



Effect of *Ziziphus jujuba* leaves extract on phagocytosis by human neutrophils

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Abstract

Objective: To study the effect of hydroalcoholic extract of *Ziziphus jujuba* leaves on neutrophil phagocytic function. **Methods:** The different concentrations (5, 10, 25, 50 and 100 µg/ml) of *Ziziphus jujuba* leaves extract was subjected, to study its effect on different *in vitro* methods of phagocytosis such as neutrophil locomotion and chemotaxis test, *in vitro* immunostimulant activity by slide method and qualitative nitro blue tetrazolium test using human neutrophils. **Results:** The *Ziziphus jujuba* leaves extract has stimulated chemotactic, phagocytic and intracellular killing potency of human neutrophils at the concentration range of 5-50 µg/ml. **Conclusion:** The hydroalcoholic extract of *Ziziphus jujuba* leaves stimulates cell-mediated immune system by increasing neutrophil phagocytic function.

Key words: Immunostimulant activity, neutrophils, phagocytosis, *Ziziphus jujuba*.

1. Introduction

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases [1]. The degree to which the patient becomes abnormally susceptible to infections by this microbial environment depends on the extent of immunosuppression. The suppression of the immune system is characterised by reduction in number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This immunosuppression allows opportunistic

pathogens to overwhelm the host to cause secondary infections [2].

This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs [3]. Many medicinal plants are known to have immunomodulatory properties and maintain organic resistance against infection by re-establishing the body's immune system such as *Azadirachta indica* [4], *Terminalia chebula* [5], *Lawsonia alba* [6] etc. The phytochemical constituents like alkaloids, terpenoids, steroids, proteins and

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polysaccharides are considered to exhibit this immunomodulatory property [1].

A number of *in vitro* and *in vivo* test systems are available for assessing immunomodulatory activity. Phagocytosis is one such widely used method for screening the immune response [7]. Phagocytosis is the primary defence mechanism against any foreign bodies entering the body, which is offered by neutrophils and macrophages. The process of phagocytosis consists of sequential stages such as motility, adhesion to microorganisms, ingestion of microorganisms, degranulation and intracellular killing of microorganisms [8].

Ziziphus jujuba of family Rhamnaceae is a small subdeciduous tree grown wild and cultivated in many parts of the India and Burma [9]. It is reported to contain saponin glycosides, alkaloids, steroids, polysaccharides and terpenoids as main constituents [10,11] and possess wide range of activities such as hypoglycaemic [12], antioxidant [13], hypolipidemic [14], antimicrobial [15] and permeability enhancement activity [16].

In our present study, we have attempted to evaluate immunomodulatory potency of *Z. jujuba* leaves extract using different *in vitro* methods for locomotion, phagocytic and intracellular killing potency of neutrophils, which are subsequent events involved in the process of phagocytosis by neutrophils.

2. Materials and methods

2.1 Plant material

The fresh leaves of *Z. jujuba* were collected from the damp fields near Belgaum, in August 2002 and were positively identified by Prof. S.B. Sasalatti, Head, Department of Botany, K.L.E.S's R.L.Science Institute, Belgaum, Karnataka, where a voucher specimen is deposited.

2.2 Preparation of extract

The leaves were shade dried at room temperature and powdered until able to pass through sieve

no. 40. The dried leaves were subjected to percolation using 70% ethanol at room temperature for 24 h. The dark green filtrate obtained was concentrated under reduced pressure at 50°C using Rota vapour apparatus to get a viscous mass, which was then lyophilized and stored at 4°C until used.

The crude extract obtained was subjected to preliminary phytochemical investigation [17], which showed the presence of alkaloids, steroids, flavonoids, proteins, triterpenoids and polysaccharides.

2.3 Preparation of test sample

Samples for *in vitro* study were prepared by dissolving 10 mg of crude extract in 0.5 ml of Dimethylsulphoxide (DMSO) and diluted with normal saline to obtain concentration ranging from 5, 10, 25, 50 and 100 mg/ml.

2.4 Study of the immunomodulatory activity

2.4.1 Neutrophil locomotion and chemotaxis test [18]

Neutrophil cell suspension was prepared in phosphate buffer saline solution (PBS) at about 10^6 cells/ml. The lower compartment of chemotactic chamber (5 ml beaker) was filled with appropriate chemotactic reagents preadjusted to a pH of 7.2 e.g. chamber 1-PBS solution (control); chamber 2-Casein 1mg/ml (standard); and chamber 3, 4, 5, 6 and 7 with different concentrations (5, 10, 25, 50 and 100 µg/ml) of test sample.

The upper compartment (1 ml syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37°C for 180 min.

The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 2

min and then stained with Haematoxylin dye for 5 min. The fixed filters were observed under microscope using 100x lens and the number of neutrophil cells reached to the lower surface of filter was counted.

2.4.2 *In vitro* immunostimulant activity studies by slide method [7]

Preparation of Candida albicans suspension

The *Candida albicans* culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button at the bottom and supernatant was discarded. The cell button was washed with sterile Hank's Balanced Salt Solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in proportion of 4:1. The cell suspension of concentration 1×10^8 was used for the experiment.

Slide preparation

Human blood (0.2 ml) was obtained by finger prick method on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. The blood clot was removed very gently and slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with predetermined concentration of test sample and incubated at 37°C for 15 min.

The PMNs were covered with *Candida albicans* suspension and incubated at 37°C for 1 h. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis evaluation

The mean number of *Candida* cells phagocytosed by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was repeated

for different concentrations (5, 10, 25, 50 and 100 µg/ml) of test sample. Immunostimulation in % was calculated by using following equation:

$$\text{Stimulation (\%)} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI (control)}} \times 100$$

2.4.3 *Qualitative nitroblue tetrazolium (NBT) test* [18]

A suspension of leucocytes (5×10^6 /ml) was prepared in 0.5 ml of PBS solution in 7 tubes. 0.1ml of PBS solution (control) and 0.1ml of endotoxin activated plasma (standard) is added to the 1st and 2nd tube respectively and to the other 5 tubes added 0.1ml of different concentrations (5, 10, 25, 50 and 100 mg/ml) of test sample. 0.2 ml of freshly made 0.15% NBT solution was added to each tube and incubated at 37°C for 20 min. Centrifuged at 400g for 3-4 min to discard the supernatant.

The cells were resuspended in the small volume of PBS solution. A thin film was made with the drop on a slide, dried, fixed by heating, counterstained with dilute carbol-fuchsin for 15 sec. The slide was washed under tap water, dried and focussed under 100x oil immersion objective. 200 neutrophils were counted for the % of NBT positive cells containing blue granules/lumps.

2.5 *Statistical analysis*

The values are expressed in mean+SEM (n=3). The results were analysed by using one way analysis of variance (ANOVA) followed by Dunnet's t-test to determine the statistical significance.

3. Results

The *Z. jujuba* leaves extract has caused a significant ($P < 0.001$) dose dependent, increase in movement of number of neutrophils from the upper compartment to lower surface of filter (Table 1), stimulation of phagocytosis of

Table 1.
Effect of *Ziziphus jujuba* leaves extract on neutrophil locomotion and chemotaxis

Treatment	Concentration (µg/ml)	Mean number of neutrophils per field
Control (PBS)	-	6.33 ± 0.87
Casein	1000	73.66 ± 1.45*
<i>Z. jujuba</i> extract	5	33.33 ± 1.28*
<i>Z. jujuba</i> extract	10	37.66 ± 1.35*
<i>Z. jujuba</i> extract	25	39.33 ± 1.45*
<i>Z. jujuba</i> extract	50	45.66 ± 1.25*
<i>Z. jujuba</i> extract	100	8.66 ± 0.22

Values are mean ± SEM (n=3); *P<0.001 compared to control group.

Table 2.
Effect of *Ziziphus jujuba* leaves extract on neutrophil phagocytosis

Treatment	Concentration (µg/ml)	% Stimulation
Control (PBS)	-	5.34 ± 0.96
<i>Z. jujuba</i> extract	5	23.66 ± 1.45*
<i>Z. jujuba</i> extract	10	25.33 ± 1.06*
<i>Z. jujuba</i> extract	25	29.65 ± 1.24*
<i>Z. jujuba</i> extract	50	30.33 ± 1.31*
<i>Z. jujuba</i> extract	100	6.66 ± 0.78

Values are mean ± SEM (n=3); *P<0.001 compared to control group.

Candida albicans by neutrophils (Table 2) and also increase in % of NBT positive cells containing the reduced NBT dye (Table 3), when compared with control samples containing PBS solution. In neutrophil locomotion and chemotaxis test and qualitative NBT test, the results obtained with *Z. jujuba* leaves extract treatment were comparable with that of standard.

These effects were observed with the concentration range of 5-50 µg/ml of test sample, however it has failed to show any significant effect at 100 µg/ml concentration.

4. Discussion

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating the non-specific immune system. However there is a need to subject such medicinal plants to systemic studies to substantiate the therapeutic claims made with regard to their clinical utility [3].

In the present study, *Z. jujuba* leaves extract significantly increased the phagocytic function of human neutrophils, when compared to control indicating the possible immunostimulating effect.

The movement of neutrophils towards the foreign body is the first and most important step in phagocytosis. The *Z. jujuba* leaves extract has significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells, reached the lower surface of filter, thereby *Z. jujuba* leaves extract acts as chemo attractant.

The ingestion of micro organisms after coming in contact with them, studied by slide method, provides a rapid and simple means of assessing the overall phagocytic process by the neutrophils. The *Z. jujuba* leaves extract displayed significant increase in ingestion of *Candida albicans* by neutrophils, thereby enhancing the phagocytic process of neutrophils.

The final step of phagocytosis is the intracellular killing of micro organisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, an activation which is also necessary for normal microbicidal activity [8].

The *Z. jujuba* leaves extract has significantly increased the intracellular reduction of NBT dye to formazan (deep blue compound) by the neutrophils confirming the intracellular killing property and overall metabolic integrity of phagocytosing neutrophils.

Table 3.
Effect of *Ziziphus jujuba* leaves extract on qualitative NBT test

Treatment	Concentration (µg/ml)	% NBT positive cells
Control (PBS)	-	23.66 + 1.12
Endotoxin activated plasma	-	72.00 + 0.82*
<i>Z. jujuba</i> extract	5	35.66 + 0.76*
<i>Z. jujuba</i> extract	10	62.33 + 0.98*
<i>Z. jujuba</i> extract	25	73.33 + 1.25*
<i>Z. jujuba</i> extract	50	82.66 + 0.87*
<i>Z. jujuba</i> extract	100	24.33 + 1.20

Values are mean + SEM (n=3)

*P<0.001 compared to control group

On the basis of results obtained from the study, we can conclude that *Z. jujuba* leaves extract stimulates cell-mediated immune system as evident by the increase in neutrophil phagocytic activity in dose dependent manner. It is logical to suggest that it may be useful as an adjuvant in several immuno-suppressed clinical conditions.

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