Immunostimulatory Potential of a Non-cytotoxic Exopolysaccharide

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Abstract

A novel exopolysaccharide (EPS) was isolated from a probiotic bacterium, *Bacillus coagulans* RK-02. *In vitro* proliferation studies showed that the EPS stimulated both the splenocytes and thymocytes. The nitric oxide induction of the EPS was tested on peritoneal macrophages. It was observed that the EPS induced macrophages to produce NO. The immunostimulatory studies of the EPS was also tested *in vivo*. The results indicated that the EPS stimulated splenocytes, thymocytes and induced the macrophages to produce NO significantly. The toxicity of the EPS was evaluated in both the *in vitro* and *in vivo* studies. Toxicity studies of the EPS clearly indicated that the purified EPS was non toxic.

Keywords: Exopolysaccharide; *B. coagulans*; splenocytes; thymocytes; proliferation

1. Introduction

The probiotic bacteria play beneficial role in the ecosystem of the human and animal gastrointestinal tract. The spectrum of the beneficial effects can be divided into nutritional, physiological and therapeutic effects. The mechanism of action of these beneficial effects relies on their metabolic end products termed as "probio-active substances" [1]. Exopolysaccharides (EPSs) are one of the important class of potential probioactive molecules. The EPSs are economically important because they can impart functional effects to foods and confer beneficial health effects on the host. These molecules have been proved to have antitumor, immunostimulatory, antioxidant, antiulcer and to lower blood cholesterol activities [2]. EPSs play a major role in the texture, mouth-feel, taste perception and stability of the dairy products and have been widely used in the production of fermented dairy products in Northern Europe, Eastern Europe and Asia [3, 4, 5]. Polysaccharides derived from diverse microbial genera that include *Saccharomyces cerivisiae*, *Ganoderma applanatum, Cordyceps sinensis* have shown very good promise in the treatment of infectious diseases [6, 7]. The fungal and bacterial polysaccharides have been reported to modulate key components of the immune system [8, 6, 9]. It has been postulated that the polysaccharides with significant antioxidant activities stimulate macrophages. This is an important positive immunomodulatory property of the polysaccharides [10, 11]. *Bacillus coagulans* is a new generation, spore bearing lactic acid bacillus with superior probiotic properties in terms of stability, ease of storage and L (+) lactic acid producing capacity [12]. *B. coagulans* is both acid and bile resistant and produces bacteriocin like inhibitory substance (BLIS). For instance, coagulin, which is produced by *B. coagulans* I_4 inhibits the growth of *Listeria* spp, enterococci and other unrelated species [13]. Considering the fact that our EPS is a potentially powerful antioxidant, emulsifying and antihyperglycemic activities [14, 15,16]. The immunostimulatory potential of the EPS was tested by studying the index of proliferations of splenocytes, thymocytes and induction of peritoneal macrophages on addition of the same *in vitro* and *in vivo.* The results of this study are presented and discussed below.

2. Materials and Methods

2.1. Microorganism, medium and cultural conditions

Bacillus coagulans RK-02 was isolated from soil sample containing dried excreta of a poultry farm [12]. The microbial culture was grown at 37 ˚C and 180 rpm in Glucose Mineral Salts Medium (GMSM). GMSM contained glucose 20 g/l, ammonium nitrate 2.2 g/l, potassium dihydrogen phosphate 0.14 g/l, dipotassium hydrogen phosphate 2.2 g/l, magnesium sulphate 0.6 g/l, sodium chloride 0.01 g/l, calcium chloride 0.04 g/l, ferrous sulphate 0.02 g/l. Final pH was adjusted to 6.7.

Isolation and estimation of the EPS

The EPS from *B. coagulans* RK-02 was isolated and purified as described previously [15].

2.2. In vitro testing of immunostimulatory activity

Splenocytes, thymocytes and peritoneal macrophages that were isolated from mice were used to test the in vitro immunomodulation of the EPS.

2.2.1. Splenocytes and thymocytes proliferation

A single cell suspension of spleen and thymus was prepared from the normal mice under aseptic conditions in complete RPMI medium. The contaminating red blood cells (RBC) from splenocytes and thymocytes were removed by hemolytic Gey's solution. The cell concentration was adjusted to 1×10^6 cells/ml. The cells (180 µl) were plated in 96 well flat bottom plates and incubated with 20 µl of various concentrations (1-500 µg/ml) of purified EPS in PBS solution. All cultures were set up in triplicate and incubated for 72 hours at 37 °C in a humidified atmosphere of 5 % CO_2. Proliferation was checked by MTT assay [17]. The % proliferation of the splenocytes and thymocytes was calculated and compared with positive control (Con A treated cells).

2.2.2. Macrophage activation (Nitric oxide (NO) assay)

The peritoneal macrophages were isolated by a protocol described by Zhang et al., 2008. Briefly, a syringe (10 ml) was filled with cold harvest medium (ice cold PBS (10 mM, pH 7.4). Needle was inserted through peritoneal wall along the mouse's left side (spleen side) and injected 10 ml of the cold harvest medium into each mouse. Using the same syringe and needle, fluid from peritoneum was aspirated. Needle was moved away from the viscera to cause tenting of the peritoneal wall, and peritoneal fluid was withdrawn slowly. Needle was removed from syringe and peritoneal fluid was dispensed into a 50-ml conical polypropylene centrifuge tube on ice. The peritoneal exudate cells were centrifuged in a refrigerated centrifuge 10 min at 1000 rpm at 4°C. Supernatant was discarded and resuspended the cell pellet in cold RPMI by gently tapping the bottom of the tube and pipetting up and down [18]. The isolated macrophages (1.5 x 10^6 cells/ml) were seeded in complete RPMI medium in 96-well plate. EPS in PBS was added to wells in different concentrations (1-500 µg/ml). The cells were cultured for 24 hours at 37 °C in humidified atmosphere of 5 % CO_2. The production of NO was estimated by measuring nitrite levels in the cell supernatant with the Griess reagent (Equal volumes of Griess reagent and cell supernatant were incubated together at room temperature for 10 min and absorbance was read at 550 nm. The NO production by EPS treated cells was compared with LPS treated cells [19, 20].

2.3. In vivo testing of immunostimulatory activity of the EPS

Male Swiss albino mice (16±4 g) were used to study the immunomodulatory activity of EPS. The animals were kept under standardized conditions (temperature 21-24 ˚C and a light/dark cycle of 12 h/12 h) and fed a normal laboratory diet. After one week of acclimatization, the mice were divided into one control group and three experimental groups with 6 animals in each group. Animals from group 1 to 3 received doses expressed on the basis of mg EPS/ kg body mass, namely 25, 50 and 100 per day of the EPS by intraperitoneal route for 14 days. The fourth group of animals was treated as control and received only saline. At the end of the 14^{th} day, experimental and control animals were sacrificed and proliferation of splenocytes, thymocytes and peritoneal macrophages was studied.

2.3.1. Splenocytes and thymocytes proliferation

A single cell suspension of spleen and thymus was prepared from the EPS treated mice under aseptic conditions. The suspension was centrifuged to obtain cell pellet. The contaminating red blood cells (RBC) from splenocytes and thymocytes were removed by hemolytic Gey's solution. The cell concentration was adjusted to 1× 10^6 cells/ml. The cells (180 µl) were plated in 96 well flat bottom plates and incubated with 20 µl of various concentrations (1-500 µg/ml) of purified EPS in PBS solution. All cultures were set up in triplicate and incubated for 72 hours at 37 °C in a humidified atmosphere of 5 % CO_2. Proliferation was checked by MTT assay [17].

2.3.2. Macrophage activation (Nitric oxide (NO) assay)

The EPS treated Swiss albino mice were sacrificed by cervical disclocation and peritoneal macrophages were isolated as mentioned above [18]. The isolated macrophages (1.5 x 10^6 cells/ml) were seeded in complete RPMI medium in 96-well plate. The cells were cultured for 24 hours at 37 °C in humidified atmosphere of 5 % CO_2. The production of NO was estimated by measuring nitrite levels in the cell supernatant with the Griess reagent. Equal volumes of Griess reagent and cell supernatant were incubated together at room temperature for 10 min and absorbance was read at 550 nm [19].

2.4. Toxicity study of the EPS

2.4.1. In vitro toxicity study of the EPS

A single cell suspension of fibroblasts was prepared from the normal mice under aseptic conditions according to Bonifacino and Dasso [21]. The cell concentration was adjusted to 1× 10^6 cells/ml. The cells were plated in 96-well flat bottom plates and incubated with various concentrations of purified EPS solution. All cultures were set up in triplicate and incubated for 24 h at 37 °C in a humidified atmosphere at 5 % CO_2. Proliferation was measured by MTT assay [17]. The cytotoxicity of the EPS was further measured by cytotoxicity detection kit (Cayman Chemical Company, MI, USA). A single cell suspension of fibroblasts was prepared from the normal mice under aseptic conditions according to Bonifacino and Dasso [3]. The cell concentration was adjusted to 1× 10^6 cells/ml. The cells were plated in 96-well flat bottom plates and incubated with various concentrations of purified EPS solution. All cultures were set up in triplicate and incubated for 24 h at 37 °C in a humidified atmosphere at 5 % CO_2. Cytotoxicity of the EPS on immune cells was measured by considering lactate dehydrogenase as a marker and LDH produced by the immune cells was measured from the standard graph as mentioned by the supplier. Triton X-100 (1%) was used as positive control.

2.4.2. In vivo toxicity study of the EPS

Male Swiss albino mice (16±4 g) were used to study the *in vivo* toxicity of the EPS. The animals were kept under standardized conditions (temperature 21-24 ˚C and a light/dark cycle of 12 h/12 h) and fed a normal laboratory diet. After one week of acclimatization, the mice were divided into one control group and three experimental groups with 6 animals in each group. Animals from group 1 to 3 received doses expressed on the basis of mg EPS/ kg body mass, namely 100, 1000 and 2000 per day of the EPS by intraperitoneal route for 3 days and mortality was recorded at the end of this period [22].

3. Results and discussion

3.1. In vitro immunostimulatory activity

3.1.1. Splenocytes and thymocytes proliferation

Proliferation of splenocytes and thymocytes is an indicator of immunoactivation. The immunostimulatory effect of EPS on both splenocytes and thymocytes was determined by studying proliferation index using MTT assay. The results as shown in Figure 1 and Figure 2 indicated that the EPS was able to stimulate the proliferation of both the murine splenocytes and

thymocytes with Concanavalin (Con A) (1 µg/ml) as positive control. The maximum splenocyte proliferation index (SPI) was 1.7 at 100 µg/ml of EPS concentration and the maximum thymocyte proliferation index (TPI) was 1.8 at 50 µg/ml of EPS concentration. The SPI and TPI of Con A were 1.7±0.09 and 2±0.06, respectively. The results thus advocated for good immunostimulatory activities of our EPS as the splenocyte and thymocyte proliferations caused by the EPS at 50 and 100 µg/ml concentrations were comparable with those caused by ConA at 1 µg/ml.

The *in vitro* splenocyte proliferation activity of the EPS was compared with that of the other reported plant and fungal polysaccharides as given in Table 1. The SPI of the fungal, plant and bacterial polysaccharides were reported to be 1.02 ±0.09 (50 µg/ml dose), 1.3±0.04 (50 µg/ml dose) and approximately 1.1 (100 µg/ml) for *Phellinum baumii* Pilat (Luo et al., 2009), *Leucaena leucocephala* (Gamal-Eldeen et al., 2007) and *Bifidobacterium* RBL strain [8] respectively and it was 1.6±0.06 for the EPS at 50 µg/ml concentration. The splenocyte proliferation activity of the EPS was found to be considerably higher than that of *Phellinum baumii* Pilat, *Leucaena leucocephala* and *Bifidobacterium*. This suggests that the EPS is a potentially better immunomodulatory agent than other polysaccharides of plant, fungal and bacterial origin as reported in literature. Our EPS produced a TPI very much comparable with that of Con A, a well known mitogenic agent.

The lymphocyte-mediated immunity plays an important role in the cellular and humoral immune responses. The capacity to elicit an effective T- and B-lymphocyte mediated immunity was shown by the stimulation of lymphocyte proliferation response [19]. Thus the proliferation assay in this study showed that the EPS significantly stimulated splenocyte and thymocyte proliferation *in vitro* in a concentration-dependent manner.

3.2. Macrophage activation (Nitric oxide assay)

Polysaccharides are good stimulators of macrophages owing to the presence of various receptors on their membranes. To check the ability of EPS to stimulate macrophages, the amount of nitric oxide evolved from the cell supernatant was measured after the addition of EPS *in vitro*. As shown in Figure 4, the peritoneal macrophages produced a maximum of 63 µM of NO at an EPS concentration of 200 µg/ml, which was exactly 50% higher than that produced by LPS (5 µg/ml), used as a positive control. The production of NO was increased with the increasing concentration of EPS (Fig. 3). The results showed that EPS stimulated the peritoneal macrophages considerably.

The *in vitro* macrophage activation by the EPS was compared with other reported plant and fungal polysaccharides in Table 2. The NO production level by other polysaccharides was 48±2.5 µM/ml for *Ribes nigrum* [23], 53±3.0 µM/ml for *Salicornia herbacea* [24], 38±2.5 µM/ml for *Glycyrrhiza uralensis* [4] and 24.39±0.45 µM/ml for *Enteromorpha intestinalis* [25]. The NO production value for our EPS at the same concentration was 58±2.7 µM/ml. Thus the macrophage activation by the EPS was found to be considerably higher than that by the above mentioned polysaccharides.

Macrophages play an important role in host defense against infectious agents and tumors through the release of effector molecules such as NO [10]. Thus the current study demonstrated that the EPS significantly stimulated NO production by peritoneal macrophages and the degree of stimulation of the EPS was comparable with that of the LPS, used as positive control.

3.3. In vivo immunostimulatory activity

3.3.1. Splenocytes and thymocytes proliferation

In order to validate the findings of the *in vitro* studies, the *in vivo* experiments in mice model were carried out. *In vivo* studies showed that the splenocyte proliferation index (SPI) and thymocyte proliferation index (TPI) were significant in EPS treated groups (with 50 and 100 mg EPS/kg body weight), when compared to the PBS treated group, used as negative control (Fig 4). The TPI was more than SPI for the animals treated with 100 g/kg body weight EPS. The SPI and TPI were almost same for the animals treated with 50 mg EPS /kg body weight. There was no significant increase in the SPI and TPI for the group, which was treated with EPS at a dose of 25

mg /kg body weight. It was also observed that the SPI and TPI of the EPS treated groups were significantly higher than those of the control group (PBS treated animals; $P < 0.05$).

The results thus indicated that EPS treatment significantly increased splenocyte and thymocyte proliferation activity in mice (groups I, II, III) in a dose-depended manner. Moreover, the EPS treatment exhibited a stronger effect on the TPI than SPI in EPS-treated mice at a dose of 100 mg/kg body weight. Thymus is the organ in which T lymphocytes develop, differentiate, and mature, while spleen contains T and B cells [18]. Polysaccharide from marine brown algae was reported to be more mitogenic for B lymphocytes than for T lymphocytes [18]. In this study, it is possible that EPS had similar effect on the B cells. The differences in the mitogenic effects of the EPS on T and B lymphocytes may have resulted in different SPI and TPI values in mice. However, elucidation of the exact mechanism needs further investigation, which is beyond the scope of this thesis.

3.4. Macrophage activation (Nitric oxide assay)

To check the ability of EPS to stimulate macrophages *in vivo*, the amount of nitric oxide produced by the macrophages was measured in case of the EPS treated mice. The peritoneal macrophages produced 82 µM of NO at an EPS concentration of 200 µg/ml. The production of NO increased with the increasing concentration of the EPS (Fig. 5).

Both the *in vitro* and *in vivo* experiments revealed that the EPS stimulated splenocytes and thymocytes and activated peritoneal macrophages. This indicates that the EPS interacts with the splenocytes and thymocytes through the carbohydrate binding pockets for immune stimulation [19]. The magnitude of stimulation was more in case of splenocytes than thymocytes in both the *in vitro* and *in vivo* studies. Macrophages play critical roles in host defense, including phagocytosis of pathogens and apoptotic cells, production of cytokines, and proteolytic processing and presentation of foreign antigens [26, 27]. Thus identification of agents that can modulate macrophages is of significant interest. Indeed a variety of polysaccharides from different sources have been reported to have modulation of macrophage function. In the present study, the EPS was able to modulate the macrophages in both *in vitro* and *in vivo* studies. Because of its low toxicity and high potency, EPS represents an ideal therapeutic candidate with immunomodulatory action. The beneficial microorganisms synthesize vitamins, amino acids, other important bioactive molecules to stimulate immunoglobulin activity and improve immune function [28]. In addition, there are some reports on the relationship between immunostimulatory and antioxidant activities of polysaccharides of plant and microbial origin [29, 30, 11]. Though the exact mechanism of the EPS is not known, based on the results presented above, we can conclude that the EPS showed very significant ($P < 0.05$) immunostimulatory activities. Further investigations on the relationship between immunomodulatory activity and characteristic structure of the EPS are in the future scopes of this thesis.

3.5. Toxicity study of the EPS

The results of the *in vitro* toxicity assay (Fig. 6) showed that the EPS neither proliferated nor killed the mouse fibroblasts. The microscopic pictures of mouse fibroblasts treated with PBS, EPS (100 ng/ml and 1 mg/ml) were shown in Figures 7 (A), (B) and (C). After 24 h of incubation, the number of cells were counted and expressed in number of cells/ 10 μm^2 (Table 3). It was observed that there was no significant difference in cells number. The results indicated that the EPS neither proliferated not killed the fibroblasts. *In vivo* acute toxicity results showed that the EPS was not lethal up to a dose of 2000 mg/kg body weight.

The non-toxic nature of the other reported EPS and their composition have been presented in Table 4. The cytotoxicity of the EPS was also studied by measuring LDH by fibroblasts. The LDH production by EPS treated cells was compared with Triton X-100 (1%) treated cells. The results clearly indicated that the EPS did not show cytotoxicity at 100 µg/ml concentration. It was also observed that the EPS showed insignificant cytotoxicity when compared to the positive

control at higher doses (1000 µg/ml). These results supported the cytotoxicity test results obtained by MTT assay.

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Table 1

Comparison of splenocyte proliferation by our EPS with that of other polysaccharides

Polysaccharide source	Concentration used for the study (µg/ml)	Splenocyte proliferation index (SPI)	Reference
Phellinus baumii	50	1.02±0.06	[31]
	100	1.18±0.09	
Leucaena leucocephala (Plant)	50	1.3±0.04	[26]
Bifidobacterium RBL strain (Bacterium)	100	~ 1.1 (taken from graph)	[8]
Bacillus coagulans RK-02 (Our strain)	50	1.6±0.06	Present study
	100	1.76±0.09	

Table 2

Comparison of degree of macrophage activation in terms of NO production by EPS with other reported polysaccharides from various sources

Polysaccharide source	Concentration of EPS (µg/ml)	NO production (µM/ml)	Reference
Ribes nigrum (plant)	100	48±2.5	[23]
Salicornia herbacea (plant)	100	53±3.0	[31]
Glycyrrhiza uralensis (plant)	100	38±2.5	[32]
Enteromorpha intestinalis (fungal)	50	15.86±1.63	[25]
	100	24.39±0.45	
Bacillus coagulans RK-02 (Our strain)	50	40±3.2	Present study
	100	58±2.7	

Table 3

Number of fibroblasts present at 24 h after treatment with EPS

EPS Concentration	No. of cells/10 µm^2
Control	1.3±0.09[a]
10 ng/ml	1.1±0.16
100 ng/ml	1.1±0.28
1 µg/ml	0.9±0.49
100 µg/ml	0.9±0.23
1 mg/ml	0.8±0.06

[a]Values are the means± S.D (n=3)

Table 4
Comparison of non-toxic activity of polysaccharides with their monomeric composition

S. no	EPS Producer organism	Monomeric composition	Toxic/Non Toxic	Reference
1.	*Lactobacillus casei LC2W*	Glucose, rhamnose and galactose	Non-toxic	[23]
2.	*Lactobacillus kefiranofaciens*	Glucose and galactose	Non-toxic	[33]
3.	*Lactobacillus pentosus LPS26*	Glucose, rhamnose and glucuronic acid	Not reported	[34]
4.	*Botryosphaeria rhodina*	Glucose	Non-toxic	[35]
5.	*L. johnsonii*	Not reported	Non-toxic	[36]
6.	*Amphidinium carterae*	Galactose and Glucose	Non-toxic	[37]
7.	*Rhodotorula glutinins*	Mannose, fucose, glucose, and galactose	Non- toxic	[38]
8.	*B. coagulans* RK-02	Galactose, mannose, Glucose, glucosamine and fucose	Non-toxic	Present study

Figures

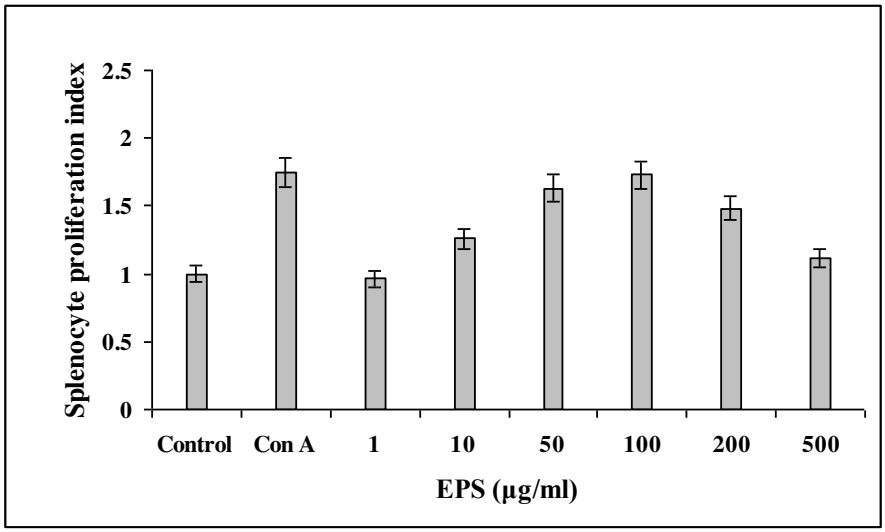

Fig. 1. Effect of different concentrations of EPS on splenocyte proliferation. Splenocytes (1 x 10^6) were stimulated by EPS from the concentrations of 1 µg/ml to 500 µg/ml were applied for 72 h at 37˚C, 5 % CO_2. Cell proliferation was measured by MTT assay method. Data reported as the mean ± S.D of 3 independent observations and compared against PBS control by using student's *t* test. *P* values < 0.05 were considered significant compared against PBS control.

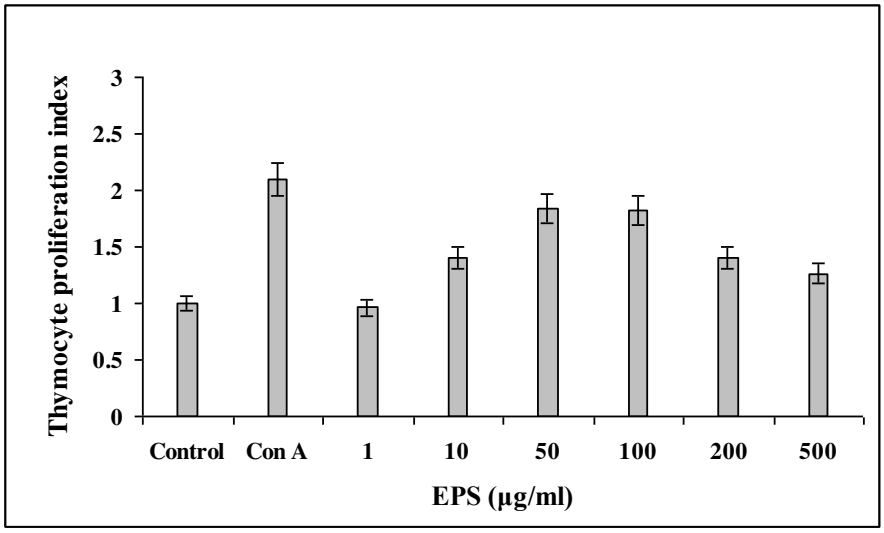

Fig. 2. Effect of different concentrations of EPS on thymocyte proliferation. Thymocytes (1 x 10^6) were stimulated by EPS from the concentrations of 1 µg/ml to 500 µg/ml were applied for 72 h at 37˚C, 5 % CO_2. Cell proliferation was measured by MTT assay method. Data reported as the mean ± S.D of 3 independent observations and compared against PBS control by using student's *t* test. *P* values < 0.05 were considered significant compared against PBS control.

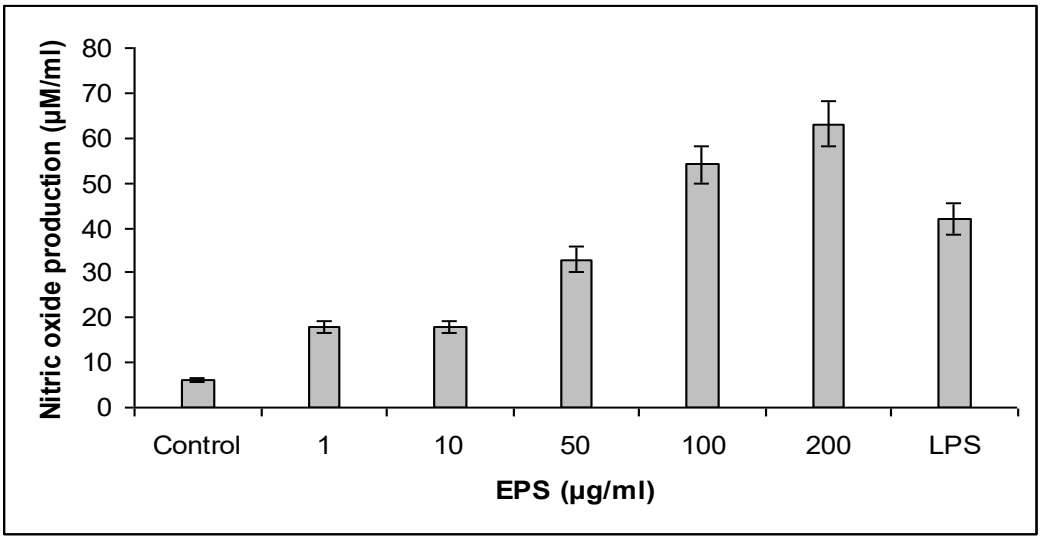

Fig. 3. *In vitro* activation of peritoneal macrophages stimulated with different concentrations of EPS in terms of NO production. Purified EPS in the concentration range 1 µg/ml to 200 µg/ml was used for the stimulation of peritoneal macrophage (1 x 10^6 cells/ml). The amount of NO was measured by Griess reagent after 24 h of incubation. Data reported as the mean ± S. D of 3 different observations. (P< 0.05 by using students's *t* test compared against PBS control).

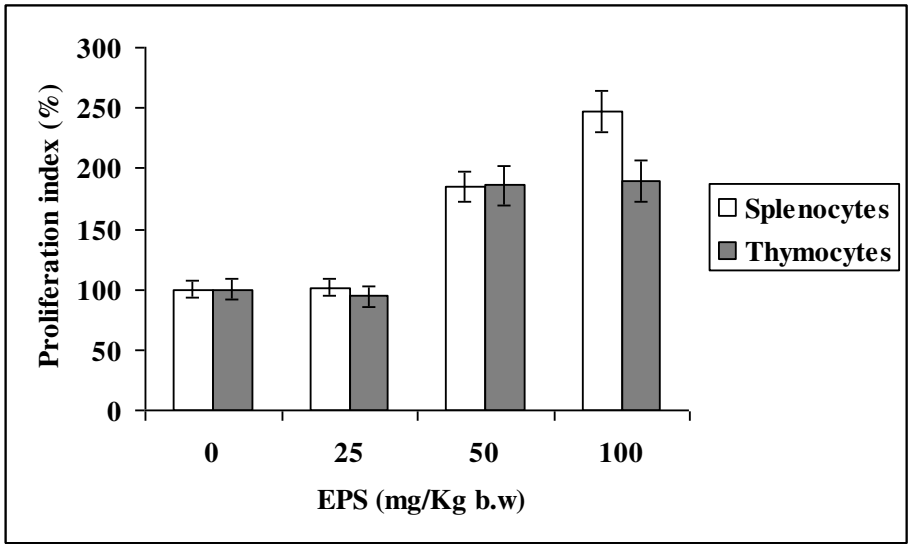

Fig. 4. The splenocyte and thymocyte proliferative effect of EPS on mice. The mice were treated with in different doses of EPS (25, 50 and 100 mg/kg body weight) for 14 days. After treatment of EPS thymocyte and splenocyte proliferation was studied by MTT assay Thymocytes and splenocytes (1 x 10 ^{6}) were seeded complete RPMI medium in 96-well plate. The cells were cultured for 72 h at 37°C in humidified atmosphere of 5 % CO_2. Cell proliferation was measured by MTT assay method. Data reported as the mean ± S.D of 3 independent observations.

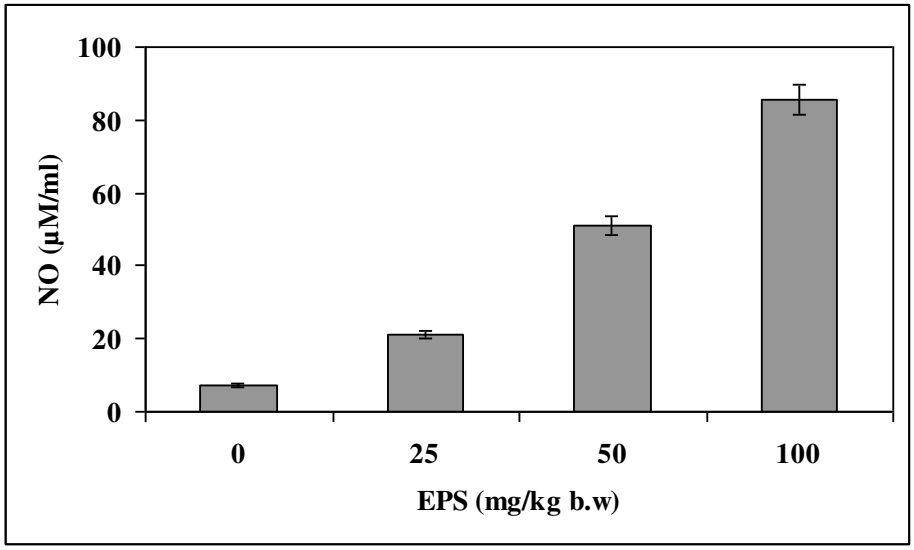

Fig. 5. The activation of peritoneal macrophages on EPS treated mice. The mice were treated with in different doses of EPS (25, 50 and 100 mg/kg body weight) for 14 days. After treatment of EPS, peritoneal macrophages (1 x 10 ^{6}) were seeded in complete RPMI medium in 96-well plate. The cells were cultured for 24 h at 37°C in humidified atmosphere of 5 % CO_2. The production of NO by macrophages was estimated by measuring nitrite levels in the cell supernatant with the Griess reagent. Cell Data reported as the mean ± S.D of 3 independent observations.

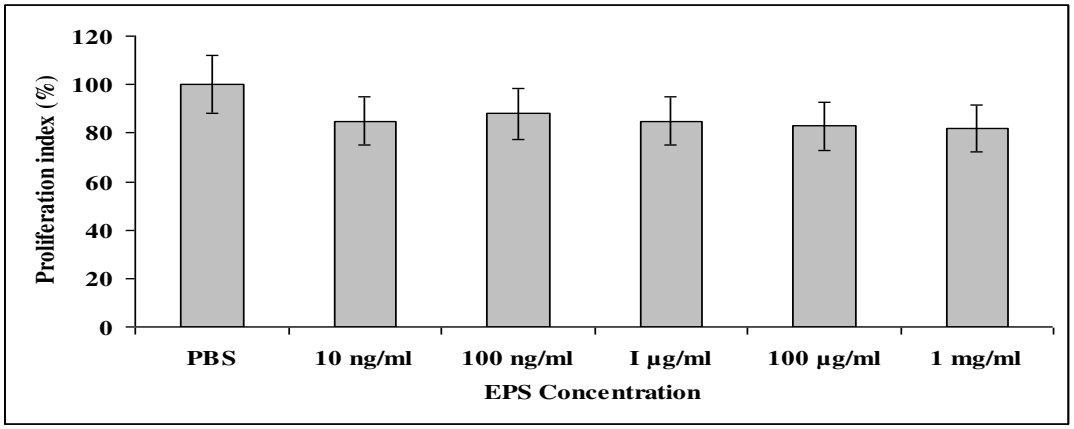

Fig. 6. Effect of different concentrations of EPS on mouse fibroblasts (1×10^6). Cell proliferation was measured by MTT assay. Data reported as the mean ± S.D of 3 independent observations

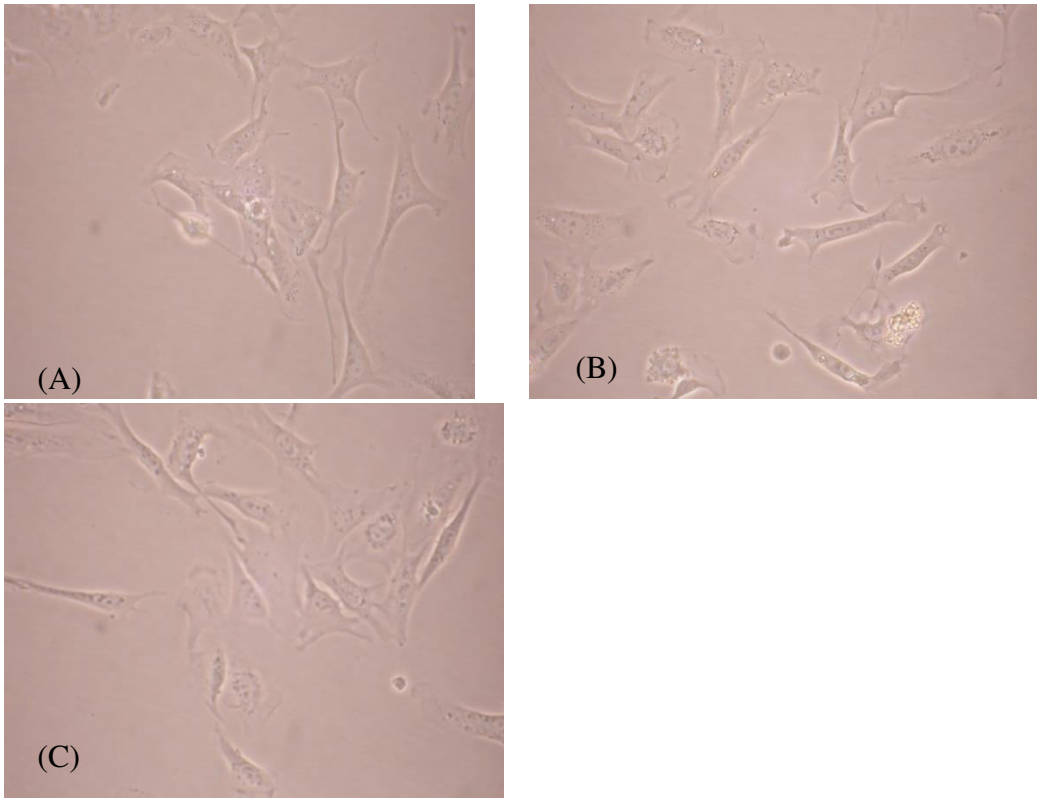

Fig. 7. Micrograph (taken in Olympus CKY 41 Inverted Microscope) of mouse fibroblasts at 24 h after treatment treated with the EPS; A-PBS treated; B-EPS at 100 ng/ml and C-EPS at 1 mg/ml concentrations.

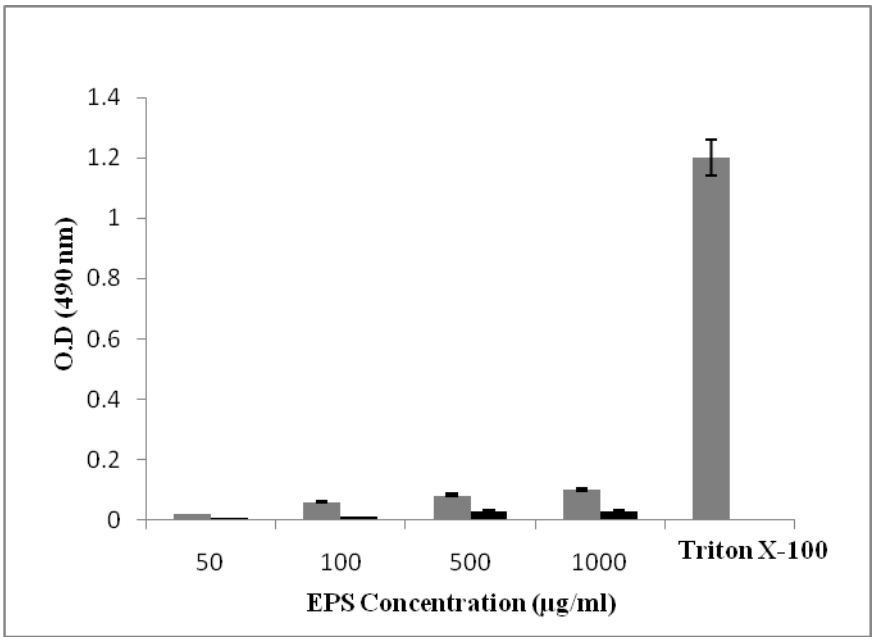

Fig. 8. Lactacte dehydrogenase (LDH) production assay on mouse fibroblasts. The fibrolasts were treated with different concentrations of EPS (1×10^6) for 24 h. Triton X-100 (1%) was used as positive control. ■-Negative control treated with PBS. Data reported as the mean ± S.D of 3 independent observations