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Research article

NATURAL IMMUNOSTIMULATORY COMPOUNDS – A STUDY ON *ZIZYPHUS MAURITIANA*

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ABSTRACT

Attack of microorganisms is inevitable, but immune system has the potential to protect the body from them. Therefore, it becomes extremely essential to maintain the integrity of immune system. Maintenance and modulation of the immune system in a positive direction is always of a great interest to the researchers. Synthetic immunostimulators are rapidly effective, but unfortunately have many side effects on various other systems of the body. On the other hand, natural immunostimulators present in various parts of medicinal plants are comparatively slow in their action but are equally effective. Most of them are free from side effects. Medicinal properties in plants are due to the phytochemicals present in them. *Zizyphus mauritiana*, Lam (Rhamnaceae) is one such medicinal plant. Extracts of the stem bark were prepared in various solvents in the increasing order of polarity. Aqueous extract was found to contain significant amount of various phytochemicals. Hence, it was selected to study immunostimulation. Eight different fractions were collected by adsorption column chromatography of the aqueous extract and the most effective fraction termed as immunostimulatory fraction (IF) was isolated. IF was tested for its ability to stimulate phagocytosis in macrophages and to stimulate proliferation of splenocytes and lymphocytes.On treatment with IF (100 µg/ml), phagocytic index of macrophages as well as lysosomal enzyme activity both were raised to more than 2 while splenocyte and lymphocyte proliferation index was found to be more than 3.5.

Key words: Phagocytosis, Lysosomal degranulation, Splenocytes, Lymphocytes, Proliferation.

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INTRODUCTION

Immune system is one of the most essential systems in vertebrates. It is involved in combating infections and protecting the body from diseases.Being a very active system, it generally controls the pathogens quite earlier; thus preventing severe infections. Phagocytes are the most efficient cells of the innate immune system and lysosomes are their powerful weapons. Phagocytes have the ability to distinguish self and nonself and thus can tackle the pathogens (Guerin I & De Chastellier C, 2000). In case, if innate immune system fails, adaptive immune system comes into action. Both T and B lymphocytes play an important role in clearing pathogens by forming cytokines and antibodies, respectively. But, sometimes, the immune system takes much more time to cure the infection. This may cause severe damages to the body. Therefore, immunostimulators are discovered and designed by researchers. But, most of the immunostimulators are synthetic and have many side effects on various other systems of the body. Therefore; researchers, from last two decades, have turned their attention in search of natural immunostimulators which are free from adverse effects (Talmale S *et al*., 2015). *Zizyphus* genus is considered as a storehouse of phytochemicals in *Ayurveda*. The stem barks of some species of *Zizyphus* have already been tested for their

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analgesic, anti-inflammatory, antidiarrheal and antioxidant activities (Adzu B *et al*., 2001; Adzu B *et al*., 2002). Schistosomiasis (intravascular trematode infection) in animals was completely cured with normal appearance of the glomeruli and tubules after the treatment with ethanol extract of root bark of *Zizyphus spina* (Ali SA and Hamed MA, 2006). Also, the spleen sections of mice treated with ethanol extract of the roots of *Zizyphus spina* showed activation of lymphocytes. Scientists have explored the presence of saponins, glycosides, essential oils, phenols and their derivatives in *Zizyphus* species. Also it is seen that *Zizyphus* is one of the richest plant in cyclopeptide alkaloids possessing antibacterial and antifungal activity (Dimitris CG *et al*., 1997). Pretreatment of Wistar rats with ethanolic extract of *Zizyphus mauritiana* leaves provide protection against carbon tetrachloride induced hepatic injury by maintaining levels of glutathione, vitamin E and decrease in lipid peroxidation levels, indicating antioxidant property of *Zizyphus mauritiana* (Dahiru D *et al*., 2005, Dahiru and Obidoa, 2007). Barks of medicinal plants are always found to be rich in active ingredients and are included in many *Ayurvedic* preparations. In the present work, aqueous extract of stem bark of *Zizyphus mauritiana* has been tested for its ability to stimulate the immune system.

MATERIALS AND METHODS

Chemicals

Dulbecco's phosphate buffered saline (DPBS), RPMI-1640, fetal calf serum (FCS) were purchased from Gibco laboratories. Antibiotic-antimycotic solution and *p*-nitro phenyl phosphate (p-NPP) was purchased from Himedia laboratories, Mumbai. Trypsin, Atropine, Zymosan A, Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino) carbonyl -2H- tetrazolium (XTT), N-methyl dibenzopyrazine methyl sulfate (PMS), Lipopolysaccharide (LPS) and nitrobluetetrazolium (NBT) were purchased from Sigma Aldrich chemical company, St. Louis, USA. DMSO, silica gel and solvents for soxhlet extraction were of analytical grade.

Plant material

Without any injury to the plant, stem barks of the plants were carefully taken out and collected from forest region of Nagpur district of Maharashtra (India). Plants were authenticated by taxonomists at University Department of Botany, RashtrasantTukdojiMaharaj Nagpur University, Nagpur and voucher specimens were deposited in herbarium. The plant was identified to be *Zizyphus mauritiana* Lam by experts (Rhamnaceae) andrelevant voucher specimen number was 9483.

Animals

Wistar albino rats of either sex were procured from National Centre for Laboratory Animal Sciences, Hyderabad, India. Animals were maintained under standard conditions (temperature 25 ± 2°C) with 12 h light/ 12 h dark cycle and fed *ad libitum* with standard pellet diet and purified water. Human care was provided to all animals and norms prescribed by Animal Ethics Committee were critically followed.

Preparation of extracts

Stem barks of *Zizyphus mauritiana* were shed dried in proper conditions for approximately two months. Dried barks were crushed in grinder. Powdered barks were extracted using soxhlet apparatus successively in the increasing order of polarity by petroleum ether (polarity = 0.0), toluene (polarity = 2.7), chloroform (polarity = 4.1), ethanol (polarity = 5.2) and water (polarity = 9.0). Solvents were evaporated by rotary vacuum evaporator (Superfit DB3135S). Dried extracts were dissolved in 0.1% Dimethyl sulphoxide (DMSO) in PBS, mixed and vortexed for 1 min. Supernatants obtained after centrifugation at 100 *g* for 2 min were used for phytochemical analysis.

Phytochemical analysis

Phytochemical analysis of all the extracts were performed as per the methods proposed in Trease and Evans(Evans 2002).

Adsorption Column Chromatography

The aqueous extract of stem bark was found to contain significant amount of various phytochemicals and thus was selected for further purification. It was further purified by adsorption column chromatography. Column (5 x 30 cm) was prepared using silica gel (100-200 mesh). Slurry of silica gel was prepared in petroleum ether and column was allowed to stand for 1 h (Bathori M *et al*. 1986; Durley RC *et al*. 1972). Dried aqueous extract powder of stem bark (20 g) was used for adsorption column chromatography. Petroleum ether (PE), toluene (To), chloroform (Ch), ethyl acetate, acetone, ethanol, water and their mixtures in various proportions in the increasing order of polarity (50 ml PE; 40 ml PE + 10 ml To, 30 ml PE + 20 ml To, 20 ml PE + 30 ml To, 10 ml PE + 40 ml To; 50 ml To, 40 ml To + 10 ml Ch and so on upto 50 ml water) were introduced successively in continuation. Fractions of 5 ml each were collected (approx. 3 ml/min.).Thin layer chromatography of each fraction was performed and similar fractions were mixed. Such eight different fractions were obtained. Dried fractions were dissolved in 0.1% DMSO in PBS and

further tested for their ability to stimulate phagocytosis. Fraction number sixwas found to be most effective in stimulating phagocytosis and was named as immunostimulatory fraction (IF). Later IF was further purified by adsorption column chromatography.

Preparation of peritoneal mouse macrophages

Fetal calf serum (FCS) was administered by intraperitoneal injection in Wistar rats. Three days later, peritoneal exudates were collected by peritoneal lavage with RPMI 1640 medium. Exudates were centrifuged at 300 *g* for 20 min at 25 °C and cell pellets were washed twice and suspended in complete RPMI 1640 medium (RPMI 1640 with 10% FCS and 1% antibiotic- antimycotic solution). Cell number was adjusted to 1 x 10^6 cells/ml with hemocytometer and cell viability was tested by trypan blue dye exclusion method (Manosroi A *et al*. 2005).

Phagocytic Index assay

Capacity of all the eight fractions obtained by adsorption column chromatography of aqueous stem bark extract to stimulate phagocytosis was tested by NBT dye reduction assay (Rainard 1986). Macrophages (1 x 10^6 cells/well) suspended in complete RPMI 1640 medium were treated with 100 µg/ml of all the eight fractions separately dissolved in 0.1% DMSO in PBS. In control, macrophages were treated with 0.1% DMSO in PBS (without plant extract). After incubation for 24 h at 37 °C in 5% CO_2 humidified atmosphere, medium was removed and adherent macrophages were washed twice with RPMI medium. ZymosanA (1 µg/ml of PBS) was introduced along with NBT solution (1.5 mg/ml of PBS) and cells were incubated for 60 min at 37 °C in 5% CO_2 humidified atmosphere (Indian Equipment Corporation 3821). After 60 min, medium was removed and cells were washed twice with PBS and air dried for 1 min. Finally, 2 M KOH and DMSO were successively added and absorbance was measured at 570 nm using micro plate reader (Thermo electron Corp., 358). Phagocytic index (PI) was calculated by following equation:

PI = O.D. of experimental / O.D. of control

Effect of purified IF (immunostimulatory fraction) on phagocytosis was also tested in a similar way with various concentrations (100, 10, 1, 0.1, 0.01 µg/ml).

Lysosomal enzyme activity assay

Ability of IF to stimulate lysosomal enzyme activity (acid phosphatase) was tested using *p*-NPP (*p*-nitrophenyl phosphate) assay (Suzuki I *et al*. 1988). Macrophages (1 x 10^6 cells/well) in complete RPMI 1640 medium were treated with IF (100, 10, 1, 0.1, 0.01 µg/ml) dissolved in 0.1% DMSO in PBS. Macrophages with 0.1% DMSO in PBS (without plant extract) were taken as control. After incubation for 24 h at 37 °C in 5% CO_2 humidified atmosphere, medium was removed and adherent macrophages were washed twice with PBS. 0.1% Triton X-100, 10 mM*p*-NPP and 0.1 M citrate buffer (pH 5.0) were then introduced and cells were incubated for 30 min at 37 °C in 5% CO_2 humidified atmosphere. After 30 min, 0.2 M borate buffer (pH 9.8) was added and absorbance was measured at 405 nm using micro plate reader. Lysosomal enzyme activity index (LI) was calculated according to equation:

LI = O.D. of experimental / O.D. of control

Preparation of rat splenocytes

Wistar albino rats were sacrificed before removal of spleen. Outer layers of spleen were removed. The inner mass is chopped finely into small pieces followed by trypsin treatment to form splenocyte suspension which was washed twice and suspended in complete RPMI 1640 medium (Manosroi *et al*. 2003). Cell number was adjusted to 1 x 10^6 cells/ml.

Splenocyte proliferation assay

Effect of IF on splenocyte proliferation was tested by XTT assay (Kainthala *et al*. 2006). Splenocyte suspension (1 x 10^6 cells/ml) in complete RPMI 1640 medium were incubated in presence of IF (100, 10, 1, 0.1, 0.01 µg/ml) dissolved in 0.1% DMSO in PBS. Splenocytes with 0.1% DMSO in PBS (without plant extract) was taken as control. One group was treated with 10 µg/ml LPS (a powerful mitogen). After incubation for 48 h at 37 °C in 5% CO_2 humidified atmosphere, the medium was removed and the adherent macrophages were washed twice with PBS. A mixture of XTT and PMS (N-methyl dibenzopyrazine methyl sulphate) was then introduced along with RPMI 1640 medium. Cells were then incubated for 4 h at 37 °C in 5% CO_2 humidified atmosphere. Absorbance was measured at 450 nm using microplate reader. Splenocyte proliferation stimulation index (SSI) was calculated as:

SSI = O.D. of experimental / O.D. of control

Isolation of lymphocytes from rat blood

Blood was withdrawn from orbital plexus of Wistar rats and lymphocytes were isolated using ficollhistopaque. Lymphocytes were washed twice with PBS and resuspended in complete RPMI 1640 medium. Cell number was adjusted to 1 x 10^6 cells/ml.

Lymphocyte proliferation assay

Effect of IF on lymphocyte proliferation was tested by XTT assay almost similarly as that of splenocyte proliferation. Lymphocyte proliferation stimulation index (LSI) was calculated as:

LSI = O.D. of experimental / O.D. of control

Statistical analysis

Statistical analysis of experimental data was performed using Sigma Plot 10 software. Data were expressed as mean ± S.D. *P*-values were determined using the unpaired student's *t*-test. *P*-values less than 0.01 and 0.05 were considered as significant.

RESULTS

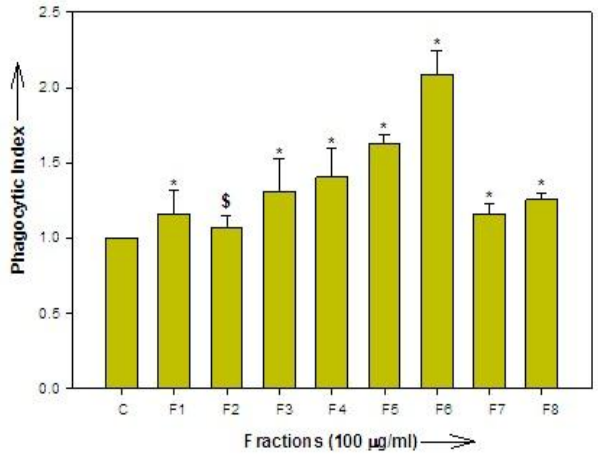
The crude aqueous extract of stem bark has shown positive phytochemical tests for alkaloids, anthracene glycosides, cardiac glycosides, cyanogenic glycosides, flavonoids, saponins and tannins mainly (Table 1). After adsorption column chromatography of the aqueous extract of stem bark, eight different fractions were collected. These fractions were tested for their ability to stimulate phagocytosis. The sixth fraction was found to be most effective with the PI of approximately 2 at 100µg/ml concentration (Fig. 1) and hence named as immunostimulatory fraction (IF). IF has shown positive test for the presence of flavonoids only. When 20 g of aqueous extract of stem bark was applied for adsorption column chromatography, 3.8 g of IF was obtained. IF was further purified by adsorption column chromatography. At 10 µg/ml concentration, IF has shown phagocytic index (PI) of approximately 1.7 (Fig. 2) while lysosomal enzyme activity index (LI) of approximately 1.4 was recorded (Fig. 3). Splenocyte stimulation index (SSI) at a concentration of 10 µg/ml of IF was found to be more than 2.4 (Fig. 4) while lymphocyte stimulation index (LSI) of approximately 3 was recorded at the same concentration (Fig. 5).

Table 1. Results of the phytochemical analysis of aqueous extract of stem bark.

Test	Phytochemical
1) Alkaloids	++
2) Anthracene glycosides	++
3) Cardiac glycosides	+++
4) Cyanogenic glycosides	++
5) Flavonoids	++
6) Saponins	+
7) Tannins	++++

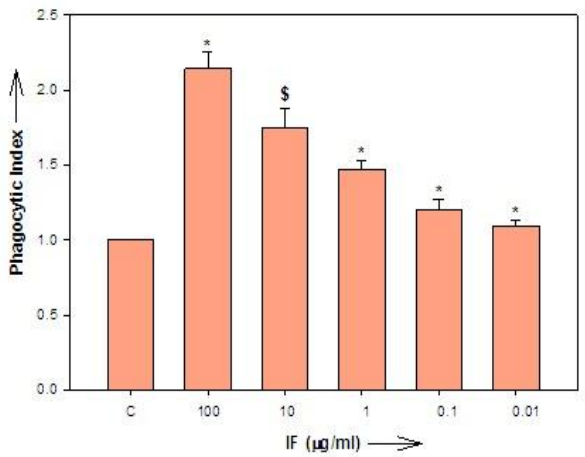
Phytochemical analysis of aqueous extract of stem bark has shown the presence of various phytochemicals like alkaloids, anthracene glycosides, cardiac glycosides, cyanogenic glycosides, flavonoids, saponins and tannins.

Fig1. Effect of all the eight fractions (100 µg/ml) obtained from aqueous stem bark extract on phagocytic activity.



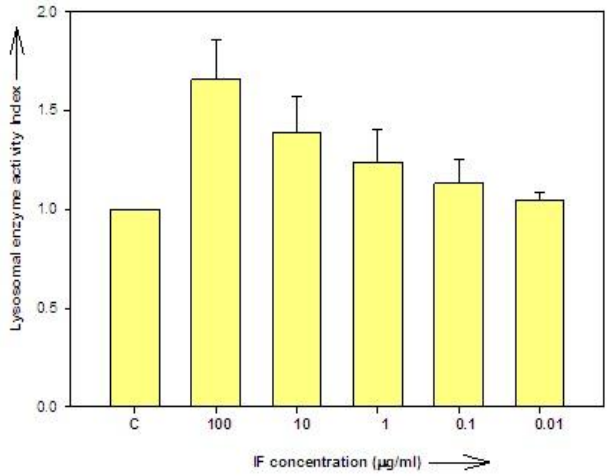
[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (**P*<0.01) and 0.05 ($*P*<0.05) were considered to be statistically significant].

Fig 2. Effect of IF (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on phagocytic activity.



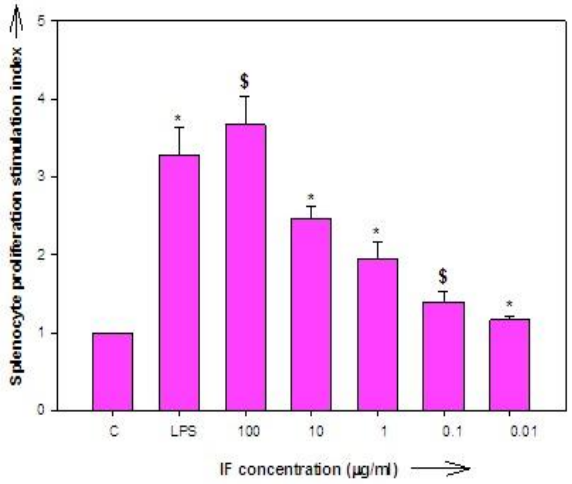
[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (**P*<0.01) and 0.05 ($*P*<0.05) were considered to be statistically significant].

Fig 3. Effect of IF (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on lysosomal enzyme activity.



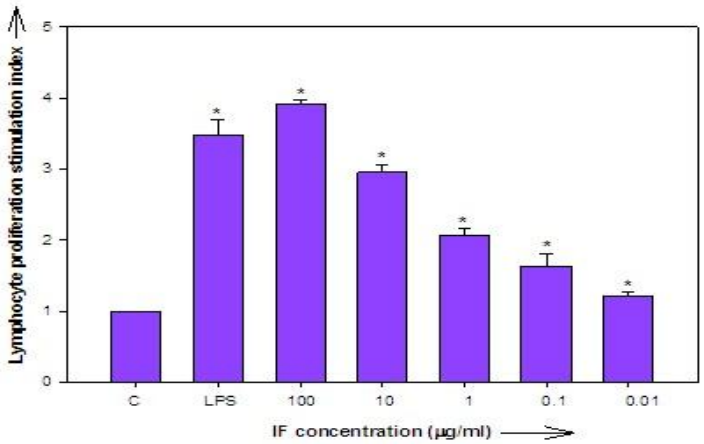
[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (**P*<0.01) and 0.05 ($^{\$}P$<0.05) were considered to be statistically significant].

Fig 4. Effect of IF (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on splenocyte proliferation.



[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (**P*<0.01) and 0.05 ($^{\$}P$<0.05) were considered to be statistically significant].

Fig 5. Effect of IF (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on lymphocyte proliferation.



[Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 (**P*<0.01) and 0.05 ($^{\$}P$<0.05) were considered to be statistically significant].

DISCUSSION

Phagocytes are the most important cells of innate immune system. They are the first to migrate to the area of infection through extravasation. Immunostimulatory fraction (IF) isolated from the aqueous extract of stem bark of *Zizyphus mauritiana*, has shown positive effect in stimulating phagocytosis in a dose dependent manner. Similarly, the lysosomal enzyme activity assay results also indicate immunostimulation in positive direction (Langermans *et al.*, 1994). Splenocytes and lymphocytes are one of the most important parts of acquired immunity and are actively involved in clearing blood born and organ born infections, respectively. Mitogenic potential of IF was clear from its ability to stimulate splenocytes and lymphocytes proliferation. Increase in the concentration of both these cells is a positive sign for immunostimulation.

Many synthetic immunostimulators are available, but along with immune system, they act on many other systems adversely. Most of the synthetic drugs accumulate slowly and steadily in the various organs of the body, causing bioaccumulation. Thus they can create any serious problem in future which can take very dangerous form. IF on the other hand is natural and thus the chances of side effects are almost nil. Hence, IF can be a perfect replacement for synthetic immunostimulators.

CONCLUSION

From the results, it is clear that the compounds present in IF manifested great potential to stimulate both innate and acquired immunity, thus IF can act as perfect immunostimulator.

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