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Screening of *Limonia acidissima* fruit pulp for Immunomodulatory activity

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ABSTRACT

The present study was aimed at investigating the immunomodulatory activity of *Limonia acidissima* fruit pulp. The dried fruit pulp was subjected to maceration with methanol. The extract was investigated for immunomodulatory activity in Swiss albino rats and mice using standard methods. The haemagglutinating antibody titre was used to assess humoral immune response. The difference between the pre and post challenge foot thickness expressed in milliliter was taken as a measure of delayed type hypersensitivity. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Newman-Keul multiple comparison test. The obtained values revealed that the extract showed significant immunostimulant properties at a dose of 400mg/kg body weight when compared to that of the standard drug, Levamisole and gum acacia in water was taken as control and vehicle for the extracts.

Keywords: *Limonia acidissima*, Immunomodulatory activity, Carbon clearance test, Delayed type hypersensitivity, Haemagglutinating titre, Levamisole, Acute toxicity.

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INTRODUCTION

Immunomodulation

In many of the diseased conditions, immune response is impaired. Hence to maintain a disease-free state, modulation of immune response by either its stimulation or suppression can be a helpful therapy. This phenomenon is termed as immunomodulation. Immunomodulators are often capable of stimulating or suppressing both the humoral and cellular immune system.

The plant *Limonia acidissima* belonging to the family Rutaceae is a small to medium-sized deciduous tree [1-2]. It is bestowed with many medicinal uses in traditional systems of medicine. Leaves are aromatic and astringent, an oil of leaves useful in relieving itching and when mixed with a pinch of black pepper used as a carminative. Fruits are useful as tonic [3], antiscorbutic, alexipharmic, astringent, stomachic and stimulant. Fruit pulp and powdered rind are externally useful for the bites of venomous insects and reptiles. Previously published scientific reports proven that the plant was reported to possess a new tyramine derivative, acidissimin from the fruits and coumarins bergapten, psoralen, xanthotoxin and ostheno in the root bark, antifungal compounds in the stem bark, root bark and unripe fruit-shells [4-5].

The review of the scientific literature did not reveal any information on the immunomodulatory studies of this plant. In this investigation, an attempt was made to assess the efficacy of this indigenous plant for its immunomodulatory activity by the haemagglutinating antibody titre used to assess humoral immune response. The difference between the pre and post challenge foot thickness expressed in milliliter was taken as a measure of delayed type hypersensitivity in experimental animals using standard methods.

MATERIALS AND METHODS

Levamisole was obtained from Khandelwal Laboratories, Mumbai. The solvents used were of Laboratory grade obtained from EMerck Ltd., Mumbai. Gum acacia

Collection of Plant Material

The fruits of *Limonia acidissima* were collected from the Forest Reserve of Karimnagar after identification by a taxonomist. A voucher specimen is preserved in the laboratory herbarium. The collected plant material was thoroughly checked and freed from foreign matter.

Preparation of the Extract

The fresh fruit pulp of *Limonia acidissima* was macerated with methanol for seven days. The obtained solvent extract was concentrated in vacuo using rotary vacuum evaporator and dried in desiccators.

Animals

The healthy Swiss albino mice of either sex, approximately the same age, weighing between 18-25 g and Swiss albino rats of either sex, approximately the same age and weighing between 180-250 g used for the study were obtained from Mahaveer Enterprises, Hyderabad. They were fed with standard chow diet and water ad libitum. The animals were housed in polypropylene cages maintained under standard environmental conditions (12h light/12h dark cycle; 25±3°C, 35-60% relative humidity). The animals were treated strictly according to the CPCSEA guidelines and the study was conducted after obtaining permission from Institutional Animal Ethics Committee (IAEC).

Acute Toxicity and Gross Behavioral Study

The rats and mice, each were divided into groups (n=6) and were orally fed with increasing doses (100, 200, 400 and 600mg/kg body weight) of methanolic extract suspended in Gum acacia in water. After administration of the extract, the animals were observed during first 2h for their gross behavioral changes and once in 30 min for next 4h and then once in 24h for next 72h to find out percentage mortality [6-8].

Evaluation of Extract

Carbon Clearance Test

The phagocytic activity of the extract was assessed by the method previously described by Biozzi *et al* [9]. For the assessment of immunomodulatory activity, the rate of removal of gelatin stabilized carbon particles from the blood circulation was determined. The animals were divided into five groups with each group containing six animals. Among the five groups of animals, Group I received G (control, vehicle for the extracts) and Group II received the standard diuretic drug Furosemide at 20 mg/kg body weight. Each proposed extract was studied at two concentrations. Groups III to VIII received petroleum ether (250 and 500 mg/kg body weight), methanol (250 and 500 mg/kg body weight) and aqueous (250 and 500 mg/kg body weight)

25µl sample was mixed with 0.1% sodium carbonate solution (2ml) and the absorbance was measured at 660nm taking 0.1% sodium carbonate solution as blank and the rate of removal of the carbon clearance was calculated using the following equation.

$$\text{Carbon clearance} = \frac{\text{LogOD}_1 - \text{logOD}_2}{T_2 - T_1}$$

where OD₁ and OD₂ are the optical densities at T₁ and T₂ respectively.
T₁ - 2min; T₂ - 15min

Delayed Type Hypersensitivity (DTH) Response

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their products (lymphokines). The cell mediated immune response was assessed by DTH reaction, i.e. Footpad reaction. [10]

Humoral Antibody (HA) Titre

Blood samples were collected in micro centrifuge tubes from individual animal by retro orbital puncture on day 7. Antibody levels were determined by the haemagglutination technique¹¹⁻¹². Briefly, equal volumes of individual serum samples of each group were pooled. To serial two fold dilutions of pooled serum samples made in 25µl volume of normal saline, in U-bottomed micro titration plates were added 25µl of freshly prepared 1% suspension of SRBCs in saline. After mixing, the plates were incubated at 37°C for 2h and examined visually for agglutination. The reciprocal of the highest dilution of the test serum causing visible haemagglutination was taken as the antibody titre.

RESULTS

Carbon Clearance Test

The phagocytic activity of tissue macrophages of the reticulo-endothelial system (RES) is determined. The maximum carbon clearance was observed with the 500 mg/kg of the extract (Refer table 1). The extract effects were compared with the standard drug, Levamisole-50 mg/kg.

Table1: Effect of methanolic extract of fruit pulp of *Limonia acidissima* phagocytic response.

Group	Treatment	Dose(mg/kg) p.o for 7days	Phagocytic response (Mean±SD)
I	Control	-	0.021±0.04
II	Levamisole	50	0.023±0.02
III	Extract	100	0.024±0.02
IV	Extract	200	0.026±0.03
V	Extract	400	0.029±0.04***

(n=6) *P<0.05(Compared to control)

DTH Response

The extract produced a significant dose related increase in DTH reaction in rats (Refer table 2). The maximum effect was observed with the 500 mg/kg of OME. The effects were compared with standard drug.

Table2: Effect of methanolic extract of fruit pulp of *Limonia acidissima* on HA titre and DTH response.

Group	Treatment	Dose(mg/kg) p.o for 7 days	HA titre Mean±SD)	DTH response(Mean±SD)
I	Control	--	6.66±0.28	0.2812±1.27
II	Levamisole	50	7.66±0.51	0.02924±1.45
III	Extract	100	8.33±0.75	0.03119±1.80
IV	Extract	200	9.58±0.35	0.03385±2.05
V	Extract	400	10.23±0.39	0.03420±1.24***

(n=6) *P<0.05(Compared to control)

HA Response

The extract produced a significant increase in humoral antibody titres in a dose-dependent manner (Refer table 2) in rats. The maximum effect was observed with the 500 mg/kg of the extract. The extract effects were compared with the standard drug.

DISCUSSION

From the above results it can be concluded that the methanolic extract of *Limonia acidissima* fruit pulp was found to stimulate the phagocytic activity of the macrophages as evidenced by an increase in the rate of carbon clearance. The DTH directly correlates with cell-mediated immunity, was found to be highest at the maximum dose of extract (500 mg/kg). The extract was tested on SRBC haemagglutination antibody titre in rats. The extract was found to significantly enhance the circulating antibody titre when compared to untreated immunized rats. This indicates the enhanced responsiveness of T and B lymphocyte subsets involved in antibody synthesis. However, the activity was not comparable in terms of quantitative activity elicited by standard drug. This could be due to the use of crude extracts. Hence, isolation of active principles will be advantageous to produce novel bioactive constituents from these extract which may possess more significant activity.

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