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Investigations into the Immunostimulatory Activities of the Compounds Isolated from *Zizyphus mauritiana*

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Abstract

Objective: Any lacuna in the immune system can cause serious problems. Many synthetic immunostimulators are available, but they show many side effects. Phytochemicals present in *Zizyphus mauritiana*, Lam (Rhamnaceae) can act as natural immunostimulators. In the present study, natural and potent immunostimulatory compounds were isolated from *Zizyphus mauritiana* and their immunostimulatory activities were studied.

Methods: Extracts of the stem bark were prepared in various solvents in the increasing order of polarity. These extracts were tested for their potential to stimulate phagocytosis. Ethanolic extract of stem bark, which was found to be effective in stimulating phagocytosis in macrophages, was further purified by adsorption column chromatography to get effective fraction termed as effective immunostimulatory fraction (EIF). EIF was tested for its potential to stimulate phagocytosis in macrophages, to induce proliferation in lymphocytes as well as splenocytes and to stimulate antibody formation in Wistar rats.

Results: Phytochemical analysis of EIF indicated that it is rich in alkaloids. On treatment with EIF (1 µg/ml), phagocytic potential of macrophages was raised 6 times, while on EIF treatment (100 µg/ml) lysosomal enzyme activity was doubled. Splenocytes and lymphocytes were found to proliferate with a stimulation index of 5 and 6 respectively, on treatment with EIF (10 µg/ml). *In vivo*, EIF was found to trigger antibody formation two times, elevate leucocyte count (by 700/Cu mm) and increase the weight of thymus (3 times) as well as spleen (1.3 times) as compared to control on oral administration to Wistar rats (10 mg/kg body weight).

Conclusions: Results of the experiments revealed that phytochemicals present in EIF appear to be excellent immunostimulators and can act as replacement for synthetic immunostimulators.

Keywords: Zizyphus; Immunostimulation; Haemagglutination; Thymus; $CD4^+$

Introduction

Immune system is involved in combating infections and protecting the body from diseases. In traditional medicines different plant parts are believed to have specific medicinal properties including the ability to modulate the body's defense mechanisms [1]. In recent years, natural products obtained from plants are being investigated for their immunomodulating potential [2]. Drugs isolated from medicinal plants promote positive health and maintain resistance against infection by re-establishing body's equilibrium. Since time immemorial several diseases have been treated with plant extracts and rasayanas based on traditional knowledge of medicine [3]. Barks of medicinal plants are always found to be rich in active ingredients and are included in many Ayurveda preparations. Dried stem bark powder of *Zizyphus mauritiana* Lam (Rhamnaceae) is being used as a spice in the preparation of food by tribal communities in some parts of India.

Scientists have explored the presence of alkaloids, essential oils, glycosides, phenols, saponins, tannins and their derivatives in *Zizyphus* genus. It has also been reported that *Zizyphus* genus is rich with cyclopeptide alkaloids [4]. Ethanolic extract of leaves and endocarp of *Zizyphus mauritiana* helps to maintain levels of glutathione, superoxide dismutase, catalase, vitamin E and decrease in lipid peroxidation levels indicating its antioxidant property [5,6]. *Zizyphus mauritiana* has been reported for antimicrobial activity against *Aspergillus niger*, *Candida albicans*, *Enterobacter* species, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus* species [7,8]. Plants rich in phytochemicals with antimicrobial and antioxidant activities are generally found to be excellent immunomodulators [9]. As far as immunomodulation is concerned, only crude extracts of leaves of *Zizyphus* were studied for stimulating cell mediated immunity [10]. In the present work, stem bark of *Zizyphus mauritiana* has been tested for its ability to modulate the immune system.

Phagocytes are extremely important part of innate immunity while lysosomes are their weapons, useful in destroying microbes [11]. In case if innate immunity fails, acquired immunity comes into action. Lymphocytes and splenocytes are the pillars of acquired immunity, involved in antibody formation. Decrease in their number may lead to many immune deficiency diseases and several infections while their stimulation for proliferation is an important aspect of immunostimulation. $CD4^+$ lymphocytes are the one which stimulate antibody formation through cytokine secretion. Increase in the count of $CD4^+$ lymphocytes is considered to be the best remedy for most of

the infectious diseases. Thymus is the primary lymphoid organ of immune system while spleen is one of the secondary lymphoid organs. Gain in the weight of thymus and spleen is a reflection of their increased cellular concentration which is a positive sign of immunostimulation.

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 nitroblue tetrazolium (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 Maharashtra (India). Plants were authenticated by taxonomists at University Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur and voucher specimens were deposited in herbarium. The plant was identified to be *Zizyphus mauritiana* Lam by experts (Rhamnaceae) and relevant 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 hour light/ 12 hour 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 (permission letter ref. no. PGTD/BC/53). During oral administration, care was taken to prevent any injury to animals. Animals were weighed on electronic weighing balance (Essae Teraoka Ltd. FB200) before experiments.

Preparation of extracts

Stem barks of *Zizyphus mauritiana* were shade dried (without direct exposure to sunlight) and extracted using soxhlet apparatus with the help of different solvents in the increasing order of polarity [12]. Dried extracts were dissolved in 0.1% Dimethyl sulphoxide (DMSO) in PBS, mixed and vortexed for 1 minute. Supernatants obtained after centrifugation at 100 g for 2 minutes were used for testing their efficiency to stimulate phagocytosis.

Adsorption column chromatography

Ethanolic extract of stem bark was found to be more effective in stimulating phagocytosis. It was further purified by adsorption column chromatography. Slurry of silica gel (100-200 mesh) was prepared in petroleum ether and introduced in a glass column (5 × 30 cm). The column was allowed to stand for 1 hour [13,14]. Dried ethanolic extract of stem bark (20 g) was used for adsorption column chromatography. Petroleum ether, toluene, chloroform, ethyl acetate, acetone, ethanol, water and their mixtures in various proportions in the increasing order of polarity (50 ml petroleum ether; 40 ml petroleum ether+10 ml toluene, 30 ml petroleum ether+20 ml toluene, 20 ml petroleum ether+30 ml toluene, 10 ml petroleum ether+40 ml toluene; 50 ml toluene, 40 ml toluene+10 ml chloroform and so on upto 50 ml water) were introduced successively in continuation. Fractions of 5 ml each were collected (approx. 3 ml/minute). Thin layer chromatography of each fraction was performed and similar fractions were mixed. Such 20 different fractions were obtained. All the fractions were dried in rotary vacuum evaporator. Dried fractions were dissolved in 0.1% DMSO in PBS and further tested for their activity to stimulate phagocytosis. Fraction number 15 was found to be most effective in stimulating phagocytosis and was named as effective immunostimulatory fraction (EIF).

Phytochemical analysis

Phytochemical analysis of EIF was performed as per the methods proposed in Trease and Evans [15].

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 minutes 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 1×10^6 cells/ml with hemocytometer and cell viability was tested by trypan blue dye exclusion method [16].

Phagocytic index assay

Phagocytosis stimulation by crude extracts was tested by NBT dye reduction assay [17]. Macrophages (1×10^6 cells/well) suspended in complete RPMI 1640 medium were treated with 1% crude plant extracts 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 hours at 37°C in 5% CO_2 humidified atmosphere, medium was removed and adherent macrophages were washed twice with RPMI medium. Zymosan A (1 µg/ml of PBS) was introduced along with NBT solution (1.5 mg/ml of PBS) and cells were incubated for 60 minutes at 37°C in 5% CO_2 humidified atmosphere (Indian Equipment Corporation 3821). After 60 minutes, medium was removed and cells were washed twice with PBS and air dried for 1 minute. 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 EIF on phagocytosis was tested in a similar way by taking various concentrations of EIF (100, 10, 1, 0.5, 0.1, 0.01 µg/ml). Here atropine solution (100 µg/ml in PBS) was considered as negative control.

Lysosomal enzyme activity assay

Ability of EIF to stimulate lysosomal enzyme activity (acid phosphatase) was tested using p-NPP (p-nitrophenyl phosphate) assay [18]. Macrophages (1×10^6 cells/well) in complete RPMI 1640 medium were treated with EIF (100, 10, 1, 0.5, 0.1, 0.01 µg/ml) dissolved in 0.1% DMSO in PBS. Macrophages with 0.1% DMSO in PBS (without plant extract) were considered as control, while macrophages with atropine (100 µg/ml in PBS) were treated as negative control. After incubation for 24 hours 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 minutes at 37°C in 5% CO_2 humidified atmosphere. After 30 minutes, 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 [19]. Cell number was adjusted to 1×10^6 cells/ml.

Splenocyte proliferation assay

Effect of EIF on splenocyte proliferation was tested by XTT assay [20]. Splenocyte suspension (1×10^6 cells/ml) in complete RPMI 1640 medium was incubated in different concentrations of EIF (100, 10, 1, 0.5, 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 while splenocyte with atropine solution (100 µg/ml in PBS) was considered as negative control. One group of splenocytes was treated with 10 µg/ml LPS (a powerful mitogen). After incubation for 48 hours 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 hours 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 ficoll histopaque. Lymphocytes were washed twice with PBS and resuspended in complete RPMI 1640 medium. Cell number was adjusted to 1×10^6 cells/ml.

Lymphocyte proliferation assay

Effect of EIF 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

Acute toxicity study

Acute toxicity study was carried out according to OECD (Organization for Economic Corporation Development) guidelines. A group of five Wistar rats (3 males and 2 females) was daily orally administered with EIF. In the first week, 1 mg/kg body weight (bw) dose of EIF was provided and animals were regularly observed for acute toxicity signs like mortality and behavioral changes 1 hour post dosing and at least twice daily. As no change in behavior was observed, dose was shifted to 2 mg/kg bw. Thus, graded doses of 5, 10, 25, 50, 100 mg/kg bw were successively provided for a week. Half of lethal dose (LD_{50}), as obtained from this experiment was found to be 100 mg/kg bw at which rats showed little bit different behavior than normal like irritability. The $1/10^{th}$ of LD_{50} value was considered as therapeutic value. Thus, dose of EIF selected, for *in vivo* studies, was 10 mg/kg bw.

Haemagglutination assay

Six Wistar rats (3 males and 3 females) were taken in each group (2 months old; average weight 200-225 g). Animals were divided into three groups; Positive Control (PC), Negative Control (NC) and Experimental (E). Sheep RBCs (SRBCs) required for the experiment were collected from the Animal Husbandry Department of Veterinary College, Nagpur. Sheep blood was withdrawn from external jugular vein of sheep with intravenous set and was directly introduced in Alsevier's solution in 1:1 proportion with gentle mixing [21]. EIF was mixed in PBS and orally administered (10 mg/kg bw) to NC and E daily from *day* -10 (10 days prior to sensitization injection) to *day* +15 (15 days after the sensitization injection). Thus total 25 doses of EIF were provided to NC and E. Intravenous injection (sensitization injection) of 0.5×10^9 SRBCs in 0.2 ml PBS was administered to PC and E on the *day* 0. Blood was withdrawn from orbital plexus of all three groups of rats on day +15. Serum was considered as a source of antibodies against SRBCs. Different dilutions of all three groups of sera were prepared and checked for haemagglutination assay by titrating serum dilutions with SRBCs (0.025×10^9). Micro titer plates were incubated at room temperatures for 2 hours and examined visually for agglutination. Reciprocal of the highest dilution of serum showing 50% agglutination was expressed as HA titer [22].

Leucocyte concentrations in blood and lymphoid organ weight

Wistar albino rats were grouped into two groups with six animals in each; Control (C) and Experimental (E). Daily oral administration of 10 mg/kg bw of EIF was provided to E. After 25 doses of EIF, animals of both groups were sacrificed. Total Leucocytes Count (TLC) and Differential Leucocytes Count (DLC) were determined by fully automated cell counter (Orphee mythic 18; Switzerland) as well as by standard manual method. Absolute $CD4^+$ count was done on fully automated $CD4^+$ analyzer (BD FACS Counter; San Jose, USA). Relative weights of spleen and thymus of each animal were determined.

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

Effect of different extracts on phagocytosis

To some or more extent all the extracts (1%) of stem bark were found to be effective in stimulating phagocytosis of macrophages. However, ethanolic extract of the stem bark was found to be more effective in stimulating phagocytosis with PI of 4 (Figure 1). Hence, ethanolic extract of stem bark was chosen for isolation of the most potent immunostimulator by adsorption column chromatography.

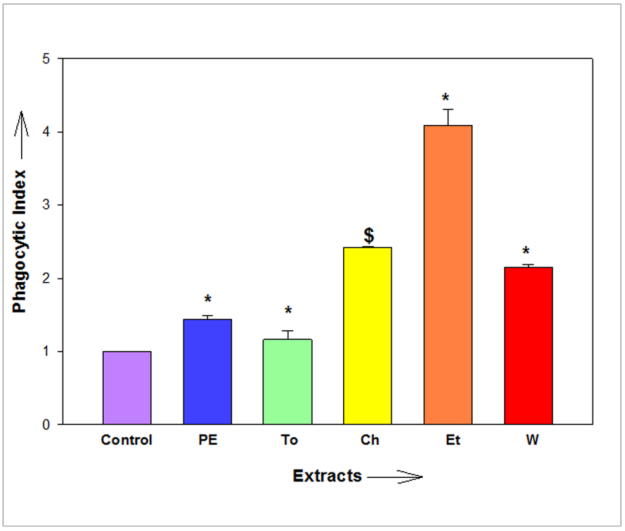

Figure 1: Effect of crude stem bark extracts (prepared in different solvents) at 1% concentration on phagocytic activity. (PE: Petroleum Ether; To: Toluene, Ch: Chloroform, Et: Ethanol, W: Water). [Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 ($^{*}P<0.01$) and 0.05 ($^{\$}P<0.05$) were considered to be statistically significant].

Purification and phytochemical analysis of EIF

When 20 g of ethanolic extract of stem bark was applied for adsorption column chromatography, 5.33 g of EIF was obtained.

Phytochemical tests of EIF had shown most prominent positive test for the presence of alkaloids, but along with that traces of anthocyanins and anthocyanidins, cardiac glycosides, emodins, tannins and triterpenoids were also present.

Effects of EIF on phagocytosis, lysosomal enzyme activity and cell proliferation

At 1 µg/ml, EIF had shown phagocytic index (PI) of approximately 6 (Figure 2). A dose dependant increase in the phagocytic activity was observed. At 100 µg/ml of EIF, lysosomal enzyme activity index (LI) of more than 2 was recorded (Figure 3).

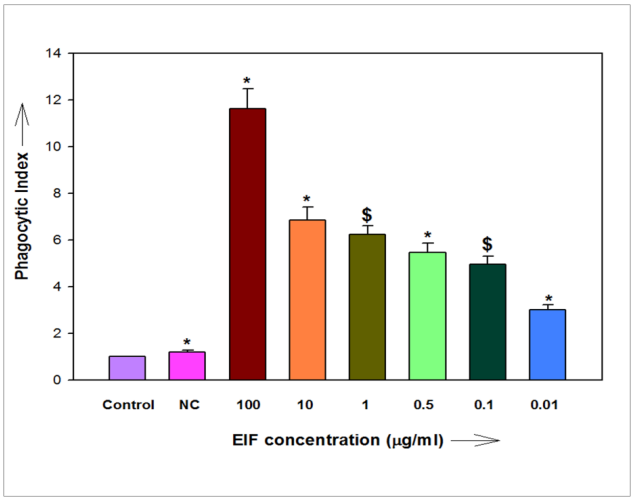

Figure 2: Effect of the different concentrations of EIF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on phagocytic activity (NC: Negative Control). [Each value has been expressed as mean ± SD of five independent experiments. P values less than 0.01 ($^{*}P<0.01$) and 0.05 ($^{\$}P<0.05$) were considered to be statistically significant].

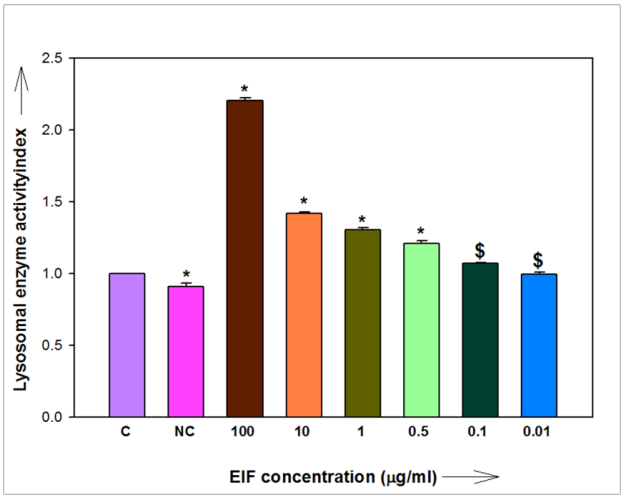

Figure 3: Effect of the different concentrations of EIF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on lysosomal enzyme activity (C: Control, NC: Negative Control). [Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 ($^{*}P<0.01$) and 0.05 ($^{\$}P<0.05$) were considered to be statistically significant].

Splenocyte stimulation index (SSI) at a concentration of 10 µg/ml of EIF was found to be more than 5, which was more effective than treatment with 10 µg/ml of LPS (Figure 4). At 10 µg/ml concentration of EIF, lymphocyte stimulation index (LSI) was recorded to be 6 and it was found to be more effective than the treatment with 10 µg/ml of LPS (Figure 5).

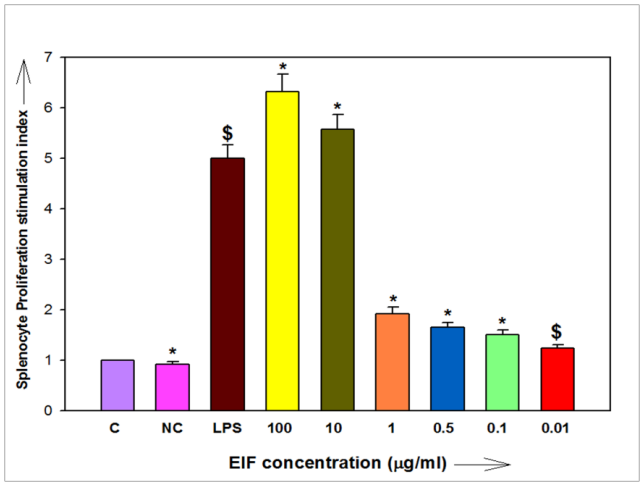

Figure 4: Effect of the different concentrations of EIF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on splenocyte proliferation stimulation index (SSI) (C: Control, NC: Negative Control, LPS: Lipopolysaccharide). [Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 ($^{*}P$<0.01) and 0.05 ($^{\$}P$<0.05) were considered to be statistically significant].

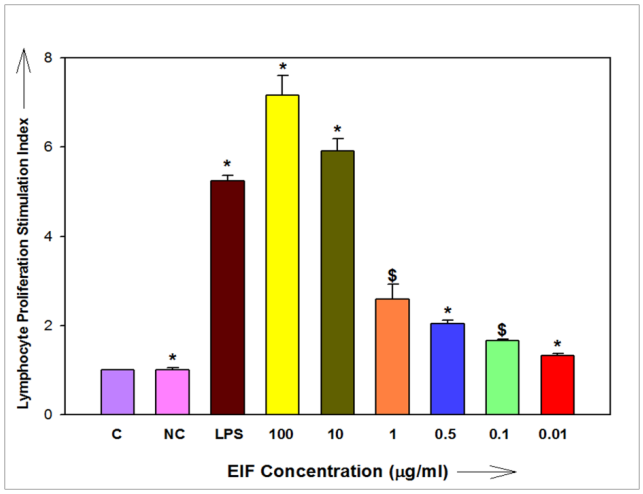

Figure 5: Effect of the different concentrations of EIF (0.01 µg/ml, 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml) on lymphocyte proliferation stimulation index (LSI) (C: Control, NC: Negative Control, LPS: Lipopolysaccharide). [Each value has been expressed as mean ± SD of five independent experiments. *P* values less than 0.01 ($^{*}P$<0.01) and 0.05 ($^{\$}P$<0.05) were considered to be statistically significant].

Effect of EIF on antibody formation in Wistar rats

Wistar rats in PC sensitized with SRBCs intravenously at *day 0* had shown haemagglutination (HA) titer of 175 ± 25. In NC, 25 oral supplementations of EIF were given but no intravenous sensitization injection of SRBCs was provided. Hence, this group did not show any antibody formation. In E, after 25 oral supplementations of EIF, including 10 doses before sensitization, animals had shown HA titer of 350 ± 50, showing effect of EIF in stimulating antibody formation (Table 1).

	Sensitizing injection (SRBCs)	Oral EIF treatment	HA Titre
PC	i.v.	-	175 ± 25
NC	-	+	-
E	i.v.	+	350 ± 50

Table 1: Effect of EIF on Haemagglutination (HA) titer in Wistar rats. i.v.: intravenous; SRBCs : Sheep Red Blood Cells. In case of PC (Positive Control), rats have shown HA titer of 175 ± 25 while in case of E (Experimental) the HA titer had jumped to 350 ± 50. Thus E is showing a great increase in antibody formation on oral treatment with EIF. However, in case of NC (Negative Control) as no sensitizing injection was given, no antibody formation was seen. Hence there was no question of HA titer.

Other *In vivo* effects of EIF on Wistar rats

As compared to C, Total Leucocyte Count (TLC) in case of E (administered with 25 oral doses of EIF), had increased by about 700/cu mm. Polymorphs (neutrophils), monocytes and eosinophils had increased by small margins but Absolute Lymphocyte Count (ALC) had increased by about 550/cu mm. Absolute $CD4^{+}$ count was found to be increased to about 500/cu mm in E (Table 2).

Test	C	E
Total Leucocyte Count (per cu mm)	6080 ± 320	6770 ± 380
Absolute Lymphocyte Count (per cu mm)	4156 ± 244	4706 ± 184
Polymorphs (%)	20.94 ± 0.46	23.06 ± 0.96
Monocytes (%)	6.14 ± 0.16	6.48 ± 0.12
Eosinophylls (%)	2.86 ± 0.24	3.18 ± 0.28
Absolute CD4 Count (per cu mm)	2729 ± 134	3249 ± 216
CD4 (%)	65.66 ± 1.24	69.04 ± 0.18

Table 2: Effect of EIF on leucocyte concentrations in Wistar rats. As compared to Control (C) rats, the Experimental (E) group has shown an increase in the Total Leucocyte Count (TLC), Absolute Lymphocyte Count (ALC), phagocytes such as polymorphs, monocytes and eosinophils and most remarkably the CD4 counts has increased to near about 4%. Values are mean ± S.E. of six rats.

E had shown an increase of about 1.3 and 3 times in the weights of spleen and thymus respectively (Table 3). Especially gain in the weight of thymus is considered to be the best part of EIF (Figure 6).

Figure 6: Photograph showing increase in the size and weight of thymus in Wistar rats where thymus of E group has shown an increase in weight more than 3 times after EIF treatment (C: Control; E: Experimental).

Organs (g)	C	E
Thymus	0.105 ± 0.02	0.353 ± 0.14
Spleen	0.448 ± 0.04	0.569 ± 0.26

Table 3: Effect of EIF on weight of lymphoid organ in Wistar rats. As compared to Control (C) rats, the Experimental (E) rats have shown an increase in the weight of thymus as well as spleen. Especially in case of thymus, the effect is quite pronounced as there is 3 times increase in weight. Values are mean ± S.E. of six rats.

Discussion

Phagocytes are the first cells to tackle the pathogens and are extremely important part of innate immunity. These are the immune cells that engulf the pathogens. Later, the endosome with pathogen (phagosome) merges with lysosome to form phagolysosome. Lytic enzymes like acid phosphatases present in lysosomes help in the digestion of pathogens [23,24]. EIF isolated from the stem bark of *Zizyphus mauritiana*, had shown a great impact in stimulating phagocytosis in a dose dependent manner. The results of phagocytic index were supported by lysosomal enzyme activity assay which indicate immunostimulation in positive direction. Spleen is an organ found in virtually all vertebrate animals. It acts primarily as a blood filter and is specialized in trapping blood born antigens; thus it can respond to systemic infections. The term splenocyte refers to any of the different white blood cell types as long as it is situated in the spleen [25]. Both B and T lymphocytes in coordination with each other help in the clearance of pathogens from the body especially through antibody formation and secretion [26]. Thus, splenocytes and lymphocytes are one of the most important parts of acquired immunity and increase in the concentration of splenocyte and lymphocyte is an important criterion of immunostimulation. Mitogenic potential of EIF was clear from its ability to stimulate splenocytes and lymphocytes. It was found to be more effective than LPS. In all *in vitro* parameters, atropine was taken as negative control. Atropine is an alkaloid but doesn't possess immunomodulatory activity [27]. Thus, it was cross checked that any alkaloid does not possess such immunostimulating potential.

Many immunostimulatory compounds are effective *in vitro* but when tested *in vivo* they do not manifest the desirable results. But EIF had proved its potential by elevating antibody formation in Wistar rats. Antibody Dependent Cell Cytotoxicity (ADCC) is the most effective and fastest mechanism of clearing pathogens. After regular doses of EIF, HA titer was found to be doubled. Thus, EIF had successfully stimulated acquired immunity by stimulating antibody formation [28]. EIF had raised the number of immune cells in Wistar rats, mainly lymphocytes and very specially $CD4^+$ lymphocytes. The $CD4^+$ lymphocytes are the main source of cytokines and play a major role in antibody formation. The ability of EIF to raise the $CD4^+$ lymphocyte count as well as its mitogenic potential can be the most probable reason to raise the antibody formation. Many immunodeficiency diseases like AIDS where $CD4^+$ count decreases, EIF can be a perfect replacement for synthetic immunostimulators or EIF can be provided as a supplement along with reverse transcriptase inhibitors. Stimulation of lymphocyte proliferation might be one of the reasons for increasing the weight of thymus and spleen by EIF [29].

Conclusion

From the results, it is clear that the compounds present in EIF manifested great potential to stimulate both innate and acquired immunity, thus EIF can act as an efficient immunostimulator to improve immunity in immunodeficient patients.

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Conflict of interest:

The authors declare no conflict of interest.

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