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EVALUATION OF *IN VITRO* IMMUNOMODULATORY EFFECTS OF SOYABEAN (*GLYCINE MAX*. L) EXTRACTS ON MOUSE IMMUNE SYSTEM

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ABSTRACT: To explore the potentially health modulatory role of most proteinaceous leguminosae plant, Soyabean, the present study was intended to evaluate the *in-vitro* immunomodulatory activity of dry seeds of *Glycine max.* L. Effect of ethanolic and methanolic extracts of soyabean was evaluated at various concentrations (800µg/ml to 6.25µg/ml) for secretion of various mediators. Immunomodulatory activity of both extracts was examined on murine macrophage phagocytosis by Nitroblue tetrazolium (NBT) dye reduction, nitric oxide, lysosomal enzyme activity and myeloperoxidase activity. Proliferation of spleenocytes and bone marrow cells was evaluated by 3 (4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with and without lipopolysaccharide (LPS) as mitogen. Both aqueous and acetone extracts presented immunomodulatory activity in peritoneal mouse macrophages, bone marrow cells and spleenocytes without clear dose response relationship. In-vitro phagocytic index and proliferation index showed significant results and thus proving the need for confirmation through *in-vivo* studies.

INTRODUCTION: The immune system is involved in the etiology as well as pathologic mechanisms of various diseases. Immune system is designed to protect the host from potentially invading pathogenic agents like Parasites, viruses, bacteria, fungi etc., to eliminate neoplastic cells and to reject foreign components. The structural and functional alterations in the immune system may lead to immunosupression and immunodepression which modifies the host's immune system against infection. [1] To overcome the immunosupression, immunostimulant drugs are require to boost the immune system to combat the consequences such as stress, chronic diseases and conditions of impaired immune response as Tuberculosis, AIDS etc.[2]

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Glycinemax. L commonly called Soya bean, belonging to the family of Leguminosae is a perrinial shrub growing to a height of 0.6mm-6.6meters. Soya bean is a widely used high nutritive food in many parts of the world. Despite of the food and traditional nutritive uses, these beans are also valued for folkloric herbal remedies for treating diseases such as head ache, constipation, blurred vision, edema, jaundice, beri-beri, enteritis, diarrhea, night blindness, sore eyes, food or drug poisoning and infantine malabsorbtion and malnutrition. [3]

Soya beans are rich in many bioactive phytochemicals which include flavonoids (Kaemferol & quercitin), isoflavones (daidzein, biochemin A, genistein, fomononetin), phytosterols (compesterol & coumesterol), Organic acids (indole -3- acetic acid, phytic acid & syringic acid) [4] and saponins. They also have a variety of potential health benefits such as anti-inflammatory [5], antioxidative [6], anti-mutagenic [7], anti-carcinogenic [8, 9 10, 11] anti-arterial contraction [12],

anti-diarrhea [13], antiviral [14] and hepatoprotective effects. [15] Soya beans, additionally have estrogenic activity play a role in cancer prevention, prevent & moderate menopausal symptoms & other health risks [16, 17, 18].

Soya is also beneficial in preventing osteoporosis. Recently, many of these phytonutrients in soya beans are reported to contribute to the immunomodulatory properties, due to the presence of immunomodulatory nutrients such as Vitamins (A , B2 , C & E) and minerals (copper , zinc , iron, selenium). [19, 20]

Phenolic compounds which include caffeic, chlorogenic, jerulic and P-Coumaric acids are the common organic acids present in soyabean which also contribute to the immunomodulatory properties of the plant. [21]

The present study was carried out to further explore the potentially health modulatory role of most proteinaceous leguminosae plant. The immunomodulatory activities of ethanol and methanol extracts of soyabean were evaluated on phagocytic response of peritoneal mouse macrophages and proliferation activity of mouse bone marrow cells and spleenocytes were studied.

MATERIALS & METHODS:

Plant material & Preparation of Extracts:

Dry seeds of *Glycine max.*L (JS -335 cultivar) were obtained and authenticated from Dr. Sukumar, Principal Scientist & Head, Dept of Plant Breeding, Regional Agriculture Research Station (RARS), Adilabad, Andhra pradesh , India . The seeds were collected in January 2013.

250gms of dry seeds of soya bean were boiled in 1000ml of distilled water for 1hr and filtered by gauze. The filtrate was then extracted by continuous extraction in sohxlet apparatus with ethanol (1000ml) at 70^{o}C & methanol (1000ml) at 80^{o}C until exhaustion. The suspension was filtered & evaporated under reduced pressure to give viscous greenish brown mass. Thus crude extracts of ethanol (SBE) & methanol (SBM) were obtained which gave the percentage yields of 2.37 & 3.76 (w/w of the dried seeds) respectively.

Experimental Animals:

Female Swiss albino mice, 4-5 weeks old (30 ± 3g) were procured from an authorized vendor (M/S Raghavendra Enterprises, Bangalore, India) and were housed (four per cage) in polypropylene cages (18" x 10" x 8") lined with paddy husk, under standard laboratory conditions (temperature 30 ± 2˚C; light and dark 12:12 h) at the Animal Facility of the Department of Biotechnology, Sri Padmavati Mahila Visvavidyalam, Tirupati.

The mice were fed on standard pellet chow (purchased from HLL Animal Feed, Bangalore, India) and water *ad libitum*. All the experiments were conducted in accordance with the guidelines for the care and use of laboratory animals (NRC 1996) and all procedures were in compliance with the laws of the country. The study was conducted after obtaining Institutional Animal Ethical Committee clearance.

Chemicals:

Fetal bovine serum (FBS) & phytohemagglutinin – M (PHA) were procured from sigma Aldrich (St. Louis, Mo, USA). Penicillin, Streptomycin, Amphotericin, Roswell Park Memorial Institute (RPMI) 1640 medium & HEPES buffer were procured from Himedia Pvt. Ltd, India. Nitroblue Tetrazolium (NBT) and Tetramethyl Benzedrine Hydrogen peroxide (TMB / H_2O_2) were purchased from Bangalore Genei, India. All other chemicals used for the experiments were of analytical grade.

Isolation of peritoneal macrophages:

2ml of 4% (W/V) fluid thioglycollate medium was injected intraperitoneally into mice as a stimulant to elicit peritoneal macrophages. Three days later, the resulting peritoneal cells were harvested by peritoneal lavage with 10ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 medium and cultured in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin & 0.25µg/ml amphotericin B. The exudate was centrifuged at 1000rpm at 25^{o}C for 20min. The erythrocytes in the cell pellets were lysed by hypotonic solution (0.2% NaCl). Isotonicity was restored with 1.6%. NaCl solution. Cell suspension was centrifuged and the cells were washed twice and resuspended in RPMI 1640.

The macrophage count was determined by using haemocytometer and cell viability was tested by trphan blue dye exclusion technique Then the cells were adjusted to required cell count per ml ($1X10^6$ cells/ml), plated on a 96 well flat bottom culture plate and incubated for 2hrs at 37^oC in 5% Co_2 humidified incubator.

After removing the non-adherent cells, the mono layered macrophages were treated with SBE and SBM extracts separately dissolved in complete RPMI medium containing 0.1% DMSO & maintained for 24 hrs at 37^0C in a 5% Co_2 humidified incubator. [22] Following in vitro assays were performed on these incubated cells. PHA (100µg/ml) was used as a positive control. All the experiments were performed in triplicate.

***In vitro* phagocytic assay on NBT dye reduction:**

The NBT dye reduction assay was carried out according to the method previously described [23]. 20µl of plant extracts (SBE & SBM), 20µl of the adherent macrophages ($1x10^6$cells/ml) and 40µl of complete RPMI were put in a flat bottom 96well plate. The final concentrations of the plant extracts were 6.2, 12.50, 25, 50, 100, 200, 400, 800 µg/ml per well. After incubation for 24hrs at 37^0C in humidified 5% Co_2, 50µl of 0.3% NBT solution in PBS (Phosphate buffered saline, P^H -7.4) was added and the mixture was further incubated at the same conditions.

After incubation for 1hr, the adherent macrophages were rinsed vigorously with complete RMI medium & washed four times with 200ml methanol. After air drying, formazan deposits were solubilized in 120 µl of 2 M KOH & 140µl of DMSO. The absorbance was read at 630 nm by using a microplate reader. Stimulation index (SI) was calculated as the OD ratio of the treated & control macrophages.

Nitrite Assay:

Nitric accumulation was used as an indicator of nitric oxide (NO) production in the medium as previously described by Lee et al. Peritoneal macrophages ($5x10^5$cells/ml) prepared as above were incubated with different concentrations (800-6.25 µg/ml) of SBE and SBM extracts for 24hrs at 37^0C in 5% Co_2 atmosphere. Cell-free supernatant (50 µl) was mixed with 50 µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min.

The optical density (OD) was measured at 540 nm with a microplate reader (ELX800MS, Bio-tek Instruments Inc., USA). PHA (100 µg/ml) was used as a positive control. Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) was calculated as the nitrite concentrations ratio of the treated and control macrophages. [24]

***In vitro* Phagocytic assay on cellular lysosomal enzyme activity:**

The cellular lysosomal enzyme activity of macrophages was evaluated by measuring acid phosphatase activity as described earlier. Briefly, 20 µl of the adherent macrophages (1×106 cells/ml) and 40 µl of complete RPMI were put in a flat bottom 96-well plate. The SBE & SBM extracts (20 ml) were added in different concentration (800 – 6.25 µg.ml). After incubation for 24hrs at 37^0C in humified 5% Co_2, the medium was removed by aspiration and 20µl of 0.1% Triton X-100 (Himedia, India) were added to each well.

After 15 minutes incubation, 100 ml of 10 mm *p*-nitro phenyl phosphate (*p*NPP) and 50 ml of 0.1 M citrate buffer (pH 5.0) were added. Further the plates were incubated for 1h and 0.2 M borate buffer (150 µl, pH 9.8) was added. The OD was measured at 405 nm by using a microplate reader. The Phagocytic stimulation index (SI) was calculated as the OD ratio of the treated and control macrophages. [25]

Myeloperoxidase Activity Assay:

Myeloperoxidase activity was evaluated on isolated macrophages as previously described [26] 24 hrs incubated peritoneal macrophages ($5x10^6$cells/ml) with different concentrations. (800 – 6-25 µg/ml) of SBE & SBM test extracts were washed thrice with complete RPMI medium.

Then the mixture of *O*-Phenylenediamine (0.4 g/ml) & 0.002% H_2O_2 in phosphate - citrate buffer (p^H -5-0) was added to each well. The reaction was

stopped after 10 min using 0.1 N H_2SO_4. The optical density (OD) was measured at 490 mm with a microplate reader. The myeloperoxidase stimulation index (SI) was calculated as the OD ratio of the treated and control cells.

Isolation of bone marrow cells and spleenocytes:
Mice were sacrificed and their femur and spleen were removed aseptically. Cell suspension was prepared by means of loose potter & flushing. [27] An amount of 1×10^7 cells was placed in a 16-mm well and incubated in 5% CO2 at 37°C for 3hr. The supernatant together with the non-adherent cells were collected by centrifugation at 1000rpm at 37°C for 10min. The cell pellets were resuspended in complete RPMI medium and adjusted to 1×10^6 cells/ml. Cell numbers and viability were determined by a hematocytometer and trypan-blue dye exclusion technique, respectively.

***In vitro* Proliferation Assay:**
The proliferation of bone marrow cells and spleenocytes was tested accordingly to the 3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by (Mossmann, 1983) , 20µl of the (800 – 6.25 µg/ml) of SBE and SBM extracts were added to 20µl of cell suspension (1 X 10^6 cells/ml) that was previously overlaid with 40µl of complete RPMI medium in a 96 well microplate reader. Proliferation of cells in the absence & presence of nitrogen lipopolysaccharide (LPS) 5µg/ml was investigated. After incubation at 37^0C in humidified 5% Co_2 atmosphere for 48 hrs, 20µl of MTT in PBS (5mg/ml) was added and overlaid with 40µl of complete RPMI medium.

The culture medium was gently removed & 100µl of 0.04M HCl in isopropanol was added to lyse the cells. Then, 100µl of deionized water was added to dilute the solution and the absorbance was measured at 570nm. The proliferation stimulation index (SI) was calculated as the absorbance ratio of treated system & nitrogen alone.

Statistical analysis: All experiments were performed in triplicate and the results were expressed as mean ± S.E. Student's *t*-test was used to analyze statistical significance of the differences between the control and the treated values.

RESULTS:

***In vitro* phagocytic Assay on Nitroblue Tetrazolium (NBT) dye reduction:**
The *in vitro* phagocytic effects of different concentrations of SBE and SBM extracts on reduction of NBT dye on peritoneal macrophages isolated from mouse are presented in **Table 1**. The ethanolic extract of soyabean (SBE) showed the significant stimulation ($p<0.05$) of NBT reduction at 100µg/ml (S.I=2.061) and 50 µg/ml (S.I= 1.603) with respect to control (S.I=1.00).

Similarly, methanolic extract of soyabean (SBM) enhanced the NBT reduction at 100µg/ml (S.I=1.962) and 50 µg/ml (S.I= 1.551) when compared to control (S.I= 1.00). PHA, the positive control also showed significant stimulation of NBT reduction (S.I= 1.872). Both extracts appeared to give phagocytic modulation without dose response relationship being ethanol the active extract, when compared to methanol.

Lysosomal enzyme activity:
The release of cellular lysosomal enzyme was found to be significant ($p<0.05$) at 200 µg/ml (S.I= 1.982) and 100 µg/ml (S.I= 1.564) of SBE and 200 µg/ml (S.I=2.075) and 100 µg/ml (S.I=1.662) of SBM with respect to controls (S.I=1.00) (**Table 1**). Positive control PHA showed significant stimulation ($p<0.05$) of lysosomal enzyme release (S.I= 1.782) for both extracts.

Nitrite assay:
The nitrite, the stable breakdown product of nitric oxide (NO) produced in cell culture supernatants was measured at 24hrs of treatment. This showed that the SBE extracts induced nitrite production in statistically significant higher amounts at 200µg/ml (S.I=2.312), 50 µg/ml (S.I=1.854) and 100 µg/ml (S.I=1.7620 and SBM extracts significantly increased the amounts at 200 µg/ml (S.I= 2.306), 100 µg/ml (S.I= 1.864) and 50 µg/ml (S.I=1.698) concentrations with respect to the control (S.I=1.00) (**Table 1)** in all three experiments performed.

PHA (positive control) showed significant increase in nitrite release (S.I=2.185). High nitric oxide (NO) production is an indication of macrophage activation.

Myeloperoxidase activity Assay:

The effect of different concentrations of SBE and SBM extracts on myeloperoxidase activity of macrophages is shown in **Table 1**. The SBE extract showed significant ($p<0.05$) stimulation of myeloperoxidase activity at 100 µg/ml (S.I=1.762), 50 µg/ml (S.I=1.451), 200 µg/ml (S.I=1.498) and in case of SBM extract at 100µg/ml (S.I=1.837), 50µg/ml (S.I=1.585), 200µg/ml (S.I=1.556) as compared to controls of both extracts (S.I=1.00). PHA (positive control) showed significant stimulation with S.I value 1.695.

TABLE: 1. *IN VITRO* PHAGOCYTIC EFFECTS OF DIFFERENT CONCENTRATIONS OF ETHANOL AND METHANOL EXTRACTS OF SOYABEAN ON PERITONIAL MACROPHAGES OF MOUSE

Sl. No	Conc. (µg/ml)	NBT Assay		Lysosomal		Nitrite Assay		Myeloperoxidase	
		SBE	SBM	SBE	SBM	SBE	SBM	SBE	SBM
1	Control	1.000±0.05	1.000±0.04	1.000±0.05	1.000±0.06	1.000±0.05	1.000±0.03	1.000±0.04	1.000±0.05
2	800	1.312±0.02	1.045±0.04	1.450±0.08	1.381±0.05	1.395±0.08	1.401±0.07	1.205±0.08	1.106±0.03
3	400	1.268±0.04	1.281±0.07	1.552±0.03	1.557±0.03	1.549±0.03	1.625±0.09	1.197±0.02	1.235±0.07
4	200	1.542±0.03	1.421±0.03	1.982±0.05	2.075±0.09	2.312±0.05	2.306±0.05	1.498±0.04	1.556±0.09
5	100	2.061±0.03	1.962±0.08	1.564±0.08	1.662±0.05	1.762±0.04	1.864±0.03	1.762±0.06	1.837±0.04
6	50	1.603±0.07	1.551±0.06	1.495±0.03	1.543±0.08	1.854±0.06	1.698±0.05	1.451±0.03	1.585±0.06
7	25	1.358±0.05	1.320±0.05	1.275±0.06	1.357±0.07	1.552±0.08	1.495±0.04	1.188±0.07	1.311±0.07
8	12.5	1.225±0.08	1.087±0.03	1.250±0.09	1.089±0.05	1.370±0.06	1.476±0.08	1.192±0.04	1.129±0.09
9	6.25	1.201±0.05	1.123±0.07	1.305±0.06	1.271±0.03	1.340±0.03	1.294±0.05	1.055±0.05	1.142±0.08
10	PHA (100)	1.872±0.04	1.872±0.01	1.782±0.07	1.782±0.05	2.185±0.05	2.185±0.07	1.695±0.06	1.695±0.05

Proliferation Assay response of plant extracts on mouse bone marrow cells and Spleenocytes:

For proliferation response, effect of different concentrations of SBE and SBM extracts on proliferation of bone marrow cells and spleenocytes in the presence and absence of LPS (mitogen) is reported. Both extracts exhibited proliferation activity of both bone marrow cells and spleenocytes. Moreover there was cell proliferation stimulation with no dose response relationship. Suppression of proliferation at higher concentration was observed. The ethanolic extract (SBE) gave the maximum proliferation enhancement both with and without mitogen, as measured by MTT assay.

In the presence of LPS, SBE extract significantly ($P<0.05$) increased proliferation of bone marrow cells at 200µg/ml (S.I=2.912) and SBM increased the activity at 400µg/ml (S.I= 2.902).

Whereas the cell proliferation in absence of mitogen was seem to be increased at 200µg/ml (S.I=2.857) with SBE and at 400µg/ml (S.I=2.651) with SBM when compared to control (S.I=1.00) (**Fig.1** and **2).** Positive control, PHA showed significant proliferation with S.I value 2.761 and 2.409 when tested with and without mitogen for both SBE and SBM.

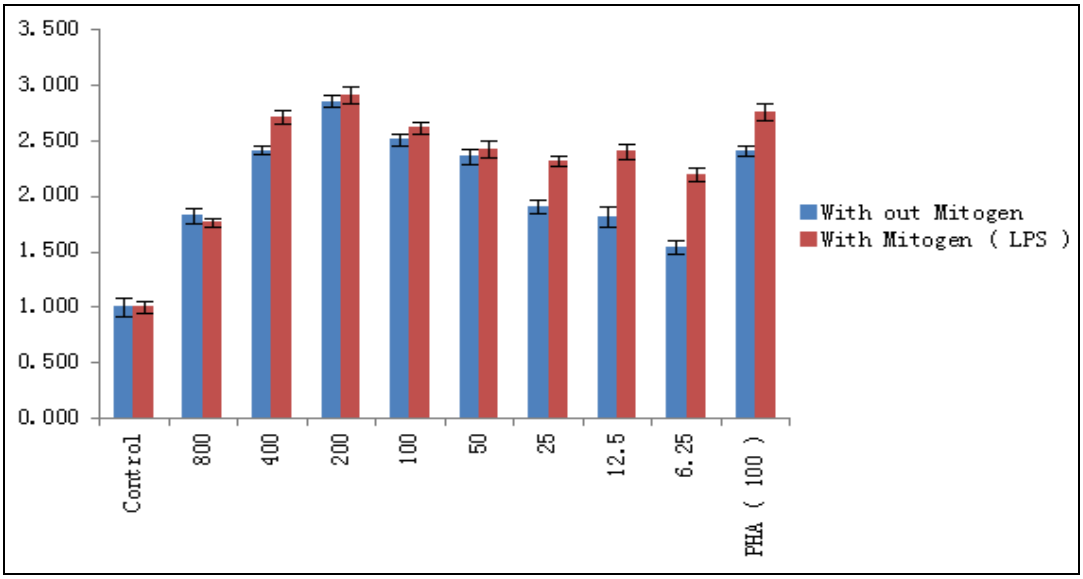

FIG. 1: PROLIFERATION RESPONSE OF MOUSE BONE MARROW CELLS WITH AND WITHOUT MITOGEN WHEN TREATED WITH SBE

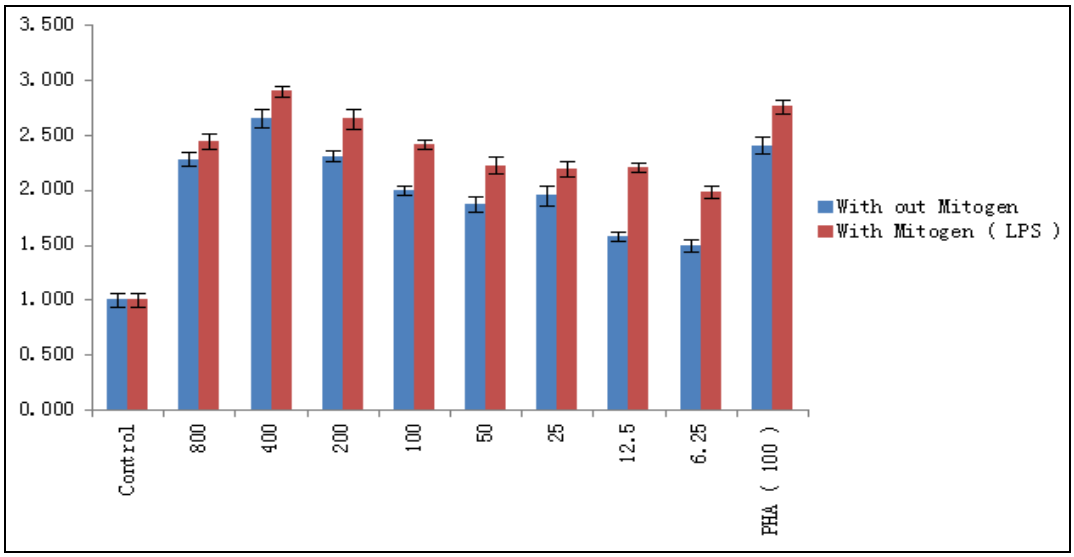

FIG. 2: PROLIFERATION RESPONSE OF MOUSE BONE MARROW CELLS WITH AND WITHOUT MITOGEN WHEN TREATED WITH SBM

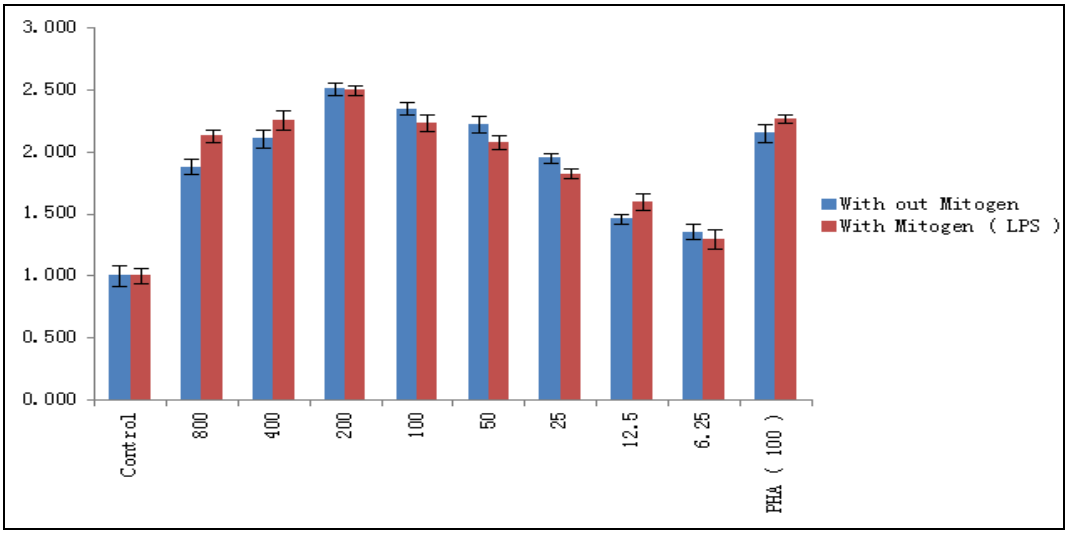

FIG.3: PROLIFERATION RESPONSE OF MOUSE SPLEENOCYTES WITH AND WITHOUT MITOGEN WHEN TREATED WITH SBE

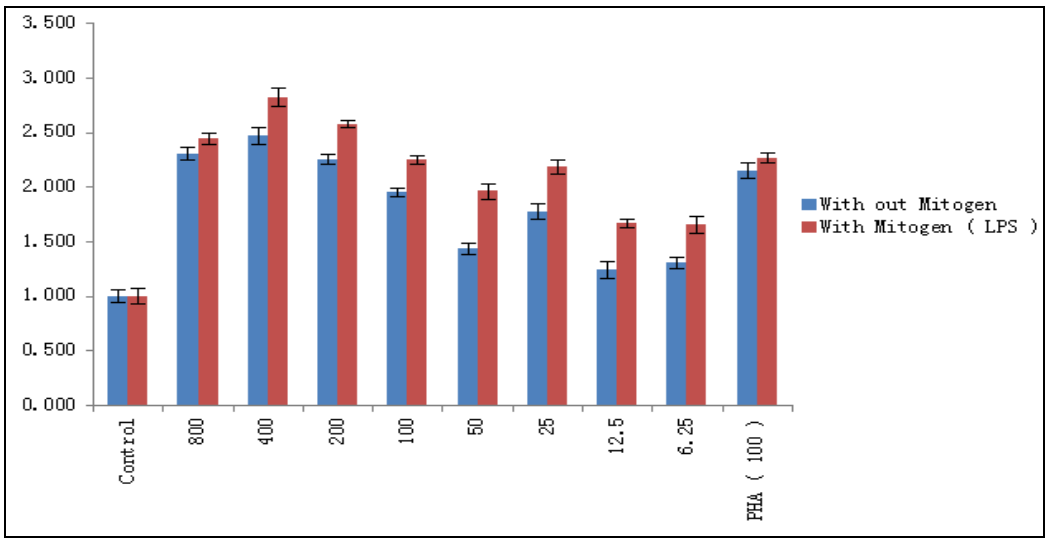

FIG. 4: PROLIFERATION RESPONSE OF MOUSE SPLEENOCYTES WITH AND WITHOUT MITOGEN WHEN TREATED WITH SBE

DISCUSSION: Immunomodulators are the substances which alter the immune response by augmenting or reducing the ability of the Immune System to produce antibodies or sensitized cells.[29] Due to increased stress conditions, mind and body are significantly showing impact on Immune response in general. There has been a tremendous increase for the drugs which are effective on immune system. Now, there is a need for the identification and development of natural and herbal Immunomodulators.[29] Specific antibodies bind to specific antigen leading to its activation or even phagocytosis. [30] Macrophages play an important role in the defence mechanism against host infection by innate and acquired immunity. Macrophages are the cells that present antigen to lymphocytes during the development of specific immunity and also increase the phagocytic activity. In the process of phagocytosis, they produce cytokines, reactive oxygen species (ROS) and nitrogen species (RNS) which are involved in the destruction of pathogen. [31]

The main objective of the study was to investigate the preliminary immunomodulatory effects of seeds of Soyabean (*Glycine max*.L) using its ethanol and methanol extracts (SBE & SBM). This study demonstrated that all extracts stimulated phagocytic activity on the tested four *In vitro* assays, NBT dye reduction, lysosomal enzyme activity, nitrite (NO) and myeloperoxidase activity on the macrophages isolated from mouse.

The phagocytosis of particles by macrophages is usually accompanied by a burst of oxidative metabolism allowing the generation of oxygen species which can be detected through reduction of NBT dye. [32] The higher reduction in NBT assay represented higher activity of oxidase enzyme reflecting the stimulation of phagocytes in proportion to the foreign particles ingested. [33] For lysosomal enzyme activity, the transformation of *p*-NPP to coloured compound by acid phosphatase of the stimulated macrophage correlates to the extent of degranulation in phagocytosis. [34]

The effects of various concentrations of ethanol and methanol extracts of soyabean (SBE & SBM) on the reduction of NBT dye reduction and lysosomal enzyme activity responses were studied by phagocytic assay. SBE and SBM extracts gave phagocytic modulation, but had no dose response relationships. Nitric oxide (NO) has been found to be the main effector molecule produced by macrophages by inducible nitric oxidosynthetase (iNOS) for cytotoxic activity and can be used as a quantitative index of macrophage activation. [33, 35]

Agents that modulate the activity of NO may be of considerable therapeutic value (Bhaskara, 2002). NO mediates diverse functions, including vasodilatation, neurotransmission, antithrombotic and inflammation. The ethanol extract of soya bean (SBE) has shown significant increase in release of nitric oxide from macrophages. This effect of SBE was not dose-proportionate. The released NO could be toxic to immune and inflammatory cells as well as microorganisms and tumour cells. Thus, NO might be regarded as both immune effector and regulatory molecules. Nitric oxide has been reported as an important mechanism for macrophages against microorganisms. [36]

Myeloperoxidase is a heme protein secreted by neutrophils and macrophages which use the oxidizing potential of H_2O_2 to convert chloride ion into hypochlorous acid (HOCl), a potent bactericidal agent. HOCl is an important component of host defense mechanism against invading pathogens. [37] On exposure of different concentrations (800-6.25µg/ml) of SBE and SBM extracts, stimulation index of myeloperoxidase increases thereby indicating the enhanced defense capability of these cells to pathogenic organisms. The mouse peritoneal macrophages were significantly stimulated by the exposure to these extracts by release of immune mediators. This suggests that SBE and SBM extracts can effectively strengthen innate immunity against foreign particles. [38]

MTT assay was used for bone marrow cells and spleenocyte proliferation, because of its ability to be cleaved by all metabolically active cells, for assaying cell survival and proliferation and the amount of MTT formazan generated is directly proportional to the cell number.[33] LPS was used for T cell independent B cell proliferation whereas PWM was used for T cell-dependent B cell proliferation. The presence of mitogens in the

system can postulate the possible activation pathway of the extracts. [39] As measured by MTT assay, the SBE and SBM extracts with and without mitogen (LPS) produced different effects on bone marrow cells and spleenocyte proliferation. With LPS both extracts enhanced the proliferation activity higher in SBE extracts when compared to SBM extracts. The results suggested that the extracts especially, the ethanol extract had specificity towards B cell proliferation through T cell independent pathway in both bone marrow cells and spleenocytes.

CONCLUSION: The present study has demonstrated the *in vitro* immunomodulatory property of ethanol and methanol (SBE & SBM) extracts of soyabean, *Glycine max*.L. The results suggest that SBE and SBM influence the non-specific immune system by the process of phagocytosis and B cell proliferation. However, it can also be suggested that the active compounds responsible for immunomodulatory activity in *Glycine max* could be extracted by either ethanol or methanol. It can be concluded that the non nutrients of soyabean such as flavonoids, plant acids and plant hormones most likely provide immunostimulation inspite of their nutrient values.

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