

# **Immunostimulatory Activity of Flavonoids Isolated From Stem Bark of *Zizyphus Mauritiana***

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**ABSTRACT:** Immune system is the maintaining authority of the living system. Both innate and acquired immunity are extremely important for the maintenance of good health. A small defect in innate as well as acquired immunity can lead to serious problems and thus modulation of immune response to prevent infections is very important part of medical science. Numbers of synthetic immunostimulants are daily consumed by patients, but along with them unnecessarily side effects are also purchased. This is the reason why for most of the people *Ayurveda* is still the first choice. *Zizyphus mauritiana*, a plant rich with phytochemicals, was selected in the present investigation for evaluating its immunostimulatory activities. Extracts of the stem bark were prepared in various solvents. Chloroform extract was found to contain significant amount of flavonoids. Hence, it was selected to study immunostimulation. Adsorption column chromatography of chloroform extract was performed and most active fraction termed as **Purified Fraction (PF)** was isolated. The PF was seen to stimulate phagocytic index, lysosomal degranulation and lymphocyte as well as splenocyte proliferation.

**KEY WORDS:** Zizyphus, Phagocytosis, Splenocytes, Lymphocytes, immunomodulation

## **I. INTRODUCTION AND BACKGROUND**

The immune system is responsible for eradication of pathogens and other non-self (foreign) substances. The immune response involves recognition of the pathogen or foreign material and mounting of a reaction to eliminate it. Thus immune system finds a vital position in combating infections and protecting the body from diseases. Its integrity and efficiency is important for maintenance of good health [1]. In traditional medicine different plant parts are believed to have specific medicinal properties including the ability to modulate the body's defense mechanisms [2, 3]. In recent years, natural products obtained from the plant kingdom are being investigated for their immune modulating potential against infections and neo plastic diseases [4].

Some of the drugs isolated from medicinal plants are believed to promote positive health and maintain resistance against infection by re-establishing body equilibrium. Since time immemorial several diseases have been treated by using plant extracts based on traditional knowledge of medicine and several medicinal plants have been reported to be used in folk medicine also for treatment of immunological disorders [5, 6].

In most of the *Ayurvedic* preparations stem bark finds an important position along with other parts of the plant. This indicates that stem barks of the plants are rich with active ingredients along with other parts of the plant. Compounds isolated from *Zizyphus* extracts have been shown to possess many therapeutic properties like treatment of malaria, fever and

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many more. The methanolic extract of the stem bark of some of the *Zizyphus* species has been recorded for anti diarrheal effect while aqueous extract of the root bark was shown to possess analgesic effect in mice [7]. The stem barks of some species of *Zizyphus* have been tested for their analgesic, anti-inflammatory, antidiarrheal and antioxidant activities [8, 9]. Scientists have explored the presence of flavonoids, 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 [10]. Hence, *Zizyphus mauritiana* is selected for immunomodulation studies.

Phagocytic cells involve mainly neutrophils and macrophages. These are the nonspecific cells with lysosomes as their weapons. Lysosomes contain lysozymes (acid phosphatases) which are useful in destroying microbes and their antigens [11]. Until and unless the phagocytes are actively working, body doesn't need to switch on the acquired immunity. Lymphocytes and splenocytes are most important parts of the acquired immunity. Main role of lymphocytes is their direct or indirect involvement in antibody formation.

In the present investigation, crude extracts of the stem bark of *Zizyphus mauritiana* were prepared successively in different solvents and tested for flavonoid content. Flavonoids, specially, are reported to have great medicinal values. Chloroform extract was found to contain large amount of flavonoids. Hence, it was further purified by adsorption column chromatography and the Purified Fraction (PF) was further tested for stimulating phagocytic index, lysosomal degranulation, lymphocyte and splenocyte proliferation.

## II. RELATED WORK

A number of Indian medicinal plants and various rasayanas have been claimed to possess immunostimulatory activity. In case of many immunodeficiencies, these immunostimulants are found to be extremely efficient [12]. Aqueous extract of fruits of *Actinidia macrosperma* (Family Actinidiaceae), commonly known as kiwifruit, has been found to significantly activate the overall immune functions. It has been found to stimulate lymphocyte proliferation, NK cell cytotoxicity as well as phagocytic activity [13]. Oral administration of both ethanolic and aqueous extracts of leaves of *Aesculus indica* (Family Sapindaceae) generally called *Bankhor* has been reported to activate the cell mediated cytotoxicity (CMC) [14]. Bulb of *Allium sativum* (Family Liliaceae), commonly referred as *lasun* in India, is referred as one of the extremely important medicinal plant in *Ayurveda*. It has been studied to stimulate Natural Killer (NK) cells to enhance cytotoxicity, T lymphocytes for enhanced production of IL-2 and macrophages for their phagocytic activity [15, 16]. *Aloe vera* (Family Liliaceae) is one of the most widely used healing plants in the history of mankind. Research has been done on its gel to stimulate phagocytic activity of the phagocytes especially macrophages as well as enhanced production of IL-1 and thus is found to be effective in case of viral infections [17]. Mice studies has indicated that ethanolic extracts of leaves of *Andrographis paniculata* (Family Acanthaceae), traditionally known as *Kalmegh*, is a potent stimulator of immune system in two ways. It can stimulate Antibody Dependant Cell Cytotoxicity (ADCC) as well as phagocytic activity of macrophages. Thus, it is effective in both innate and acquired immunity [18, 19].

Most of the above mentioned plants and their compounds are found to be as effective as the synthetic medicines but are not found to possess a single side effect. Many of them are easily available.

## III. MATERIALS AND METHODS

### 3.1 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

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and solvents for Soxhlet extraction were of analytical grade. HPLC grade solvents were purchased from authorized standard companies especially for column chromatography.

### 3.2 Plant material

Stem bark of the plant was collected in May 2009 (summer). The plants were authenticated at University Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur and voucher specimen no. 9483 (Date: 09 October, 2009) was deposited in the herbarium. The plant was identified to be *Zizyphus mauritiana* (Family Rhamnaceae).

### 3.3 Animals

Wistar albino rats (200-225g) of either sex were procured from National Centre for Laboratory Animal Sciences, Hyderabad, India. Animals were maintained at standard conditions (temperature  $25 \pm 2^{\circ}\text{C}$ ) with 12 h light/ 12 h dark cycle and fed ad libitum with standard pellet diet (Hindustan Lever) and purified water with free access to food and water. All the norms prescribed by Animal Ethics Committee were critically followed (vide the permission letter from Animal Ethics Committee dated: 24/11/2010).

### 3.4 Preparation of extracts

Dried stem bark of *Zizyphus mauritiana* was crushed in grinder, strained through the strainer to remove any hard part of the bark escaped during grinding. The powder of stem bark was stored in airtight brown plastic containers to protect from moisture and direct sunlight. Powdered bark were extracted successively in the increasing order of polarity using Soxhlet apparatus using petroleum ether (polarity 0), toluene (polarity 2.7), chloroform (polarity 4.1), ethanol (polarity 5.2) and water (polarity 9). All the extracts were concentrated by using rotary vacuum evaporator (Superfit DB3135S). The dried extracts were dissolved in 0.1% Dimethyl sulphoxide (DMSO) in PBS (phosphate buffered saline), mixed and vortexed for 1 min. The supernatants obtained after centrifugation at 100g for 2 min were used for further studies.

### 3.5 Phytochemical Screening

Phytochemical analysis of all the extracts were performed by standard methods proposed in Trease and Evans [20]. Flavonoids were detected by the method of Shinoda.

### 3.6 Adsorption Column Chromatography

Chloroform extract was found to contain significant amount of flavonoids and was further purified by adsorption column chromatography on silica gel column (5 x 30cm) using silica gel (100-200 mesh). Slurry of silica gel was prepared in petroleum ether and column was allowed to stand for 1h [21, 22]. Dried chloroform extract powder was applied. Petroleum ether (PE), toluene (To), chloroform (Ch), ethyl acetate, acetone, ethanol, water and their mixtures in various proportions in the increasing order of polarity (50ml PE; 40ml PE + 10ml To, 30ml PE + 20ml To, 20ml PE + 30ml To, 10ml PE + 40ml To; 50ml To, 40ml To + 10ml Ch and so on upto 50ml Water) were introduced one after another in continuation. Fractions of 5ml were collected (4ml/min.). Thin layer chromatography of each fraction was performed and similar fractions were mixed. Such 11 different fractions were obtained. Dried fractions were dissolved in 0.1% DMSO in PBS and further tested for stimulating phagocytosis.

### 3.7 Preparation of peritoneal mouse macrophages

Fetal calf serum (FCS) was administered by intraperitoneal injection in rats. Three days later, peritoneal exudates were collected by peritoneal lavage with RPMI 1640 medium. The exudates were centrifuged at 300g for 20 min at  $25^{\circ}\text{C}$  and the 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  $1 \times 10^6$  cells/ml with hemocytometer and cell viability was tested by the trypan blue dye exclusion method [23].

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### 3.8 Phagocytic Index Assay

Ability of the various fractions of chloroform extract to stimulate phagocytosis was tested by NBT dye reduction assay [24]. Macrophages ( $1 \times 10^6$  cells/well) suspended in complete RPMI 1640 medium were treated separately with 100 $\mu$ g/ml of each fraction dissolved in 0.1% DMSO in PBS in 5% CO<sub>2</sub> humidified atmosphere (Indian Equipment Corporation 3821). DMSO (0.1%) in PBS (without any fraction) was considered as control. Zymosan A (1 $\mu$ g/ml of RPMI) was introduced as a stimulant. Absorbance was measured at 570 nm using micro plate reader (Thermo electron Corp., model 358). Phagocytic index (PI) was calculated by the following equation:

$$PI = \text{O.D. of experimental} / \text{O.D. of control}$$

Effect of PF to stimulate phagocytosis was tested separately in a similar way as mentioned above with different concentrations (100, 10, 1, 0.5, 0.1, 0.01  $\mu$ g/ml).

### 3.9 Cellular lysosomal enzyme activity assay

The ability of PF to stimulate lysosomal degranulation was tested using *p*-NPP (*p*-nitrophenyl phosphate) assay [25]. Macrophages ( $1 \times 10^6$  cells/well) in complete RPMI 1640 medium were treated with PF (100, 10, 1, 0.5, 0.1, 0.01  $\mu$ g/ml) dissolved in 0.1% DMSO in PBS in 5% CO<sub>2</sub> humidified atmosphere. The 0.1% DMSO in PBS (without plant extract) was taken as control. Absorbance was measured at 405nm using micro plate reader. The lysosomal enzyme activity index (LI) was calculated according to the following equation:

$$LI = \text{O.D. of experimental} / \text{O.D. of control}$$

### 3.10 Preparation of rat splenocytes

Wister albino rats were sacrificed and spleens were removed aseptically and splenocyte suspensions were prepared [26]. The splenocytes were washed twice and suspended in complete RPMI 1640 medium. The cell number was adjusted to  $1 \times 10^6$  cells/ml by counting in hemocytometer and cell viability was tested by the trypan blue dye exclusion technique.

### 3.11 Splenocyte proliferation assay

Effect of PF on splenocyte proliferation was tested according to XTT [Sodium 2, 3,-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-(phenylamino) carbonyl -2H- tetrazolium] method [27]. Splenocyte suspension ( $1 \times 10^6$  cells/ml) in complete RPMI 1640 medium were incubated in presence of PF (100, 10, 1, 0.5, 0.1, 0.01  $\mu$ g/ml) dissolved in 0.1% DMSO in PBS in 5% CO<sub>2</sub> humidified atmosphere. The 0.1% DMSO in PBS (without plant extract) was taken as control. One set was treated with mitogen LPS (10  $\mu$ g/ml in PBS). Absorbance was measured at 450nm using microplate reader. The splenocyte proliferation stimulation index (SSI) was calculated according to the following equation:

$$SSI = \text{O.D. of experimental} / \text{O.D. of control}$$

### 3.12 Isolation of lymphocytes from rat blood

Blood was withdrawn from orbital plexus of Wistar albino rats and lymphocytes were isolated using Ficoll histopaque. The lymphocytes were washed twice with PBS and resuspended in complete RPMI 1640 medium (RPMI 1640 with 10% FCS and 1% antibiotic- antimycotic solution). The cell number was adjusted to  $1 \times 10^6$  cells/ml by counting in hemocytometer and cell viability was tested by the trypan blue dye exclusion technique.

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### 3.13 Lymphocyte proliferation assay

Effect of PF on lymphocyte proliferation was carried according to XTT in almost similar manner as that of splenocyte proliferation [27]. The lymphocyte proliferation stimulation index (LSI) was calculated according to the following equation:

$$LSI = \text{O.D. of experimental} / \text{O.D. of control}$$

### 3.14 Statistical analysis

Statistical analysis of experimental data was performed using Sigma Plot 10 software. Data were expressed as mean  $\pm$  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.

## IV. RESULTS

### 4.1 Qualitative phytochemical analysis of all the extracts for flavonoid content

Result of the phytochemical analysis for flavonoids is shown in table 1. The petroleum ether extract has not shown any flavonoid content while toluene extract has shown the minimum of it. Ethanol and water extracts contain significant amount of flavonoids but chloroform extract has shown the highest flavonoid content.

Test	Petroleum Ether	Toluene	Chloroform	Ethanol	Water
Flavonoids (Shinoda Test)	-	+	+++	++	++

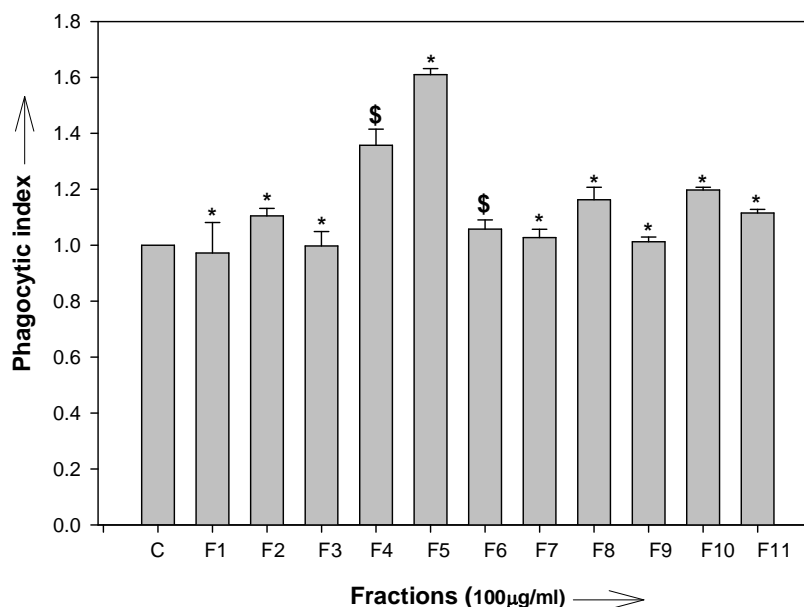
**Table 1. Qualitative analysis of flavonoid content of all the extracts.** Chloroform extract is showing highest flavonoid content.

### 4.2 Fractionation of chloroform extract by adsorption column chromatography

On fractionation of 8 g of chloroform extract by adsorption column chromatography on silica gel column, 2.03 g of the Purified Fraction (PF) (5<sup>th</sup> fraction) was obtained.

### 4.3 Effect of the various fractions of chloroform extract on phagocytic activity

All the 11 fractions collected from chloroform extract of stem bark were tested for stimulating phagocytosis at 100 $\mu$ g/ml concentration (Fig. 1). The 5<sup>th</sup> fraction, named as Purified fraction (PF) was found to be most active in stimulating phagocytosis nearly 1.6 times more than the control.

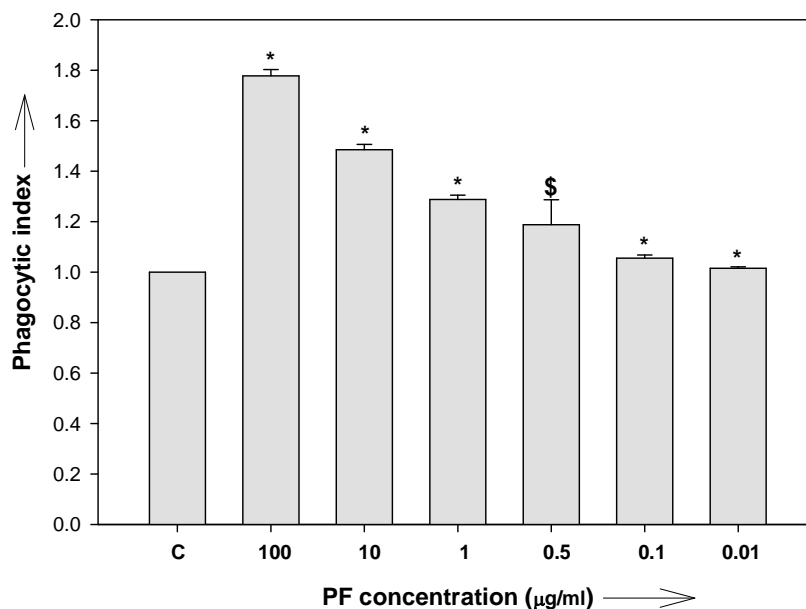


**Fig. 1.** Effect of various fractions of chloroform extract of stem bark at 100µg/ml concentration on phagocytic activity. The 5<sup>th</sup> fraction was found to be most active.

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

#### 4.4 Effect of PF on phagocytic index

PF was explored for its phagocytosis stimulating activity at different concentrations (100, 10, 1, 0.5, 0.1, 0.01µg/ml) by dissolving it in 0.1% DMSO in PBS. Even at 1µg/ml concentration, it has shown a phagocytic index of approximately 1.3 (Fig. 2). But, PF was not found to be much effective at low concentrations like 0.1µg/ml and 0.01µg/ml.

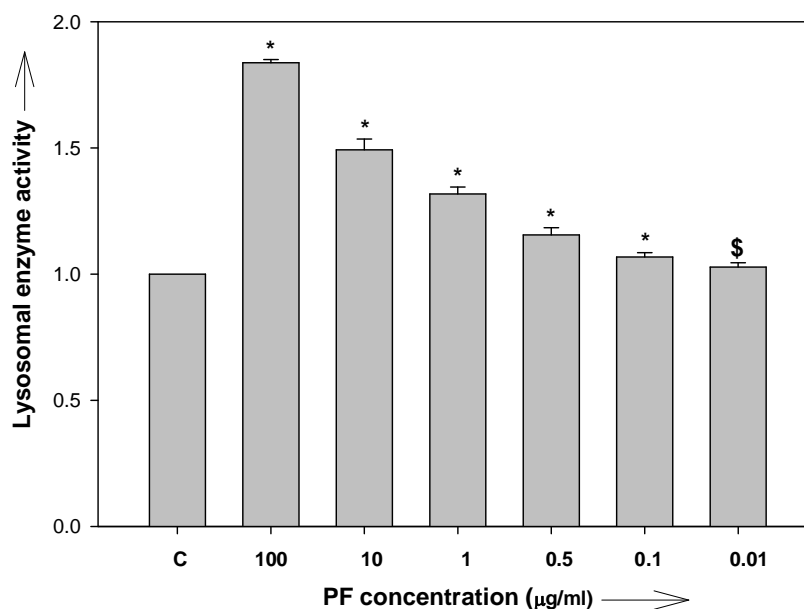


**Fig.2.** Effect of different concentrations of PF (0.01µg/ml, 0.1µg/ml, 0.5µg/ml, 1µg/ml, 10µg/ml and 100µg/ml) on phagocytic activity. Dose dependent increase in PI was observed.

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

#### 4.5 Effect of PF to stimulate lysosomal degranulation

PF has shown a dose dependent increase in the activity on lysosomal enzymes. At 100 µg/ml concentration, the lysosomal enzyme activity (LI) has shown an increase of about 1.85 times while at 10 µg/ml concentration it was approximately 1.5 times (Fig. 3).



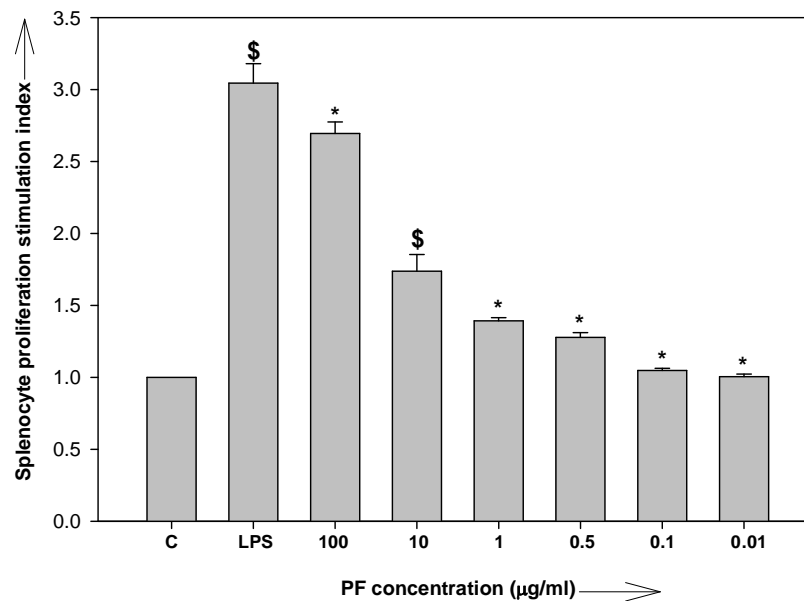
**Fig.3.** Effect of different concentrations of PF (0.01µg/ml, 0.1µg/ml, 0.5µg/ml, 1µg/ml, 10µg/ml and 100µg/ml) on lysosomal enzyme activity. Dose dependent increase in LI was observed.

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

#### 4.6 Effect of PF to stimulate splenocyte proliferation

PF was found to be quite effective in stimulating splenocyte proliferation. The splenocyte stimulation index (SSI) even at a concentration of 10µg/ml was found to be more than 1.75. So, the result was found to be significant as compared to the result shown by LPS which is a very effective mitogen (Fig. 4).

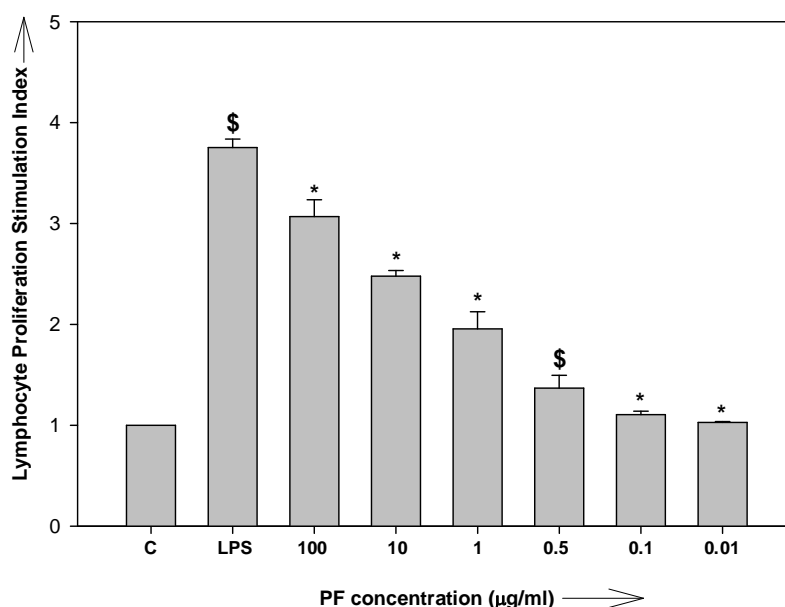




**Fig.4.** Effect of different concentrations of PF (0.01µg/ml, 0.1µg/ml, 0.5µg/ml, 1µg/ml, 10µg/ml and 100µg/ml) on splenocyte proliferation stimulation index (SSI). Dose dependent increase in SSI was observed. [Each value has been expressed as mean  $\pm$  SD of five independent experiments. *P* values less than 0.01 (\**P*<0.01) and 0.05 (<sup>\$</sup>*P*<0.05) were considered to be statistically significant].

#### 4.7 Effect of PF to stimulate lymphocyte proliferation

As for splenocytes, PF was found to be more effective in lymphocyte proliferation too. Even at 1 µg/ml concentration, the lymphocyte stimulation index was found to be approximately 2. (Fig. 5).



**Fig.5.** Effect of different concentrations of PF (0.01µg/ml, 0.1µg/ml, 0.5µg/ml, 1µg/ml, 10µg/ml and 100µg/ml) on lymphocyte proliferation stimulation index (LSI). Dose dependent increase in LSI was observed. [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].

## V. DISCUSSION

Immunostimulants are substances, which can stimulate either innate or adaptive arms of immune system. Many synthetic immunostimulants are launched by pharmaceutical companies but with many side effects.

Phagocytosis is the first weapon of innate immunity, governed by the phagocytic cells like neutrophils, macrophages etc. Even a small decrease in the ability of phagocytosis can cause serious infections. PF, a fraction rich in flavonoids obtained from chloroform extract of stem bark by adsorption column chromatography had shown a great impact in stimulating phagocytosis in a dose dependent manner. Even at low concentrations, PF has shown significant increase in Phagocytic Index (PI). These results were supported by lysosomal enzyme (acid phosphatase) activity assay indicating a very positive direction.

Spleen is an important organ of immune system. It mainly takes care of the blood born infections. So, increase in the splenocyte count is a good indicator of immunity. Similarly, lymphocytes are the pillars of acquired immunity. B lymphocytes are involved in antibody formation while CD<sub>8</sub> (T<sub>C</sub>) cells are engaged in direct cytotoxic activity but the most important of these are CD<sub>4</sub> (T<sub>H</sub>) cells which are constantly involved in boosting the immunity up. They do so by secreting a number of different cytokines. The mitogenic potential of PF can be seen by its ability to stimulate splenocytes and lymphocyte proliferation.

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## VI. CONCLUSION

The results presented here indicate that Purified Fraction (PF) isolated from *Zizyphus mauritiana* stem bark has ability to stimulate immune system in a positive direction. PF has shown a great impact on both the parts of immunity; innate and acquired. PF had stimulated macrophages for phagocytosis while lymphocytes and splenocytes had been stimulated for proliferation.

To the best of the knowledge of authors the present report is the first of its kind on isolation of natural immunostimulant compounds, especially flavonoids from *Zizyphus mauritiana*.

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