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Research Article

# ESTIMATION OF IMMUNOMODULATORY ACTIVITY OF *LIMONIA ACIDISSIMA* LINN.

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**ABSTRACT**

Objective: Plant derived substances have recently become of great interest owing to their versatile applications. Attention has been drawn towards finding the *in-vitro* immunomodulatory activity of the methanolic pulp extract from *Limonia acidissima* Linn.

Methods: The dried powdered pulp of *Limonia acidissima* Linn. extracted with methanol using Soxhlet apparatus. The *in-vitro* immunomodulatory activities were screened by different assays: *in-vitro* phagocytic assay, proliferation of splenocytes by inducing with mitogen, and cellular lysosomal enzyme activity assay.

Results: The methanolic extract of *Limonia acidissima* Linn. enhanced the NBT reduction at 0.01, 0.1 and 1mg/ml by 42% ($p < 0.01$), 69% ($p < 0.01$) and 88% ($p < 0.01$), respectively, with an $EC_{50}$ value of 0.04mg/ml. Lysosomal enzyme activity was also activated by the extract by 63% at 0.1mg/ml and by 89% ($p < 0.05$) at 1mg/ml with an $EC_{50}$ of 0.38mg/ml. The plant extract (1mg/ml) enhanced the proliferation by 184% ($p < 0.01$) compared to the control, in the absence of mitogen.

Conclusion: Our results suggest that methanolic extract of pulp of *Limonia acidissima* Linn. possess significant immunomodulatory activity *in-vitro*. Pulp of *Limonia acidissima* Linn. therefore is a good source as an immunomodulator and a possible pharmaceutical supplement.

**Keywords:** *Limonia acidissima* Linn.; Immunomodulating activity; Phagocytosis; Proliferation

## INTRODUCTION

*Limonia acidissima* Linn., syn. *Feronia limonia* (Rutaceae) is a moderate-sized deciduous tree grown throughout India. Ayurveda, the traditional medicinal system in India describes certain plants which strengthen the host immune system [1]. The fruits are woody, rough and used as a substitute for bael in diarrheoa and dysentery [2,3]. The bark and leaves of the plant are used for vitiated conditions of vata and pita while the fruits are used for treating tumors, asthma, wounds, cardiac debility and hepatitis. The fruit contains flavonoids, glycosides, saponins and tannins [4]. Some coumarins [4,5] and tyramine derivatives [6] have also been isolated from the fruits of limonia. The leaves are reported to possess hepatoprotective activity [7] while the fruit shells contain antifungal compounds,namely-psoralene,xanthotoxin,2,6-dimethoxybenzoquenone and osthenol [6]. The fruit pulp extract is also reported to possess antiproliferative activity[7,8].

## MATERIALS AND METHODS

### Collection of Plant Material

Ripe fruits of *Limonia acidissima* Linn.. were collected from the forest of Bhubaneswar hill situated in the eastern part of India in the month of May and identified by Dr SK Sahu, a taxonomist at Utkal University, Vanivihar, Orissa, India. A voucher specimen (no. 161) was deposited in the herbarium of the Department of Botany, Utkal University.

### Preparation of Extracts

The fruits were cut into small pieces and the pulp was separated. It was then shade dried, powdered and extracted with methanol using Soxhlet apparatus for 6 hours [9]. The extracts were filtered and the filtrate was concentrated under reduced pressure at 40°C using a rotary evaporator.

### Experimental animals

Swiss albino mice were obtained from a local supplier. The animals were acclimatized for 10 days before being used for the experiments. They were housed in a room with controlled temperature (23±2$0^0$C) and a 12-h light/ 12-h dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee of institute and conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

### Peritoneal macrophage preparation:

To elicit peritoneal macrophages 5 ml of ice cold PBS was injected into the peritoneal cavity of mice (with 3% FBS) as a stimulant. Collection of peritoneal exudate was done by peritoneal lavage with 8ml of RPMI 1640 medium (Sigma, Bangalore) supplemented with 10% heat-inactivated FBS, 50µM 2-mercaptoethanol (Sigma, Bangalore), 100U penicillin, 100µg streptomycin and 0.25 µg/ml amphotericin B (Sigma, Bangalore) after three days of stimulation. The erythrocytes in the cell pellets were lysed by 0.2% NaCl, after centrifugation of the exudates at 300g at $25^0$C for 20min. It was made isotonic by 1.6% NaCl. The cell suspension was centrifuged, washed twice and re-suspended in the medium. The cell number was adjusted to $1\times10^6$ cell/ml. The cell number was determined by counting in a hemocytometer and cell viability was tested by the trypan-blue dye exclusion technique.

### *In-vitro* Phagocytic assay

It was done by NBT dye reduction assay method [8]. In a 96-well microtitre culture plate 20 µl of the macrophage suspension and 40 µl of RPMI 1640 were mixed. The plant extract (20 µl) dissolved in 0.1% Dimethysulfoxide (DMSO) in phosphate buffer saline (PBS) solution. This content was added in each well to give final extract concentrations of 0.001, 0 .01, 0.1 and 1mg/ml. The 0.1% DMSO in PBS (without the plant extract) was used as a control. The content was then incubated for 24 h at $37^o$ C in 5% $CO_2$ humidified atmosphere. Then 20 µl of the heated inact ivated yeast suspension ($5\times10^7$ particles/ml) and 20 µl of NBT (Sigma, Bangalore) solution in PBS (1.5mg/ml) were added and the mixture was further incubated under the same conditions for 60min. The adherent macrophages were rinsed vigorously with RPMI medium and washed four times with 200 µl methanol. After they were air-dried, 120 µl of 2M KOH and 140 µl of DMSO were added. The absorbance was measured at 570 nm by a microplate reader and the percentage of NBT reduction was calculated by the following equation:

$$\text{NBT reduction (\%)} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

Results are presented as $EC_{50}$ value which represents the effective concentration required for 50% enhancement of oxidative burst reduction activity.

Mouse splenocytes preparation

Spleens of the mice were collected aseptically by sacrificing the animals. Preparation of the cell suspension was done by means of loose potter and flushing method which was taken after centrifugation at 300g, 37$^0$C for 10 min, erythrocytes and were lysed by hypotonic solution. The cell pellets were washed twice with RPMI 1640. RPMI medium was used for the re-suspension of cells and the cell number was adjusted to 1×10 $^6$ cell/ml. The viability of splenocytes was determined by the trypan-blue dye exclusion technique.

Assay in Cellular lysosomal enzyme activity

The cellular lysosomal enzyme activity was used to determine acid phosphatase in macrophages as previously described [10]. Briefly, 20 µl of macrophage su spension (1×10 $^6$ cells/ml), 40 µl of RPMI medium and 20 µl of the plant extracts dissolved in 0.1% DMSO in PBS were added in each well of a 96-well microtitre culture plate to obtain final concen trations of 0.001, 0.01, 0.1 and 1mg/ml. In some wells 0.1%DMSO in PBS was used as a control. The culture was incubated at 37$^0$C in 5% $CO_2$ humidified atmosphere for 24 h. The medium was removed by aspiration and in each well 20 µl of 0.1%

Triton X-100 (Merck, Mumbai), 100 µl of 10mM p-nitrophenyl phosphate (p-NPP) (Merck, Mumbai) solution and 50µl of 0.1M citrate buffer (pH 5.0) were added. Further the mixture was incubated for

30min. 150µl of 0.2M borate buffer (pH 9.8) was then added and the absorbance was measured at 405 nm. The percentage of lysosomal enzyme activity was calculated by the following equation:

$$\text{Lysosomal enzyme activity (\%)} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD negative control}} \times 100$$

Results are presented as $EC_{50}$.

Proliferation of Splenocytes by inducing with mitogen

The lymphocyte proliferation assay was carried out according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, Bangalore) assay method [12]. In 0.1% DMSO in PBS, 20 µl of various concentrations (0.001, 0.01, 0.1 and 1mg/ml) of the plant extracts were dissolved. It was then added to the mixture of 20 µl of the splenocyte suspensions (1×10 $^6$ cells/ml) and 40 µl of RPMI medium in a 96-well plate. Mitogens were preliminary tested for the optimum dose of lipopolysaccharide (LPS) and pokeweed mitogen (PWM) at 5 µg/ml. 0.1% DMSO in PBS was used as a control. The mixture was then incubated at 37$^0$C in humidified 5% $CO_2$ atmosphere for 48 h. Then 20 µl of MTT (5mg/ml) in PBS and 40 µl of RPMI media were added. The culture medium was removed by aspiration and 100 µl of 0.04M HCl in isopropyl alcohol were added to lyse cells. Dilution of the solution was done with 100 µl of distilled water and the absorbance was measured at 570 nm. Proliferation percentage was calculated by the following equation:

$$\text{Proliferation (\%)} = \frac{\text{OD sample} - \text{OD control}}{\text{OD control}} \times 100$$

Results are presented as $EC_{50}$.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean±S.E. Student's t-test was used for analyzing statistical significance and results with p< 0.05 were considered significant.

## RESULTS AND DISCUSSION

*In-vitro* Phagocytic activity

The methanolic extract of *Limonia acidissima* Linn.. enhanced the NBT reduction at 0.01, 0.1 and 1mg/ml by 42% (p < 0.01), 69% (p < 0.01) and 88% (p < 0.01), respectively, with an $EC_{50}$ value of 0.04mg/ml. Lysosomal enzyme activity was also activated by the extract by 63% at 0.1mg/ml and by

89% (p < 0.05) at 1mg/ml with an $EC_{50}$ of 0.38mg/ml. The dose response curve was significantly presented.

Proliferation of Splenocytes by inducing with mitogen

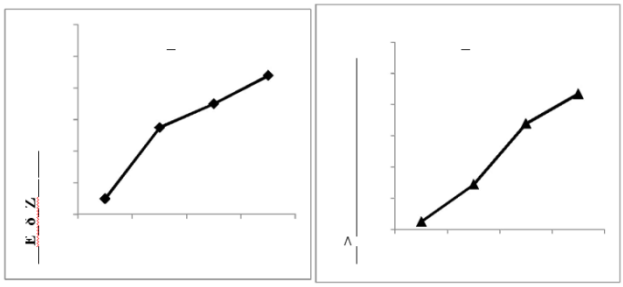

**Fig.1. Effects of a methanolic *Limonia acidissima* pulp extract on *in-vitro* phagocytic assay of normal Balb/c macrophages: (A) NBT Assay and (B) lysosomal enzyme activity test. Each value represents the mean±S.E. of the triplicates comparing to the control; p < 0.05.**

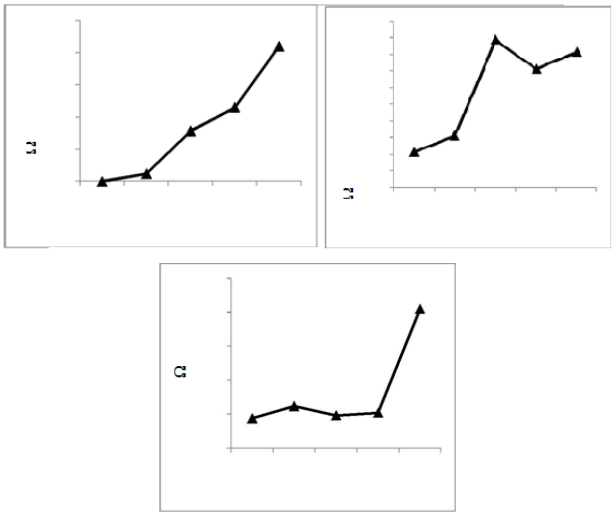

**Fig.2. Effects of a methanolic pulp extract of *Limonia acidissima* on *in-vitro* proliferation of normal Balb/c splenocytes: (A)without mitogen, (B) with lipopolysaccharide (5µg/ml) and (C) with pokeweed mitogen 5µg/ml). Each value presents the mean±S.E. of the triplicates comparing to the control; p < 0.05.**

The plant extract (1mg/ml) enhanced the proliferation by 184% (p < 0.01) compared to the control, in the absence of mitogen. The plant extract produced an increase of splenocyte proliferation by 171% (p < 0.05), 159% (p < 0.01) and 178% (p < 0.01) at 0.01, 0.1 and 1mg/ml, respectively in the presence of LPS. The extract at 0.1 and 1mg/ml elicited the proliferation by 56% (p < 0.01) and 191% (p < 0.05) of the control, respectively (Fig 2) with PWM. The $EC_{50}$ values of stimulation of splenocyte proliferation with LPS, PWM and without mitogen were 0.41, 0.01 and 0.02 mg/ml respectively. The extract in concentration up to 1mg/ml was not toxic to mouse macrophages and splenocytes (survival rates higher than 90 and 80%, respectively). The methanolic pulp extract of *Limonia acidissima* Linn.. showed stimulation of phagocytes in proportion to the foreign particles ingested which is represented by the higher reduction in NBT assay due to the higher activity of oxidase enzyme [9]. In the lysosomal enzyme activity assay, the increased transformation of p-nitrophenyl phosphate (p-NPP) to a p-nitrophenol (a coloured compound) by the membrane associated acid phosphatase activity of the treated macrophages is related to the stimulation of macrophages. The mitogenic responses of mouse splenocytes by the extract together with the optimum dose of LPS (a

mitogen for T-cell independent B-cell proliferation) and PWM (a mitogen for T-cell dependent proliferation) were also evaluated. The presence of mitogens in the system can postulate the possible proliferation activation pathway of the extracts [12]. In the present study, the maximum phagocytic activity of the extract was the same on the NBT dye reduction as well as the lysosomal enzyme activity assay. However, the $EC_{50}$ value from the former was less than the latter indicates a stronger potency on superoxide production than lysosomal enzyme activity. It should be stated that the extract might contain some phytoconstituents responsible for intracellular killing more than degranulation. These constituents may be phenolic compounds since *Limonia acidissima* Linn.. contains a greater proportion of it. Compounds such as flavonoids, glycosides, saponins and tannins from the methanolic extract of *Limonia acidissima* Linn.. were found as main constituents [4]. The phenolic compounds can stimulate or suppress the immune system due to the hydroxyl groups in the structure. These groups can affect the enzyme or electron-transferring system resulting in an immunomodulating property, especially phagocytic activity.

## CONCLUSION

The extract exhibited high activity on the oxidative burst reduction, presenting intracellular killing, and the enhancement of lysosomal enzyme activity, showing the activity on degranulation of macrophages. The $EC_{50}$ value of the extract was more with LPS than with PWM. Some active compounds in the extract may involve in B-cell proliferation stimulation, related to the humoral mediated immunity (HMI) [13]. In case of LPS addition the extract demonstrated stronger effect on the T-cell independent than in the case of PWM addition in the T-cell-dependent pathway. The phenolic compounds in the extracts might be assumed to be responsible for the immunomodulating activities found in this study.

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