kg body weight to healthy rats and levamisole, 2.5mg/kg was used as a standard drug. The assessment of immunomodulatory activity was carried out by testing, Delayed type hypersensitivity and Haemagglutination antibody titre and Neutrophil adhesion test. Oral administration of the extracts significantly increased in cell mediated immunity and humoral immunity by facilitating the foot pad volume in response to the sheep RBCs in sensitized rats and significant increase in circulating antibody titre and significant increase in neutrophil adhesion to nylon fibres respectively. Hence it was concluded that *C. auriculata* increases both cell mediated and humoral immunity.

KEYWORDS: Levamisole, Delayed type hypersensitivity, Neutrophil adhesion test, Haemagglutination antibody titre, Cell mediated immunity, Humoral immunity.

INTRODUCTION

Our environment contains a great variety of infectious microbes such as viruses, bacteria, fungi, protozoa and multi cellular parasites. These can cause diseases and if they multiply unchecked will eventually kill their hosts.[1] The immune system is a remarkably adaptive defence system, which is able to generate a variety of cells and molecules capable of specifically recognising and eliminating a variety of limitless foreign invaders from the system.[2] The immune system is involved in the etiology as well as pathophysiological mechanisms of many diseases like AIDS, autoimmune disorder and many infections, and main cause being the lack of immunity.

There are many researches studies going on for development of drugs to prevent immunosuppression and modulation of immune system for particular diseases.[3,4] The coordinated reaction of the immune system against infections is known as the immune response.[5] Immunomodulator is defined as biological or synthetic substance that can stimulate or suppress either innate or adaptive or both arms of the immune system. Immunomodulators modulate and potentiate the weapons of the immune system keeping them in a highly prepared state for any threat it may encounter, many proteins, amino acids, and natural compounds have shown a significant ability to regulate immune responses, including interferon-γ (IFN-γ), steroids, these are biological or synthetic substances, which can stimulate, suppress or modulate any of the immune system including both adaptive and innate arms of the immune response.[6,7] Immunostimulants are substances which enhance the humoral and cellular immune response both by specific and non -specific way there by reducing the risk of diseases. In healthy individuals, the Immunostimulants serve as prophylactic and promoter agents, i.e., as immunopotentiators by enhancing the basic level of immune response. In the individual with impairment of immune response, they act as immunotherapeutic agents.[8] Immunosupressants are agents which suppress the immune system and are used for the control of pathological immune response in autoimmune disease, graft rejection etc.[9] Immunoadjuvants are used to enhance the efficacy of vaccines and therefore could be considered specific immune stimulants, thus hold the promise of being the true modulators of the immune response.[10] The plant *Cassia. auriculata* contains chemical constituents tannins, β-sitosterol, leucoanthocyanin (4', 5, 7-trihydroxyflavone-3, 4-diol), auriculacidin (5, 2, 4'-trihydroxy flavon-3, 4-diol. Leaves consists of carbohydrates, flavonoids, anthracene derivatives, rutin ketoalcohols and emodin. Flowers consists of β-sitosterol, kaempferol, flavonoids, phenolic acids, steroids, terpenoids tannins and anthocyanins. Bark consists of tannins[11,12] and these chemical constituents extracted from other medicinal plants have reported for their immunomodulatory activity. The plant described for its anthelmintic[13], anti-inflammatory[14],

antibacterial[15], antihyperglycemic[16], renoprotectivity[17], wound healing[18] and antioxidant activity.[19] activities which are good candidates for screening of immunomodulatory activity.

MATERIALS AND METHODS

Chemicals: Levamisole (Khandelwal laboratories Mumbai, India) Ethanol, (Nice, Cochin, India) Leishman stain I and II (Sd. fine chem. Ltd. Boisar, India) and Anaesthetic ether (TKM Pharma-Hyderabad, India).

Antigen: Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBC) were washed three times in large volumes of Alsever's solution and adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenge.

Collection of plant material: The leaves of *C. auriculata* were collected in Raichur Dist. Karnataka, India, during July 2016.

Preparation of AQELCA and AELCA extracts[20]

1. Preparation of aqueous extract

The leaf powder was defatted with petroleum ether (1:4) to remove chlorophyll and fatty material. Then about 100 g of dried powder was taken in a round bottom flask (2000 ml) and macerated with 500 ml of distilled water with 10 ml of chloroform (Preservative) for 7 days with occasional shaking for every hour in a closed vessel. Then the marc was removed by filtering the extract and then it was concentrated on a water bath maintained at < 50° C to get aqueous (AQELCA) extract.

2. Preparation of alcoholic extract

The leaf powder was defatted with petroleum ether (1:4) to remove chlorophyll and fatty material. The dried leaf powder was packed in a soxhlet apparatus and extracted with 95% alcohol for 18 h. Appearance of colourless solvent in the siphon tube was taken as the termination of extraction. The extract was then transferred into a previously weighed empty beaker and evaporated to a thick paste on the water bath, maintained at < 50° C to get alcoholic (AELCA) extract. The extract was finally air dried thoroughly to remove all traces of the solvent Aqueous and alcoholic extracts of *C. auriculata* were subjected to Preliminary phytochemical screening.

Experimental animals

Albino rats (Wistar strain) of either sex weighing between 150-200 g All the animals were acquired from Sri. Venkateswara Enterprises, Bengaluru for experimental purpose. After that, all the animals were made accustomed to lab conditions for 7 days, housed in groups of six under standard husbandry conditions like room temperature 26 ± 2^0C, relative humidity 45-55% and light/ dark cycle of 12:12 h.

All the animals were nourished with synthetic standard diet (Amrut Laboratories, Pranava Agro Industries Ltd., Sangli. Maharashtra) and water was supplied *ad libitum* under strict hygienic conditions. After obtaining authorization from Institutional Animal Ethical Committee (IAEC) of V. L. College of Pharmacy, Raichur (Karnataka), animal studies were performed as per rules and regulations in agreement to the guidelines of CPCSEA with registration number 557/02/c/CPCSEA.

Determination of LD_{50} of AQELCA and AELCA

The acute toxicity of all the four extracts was determined by using albino mice of either sex (18-22 g) those kept under standard husbandry conditions. The animals were fasted 3 h prior to the experiment**.** Animals were administered with single dose of extract and mortality was observed for a period of 48 h (short term toxicity). Based on short-term toxicity profile of extract the dose for the next animals was determined as per as OECD guideline No. 420.

Neutrophil adhesion test[21]

Albino rats weighing between 150-200 g and each group comprising six animals were divided into 8groups. The control group I received 0.5 ml of 1% gum acacia, the standard group II received levamisole 2.5 mg/kg, while animals of treatment groups received AQELCA and AELCA extracts at a dose of 100, 200, 400 mg/kg/day p.o daily for 14 days. On the 14th day i.e., after drug treatment, under mild ether anaesthesia blood samples from all the groups were collected by puncturing the retro-orbital plexus. Blood was collected into vials pre-treated with disodium EDTA. Blood samples were analysed for total leukocyte count (TLC) and differential leukocyte count (DLC), by fixing blood smears and staining with Field stain I and Leishman's stain. After the initial counts, blood samples were incubated with nylon fibre (80 mg/ml of blood sample) for 15 min at 37°C. The incubated blood samples were again analysed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percent of neutrophil adhesion was calculated as follo**ws.**

$$\% \text{ Neutrophil adhesion} = \frac{NIu - NIt}{NIu} \times 100$$

Where

NIu = Neutrophil index of Untreated blood sample.

Nit = Neutrophil index of Treated blood sample.

Delayed type hypersensitivity[22]

Six animals per group Control and treated were immunized on day 0 by i.p. administration of 0.1 ml of 20% v/v SRBC and challenged by subcutaneous administration of 0.1ml of 1% SRBC/ml in to right hind foot pad on day 14. The AQELCA, and AELCA was administered orally from day-14 until day +13. DTH responses were measured at 24 h after SRBC challenged on day +14 and expressed as mean percent increase in paw volume plethysmometrically.

Haemagglutination antibody titre[23]

Albino rats weighing between 150-200 g and each group comprising six animals were divided into 8 groups. The control group I received 0.5 ml of 1% gum acacia, the standard group II received levamisole 2.5 mg/kg, while animals of treatment groups received AQELCA, and AELCA extracts at a dose of 100, 200, 400 mg/kg/day p.o daily for 21 days. On 7th and 14th days of study rats from all the groups were immunized and challenged respectively, with 0.1 ml of 20% sheep red blood cells (SRBC) in normal saline, intraperitoneally. Blood was withdrawn from all animals on the 21th day, from the retro-orbital plexus, under mild ether anaesthesia, and centrifuged to obtain the serum. The antibody titre was determined using Microtitre plates. Each well of a micro titre plate was filled initially with 25 µl of normal saline and 25 µl of serum was mixed with 25 µl of normal saline in the first well of the micro titre plate. Subsequently the 25 µl diluted serum was removed from the first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were carried out till the last well of the second row (twenty-first well), so that the antibody concentration of any of the dilutions is half of the previous dilution. 25 µl of 1% SRBC was added to each well and the micro titre plates were incubated at 37°C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre.

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The values are expressed as mean±SEM and $P<0.05$ was considered significant.

RESULTS

Neutrophil adhesion test: When blood samples were incubated with nylon fibres, a reduction in neutrophil percentage due to the adhesion of neutrophils to the nylon fibres was observed. Levamisole showed significant increase in neutrophil adhesion with the experimental study (fig1,2&3). The AQELCA showed significant increase in neutrophil adhesion in medium ($p<0.05$) and high ($p<0.01$) doses but not with low dose, whereas the AELCA showed significant increase in neutrophil adhesion only at high ($p<0.01$) dose but not with low and medium doses when compared to control group (Table 1).

Delayed type hypersensitivity: DTH response was measured by change in foot pad volume. An increase in DTH response indicates the stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction. The standard levamisole showed a significant increase in foot pad volume (Fig 4). Medium ($p<0.01$) and high ($p< 0.001$) doses of AQELCA and AELCA showed a significant increase in paw volume except the low dose when compared to control. (Table 2).

Haemagglutination antibody titre: Levamisole showed a significant increase in HA titre AQELCA showed a significant increase in HA titre only at high ($p<0.001$) dose but not with low and medium (Fig 5). AELCA showed a significant increase in HA titre with both medium ($P<0.05$) and high ($P<0.001$) doses but not with the low dose when compared to control. (Table 3).

Table I: Effect of Standard (Levamisole), AQELCA and AELCA on neutrophil adhesion test in rats (mean±SEM).

ANIMALS	TLC X 10^3		DLC		NEUTROPHIL INDEX		% NEUTROPHIL ADHESION
	Untreated	Treated	Untreated	Treated	Untreated	Treated	
Control	8.62 ± 0.19	8.10 ±0.21	37.50 ±0.92	32.17 ± 0.87	323.00 ± 9.89	260.20 ±7.84	19.39 ± 1.27
Standard	11.95 ± 0.44	10.37 ± 0.34	54.83 ± 1.89	42.50 ± 1.89	655.7 ±36.01	440.0 ±23.50	32.76 ± 1.66***
AQELCA 100 mg/kg	9.17 ± 0.30	8.48 ±0.28	36.17 ± 0.65	31.17 ± 0.40	331.9 ± 14.28	264.80 ±11.12	20.18 ±0.96
AQELCA 200 mg/kg	10.15 ± 0.26	9.22 ±0.29	41.83 ± 1.20	34.00 ± .27	424.60 ± 15.57	313.40 ±15.30	26.16 ± 2.36 *
AQELCA 400 mg/kg	10.68 ± 0.20	9.53 ±0.16	48.00 ± 1.16	38.00 ±1 .29	513.80 ± 21.57	362.70 ±15.90	29.41 ± 1.17**
AELCA 100 mg/kg	8.93 ± 0.29	8.23 ±0.17	37.50 ± 0.67	32.67 ± 0.71	334.90 ± 11.74	268.70 ±6.12	19.56 ± 1.36[ns]
AELCA 200 mg/kg	10.03 ± 0.17	9.08 ±0.24	42.17 ± 1.45	35.00 ± 0.82	423.90 ± 19.83	317.90 ±11.04	24.75 ± 1.23 [ns]
AELCA 400 mg/kg	11.10 ± 0.11	9.82 ±0.14	49.33 ± 0.71	40.50 ± 1.12	547.20 ± 3.64	397.10 ±8.44	27.42 ± 1.64**

n = 6, Significant at *$P<0.05$, ** $P<0.01$, *** $P<0.001$, ns = not significant
AQELCA= Aqueous extract of leaves of *C. auriculata*, AELCA= Alcoholic extract of leaves of *C. auriculata.*

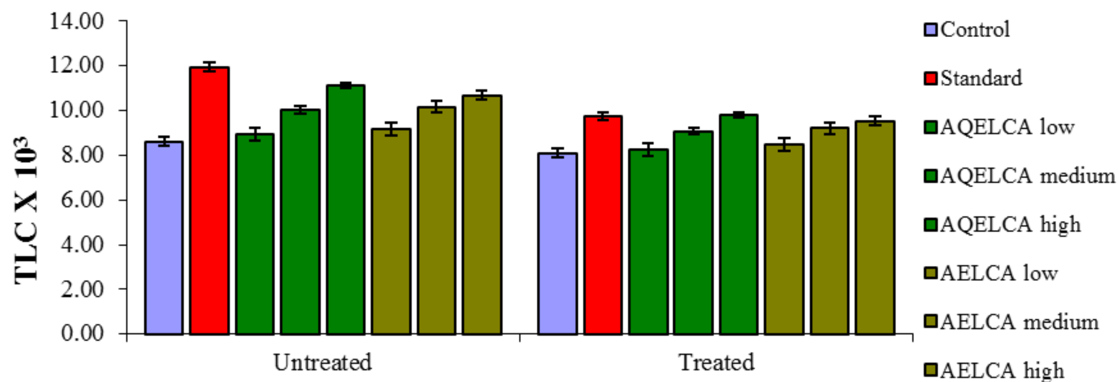

Figure 1: Effect of Levamisole, AQELCA, AELCA on TLC in Neutrophil adhesion test in rats (mean±SEM).

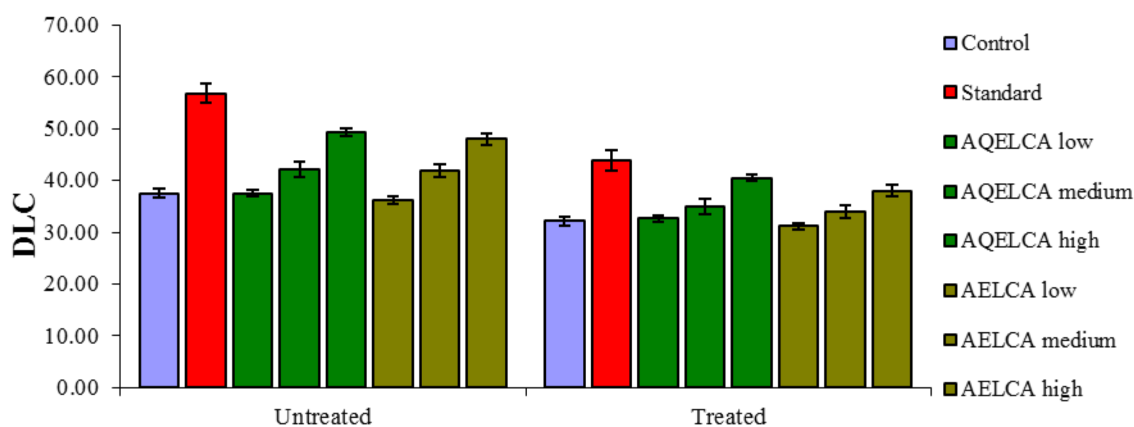

Figure 2: Effect of Levamisole, AQELCA, AELCA on DLC in Neutrophil adhesion test in rats (mean±SEM).

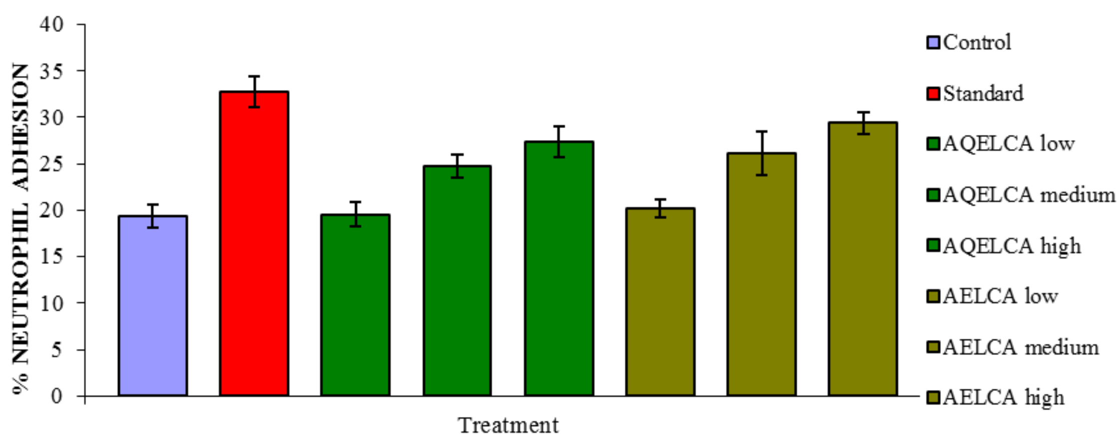

Figure 3: Effect of Levamisole, AQELCA, AELCA on % Neutrophil adhesion test in rats (mean±SEM).

Table II: Effect of Levamisole, AQELCA, AELCA, on foot pad volume in Delayed type hypersensitivity response in rats (mean±SEM).

Groups	Foot pad volume (ml)		% increase in foot pad volume
	0 h	24 h	
Control	0.61±0.04	0.71±0.04	14.26±0.90
Standard	0.68±0.04	1.23±0.04	44.75±2.75***
AQELCA 100 mg/kg	0.58±0.06	0.70±0.06	16.99±2.06ns
AQELCA 200 mg/kg	0.68±0.04	0.95±0.05	28.06±2.98**
AQELCA 400 mg/kg	0.65±0.05	1.06±0.07	38.92±3.24***
AELCA 100 mg/kg	0.68±0.04	0.79±0.04	13.91±3.01ns
AELCA 200 mg/kg	0.65±0.05	0.98±0.05	34.36±2.69***
AELCA 400 mg/kg	0.63±0.04	1.05±0.05	40.04±1.82***

n = 6, Significant at **P*<0.05, ** *P*<0.01, *** *P*<0.001 & ns = not significant
AQELCA= Aqueous extract of leaves of *C. auriculata*, AELCA= Alcoholic extract of leaves of *C. auriculata.*

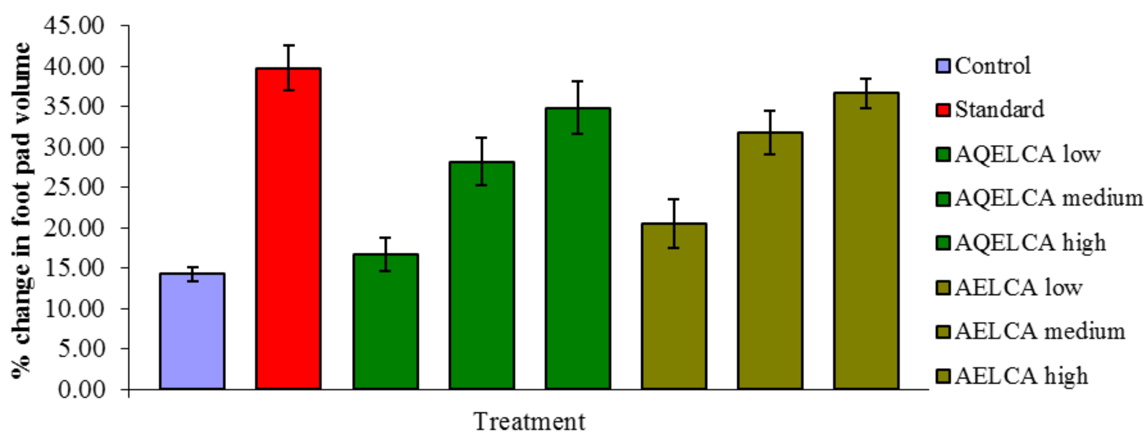

Figure 4: Effect of Levamisole, AQELCA, and AELCA on foot pad volume in Delayed type hypersensitivity response in rats (mean±SEM).

Table III: Effect of Levamisole, AQELCA and AELCA in haemagglutination antibody titre in rats (mean±SEM).

Groups	HA titre
Control	4.17 ±0.48
Standard	11.67 ±0.61***
AQELCA 100 mg/kg	4.33 ±0.49ns
AQELCA 200 mg/kg	6.17 ±0.60 ns
AQELCA 400 mg/kg	10.00 ±0.73***
AELCA 100 mg/kg	5.00 ±0.58 ns
AELCA 200 mg/kg	6.83 ±0.60 *
AELCA 400 mg/kg	9.50 ±0.43***

n = 6, Significant at **P*<0.05, ** *P*<0.01, *** *P*<0.001 ns = not significant
AQELCA= Aqueous extract of leaves of *C. auriculata*, AELCA= Alcoholic extract of leaves of C. *auriculata.*

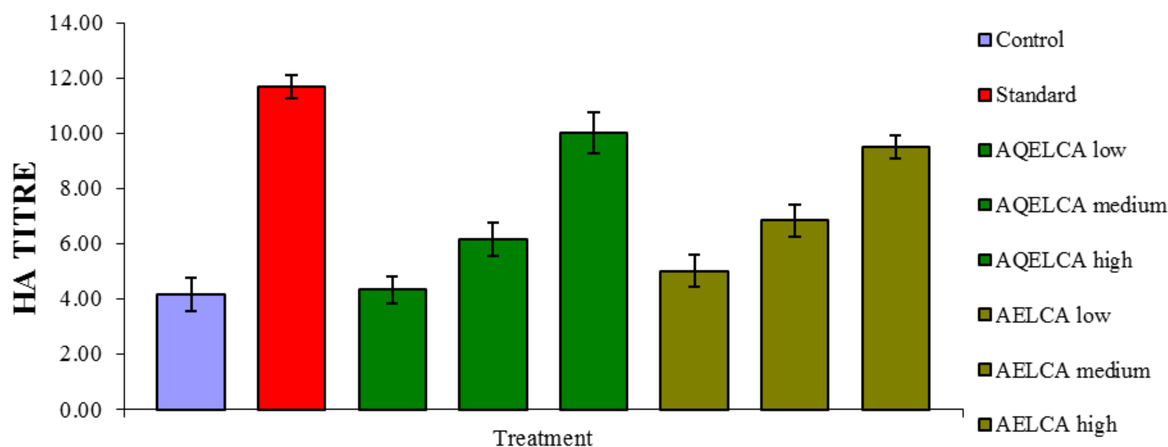

Figure 5: Effect of Levamisole, AQELCA, AELCA in haemagglutination antibody titre in rats (mean±SEM).

DISCUSSION

The results of the present study suggests that leaf extracts of the plant *C. auriculata* may stimulate cell mediated immunity as shown by increase in neutrophil adhesion to nylon fibres and increase in foot pad volume in response to the sheep RBCs in sensitized rats. It also stimulates humoral immunity indicated by increase in serum immunoglobulin levels.

In neutrophil adhesion test, adhesion of neutrophils to the nylon fibres describes the migration of polymorphonuclear lymphocytes, macrophages through the blood vessel walls reaching the site of inflammation. *C. auriculata* showed a significant increase in neutrophil adhesion to nylon fibres this might be due to the up regulation of the β-2 integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres.[3]

Delayed type hypersensitivity reaction has been widely used as one of the parameter to measure cell mediated immune response of the animal, DTH is an antigen specific and causes erythema and induction at the site of antigen infection in immunized animals, further T-cells are required to initiate the reaction the general characteristics are an influx of immune cells, macrophages and basophils at the site of injection and induction becomes apparent within 24–72 h.[24,25] medium and high doses of all the four extracts of *C. auriculata* showed a significant increase in paw volume due to the activation of T lymphocytes which subsequently proliferate and release inflammatory mediators including cytokines and histamines. These in turn increase the vascular permeability, induce vasodilatation, macrophage accumulation.[21]

Haemagglutination antibody titre assay is one of the key parameter used to assess the humoral immune response of the animal. When animal hosts are non- intravenously sensitized with sheep red blood cells (SRBC), this antigen diffuses into the lymph nodes where macrophages phagocytose the antigen, process it for presentation and becomes antigen presenting cells (APC). Once the antigen has been fragmented and processed, helper TH2 T- lymphocytes interact to stimulate the B- lymphocytes to produce antibodies against the RBC. 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 complement activation, opsonisation and neutralization of toxins etc.[26] In the present study the plant *C. auriculata* showed increase in antibody titre by conduction haemagglutination antibody titre which involves preparation of serum samples and addition of constant amount of SRBC. If the serum contains antibodies to the SRBC there will be agglutination because of the formation of antibody bridges with the neighbouring erythrocytes and these settle at the bottom as latex. Unagglutinated red blood cells appper at the bottom of the well as a button.[27]

CONCLUSION

The preliminary phytochemical investigation of leaf extracts i.e. AQELCA, AELCA were noted with carbohydrates, proteins, amino acids, steroids, tannins, triterpenes, flavonoids, saponins and glycosides. No mortality or behavioural abnormality was recorded in mice at the highest dose level tested i.e. 2000 mg/kg.

The immunomodulatory activity of leaf extracts were evaluated in neutrophil adhesion test, haemagglutination antibody titre and cell mediated immunity in rats. Standard Levamisole showed a significant immunomodulatory activity in selected models. From the studies it can be concluded that AQELCA and AELCA showed a significant immunomodulatory activity in neutrophil adhesion test only at high dose by enhancing the neutrophil and leukocyte counts i.e. it shows a significant immunomodulatory activity on non-specific immune response.

In haemagglutination antibody titre AELCA showed a significant increase in HAT at both medium and high

doses whereas AQELCA showed a significant increase in HAT with high dose only. This shows the two extracts exhibited significant immunomodulatory activity on humoral immune response.

In Delayed type hypersensitivity response extracts AQELCA and AELCA showed a significant increase in foot pad volume at medium and high doses only. An increase in DTH response indicates a stimulatory effect on cell-mediated immunity.

Phytochemical constituents like flavonoids, steroids, triterpenes, saponins, carbohydrates are already reported for their immunomodulatory activity and these constituents are present in the above two extracts. Hence these chemical constituents can be accounted for the observed immunomodulatory activity in different experimental models used in the study. So leaf extracts of *C. auriculata* were found to exhibit a significant immunomodulatory activity on both humoral and cell-mediated immunity in experimental animal models.

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