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Anti-inflammatory activity of aqueous extract of leaves of *Prosopis spicigera* using flow cytometry

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A B S T R A C T

Anti-inflammatory activity of aqueous extract of leaves of *Prosopis spicigera* (50, 100 and 300 mg/kg body weight) in Swiss mice using cyclophosphamide as an immunosuppressant and was evaluated the antibody (IgG) titre, peritoneal macrophages activation and estimates the T cell surface markers i.e. CD3, CD4 and CD8 and also analyzed the Th1 type of cytokines (IFN-gamma and TNF alpha) in serum. Mice were immunized with cyclophosphamide two days prior to immunization with antigen bovine serum albumin (BSA, 1 mg/ml). The results showed that the aqueous extract of *Prosopis spicigera* showed a significant decrease in the antibody (IgG) titre, peritoneal macrophage activation and T cell surface markers i.e. CD3, CD4 and CD8 at a dose range of 300 mg/kg as compared to control. No mortality was occurred in all the tested drug samples. Overall, the aqueous extract showed anti-inflammatory effect on the cell mediated immune (T cell surface markers) response, macrophage activation and Th1 type of immune cytokines functions in mice.

Keywords: *Prosopis spicigera*, bovine serum albumin, cyclophosphamide

A R T I C L E I N F O

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1. Introduction

Medicinal plants played a significant role in the prevention of human being from various pathogenic microorganisms and the diseases. In nature there are numerous medicinal plants which are used as immunomodulator agents [1, 2, 3]. Immunomodulation means to modulate the immune system either stimulatory e.g. can be used to help combat viral infection such as AIDS and cancer or suppressive e.g. preventing rejection of transplanted organs [4]. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases especially autoimmune disorders where selective immunosuppressant is desired. It appears that the normal way by which the immune system works is through its own modulation by factors usually synthesized by the immune cells. It is tempting to speculate that the restorative and rejuvenating power of these herbal remedies might be due to their action on the immune system and some of the medicinal plants are believed to enhance the natural resistance of the body to infections [5, 6].

Plant derived materials (glycosides, flavonoids, polysaccharides, etc.) isolated from medicinal plants have been shown to stimulate the immune system. According to Ayurveda and other Indian literature mention the use of plants in the treatment of various human infections or diseases [5, 6, 7]. It is estimated that about 25 – 30 % of all modern medicines for bacterial or viral infections are directly or indirectly derived from higher plants. The medicinal plants of India can open avenues of economic growth in the emerging world market, it has been realized that most of the medicinal plants of the Indian Himalayan region offer an advantage in having much greater possibilities of providing novel biomolecules in view of the environmental stress. Generally, most of the medicinal plants constitute a common alternative treatment of immunocompromised patients in many countries around the world. Approximately, most of the anti-inflammatory drugs currently used and have been isolated from the plants.

At this time, more than 3000 plants worldwide have been reported to possess anti-inflammatory properties [5, 6]. Out of these, one of the medicinal plant i.e. *Prosopis spicigera* leaves have been commonly used in traditional medicine for the treatment of various human ailments for many years. *Prosopis spicigera* (commonly known as shami) belonging to the family Fabaceae [8], is commonly found in Baramati region, Maharashtra. Due to the lack of awareness about its medicinal properties of shami, it is used very less for therapeutic purposes.

Generally, this plant is densely branched thorny tree and is grown abundantly in dry and arid regions of India. Most of the tribal people used this plant as fodder and source of wood. According to the literature of this plant shami which is already reported as anti-bacterial, anti-hyperglycemic and anti-oxidative activities [9, 10, 11, 12 and 13]. In this study, the leaves of shami were to investigate the anti-inflammatory activity in immunocompromised mice using flow cytometry.

2. Materials and Methods

2.1. Plant material

The plant *Prosopis spicigera* was collected from Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, Maharashtra, India. The plant was washed with sufficient quantity of distilled water. For the preparation of aqueous extract, leaf sample was macerated to finely powder form and this was used for the immunological studies. The aqueous extraction usually done in phosphate buffered saline and crushed in a mortar pestle and the extract was centrifuged at 10000 rpm at 4 °C for 10 minutes. The supernatant was collected and was used for various *in vitro* and *in vivo* immunological assays.

2.2. HPTLC analysis and bio-inorganic fingerprinting of leaves of *Prosopis spicigera*

The aqueous extract of leaf of *Prosopis spicigera* was subjected to various qualitative and quantitative investigations of secondary metabolites i.e. alkaloids, terpenoids, glycosides, phenolics and saponins determined through HPTLC. The solvents and other purified reagents, HPTLC plates (10 x 10 cm) were purchased from Qualigens and Merck. The stock solution of the leaves of *Prosopis spicigera* was prepared for HPTLC studies and dissolved the leaves in phosphate buffered saline or with different solvents. Plate was scanned by using densitometric scanner. The aqueous extract of leaf of *Prosopis spicigera* showed the presence of glycosides (RF value 3.26), terpenoids (RF value 0.96) and saponin (RF value 0.34 – 0.46).

Bio-inorganic fingerprinting of *Prosopis spicigera* is done by using atomic absorption spectroscopy (AAS). *Prosopis spicigera* plant leaves are analyzed for trace metal contents-iron (Fe), copper (Cu), Magnesium (Mg), Manganese (Mn), Calcium (Ca) and Zinc (Zn). Sample preparation was done in both water and HCl. Data is recorded in ppm. The aqueous extract of *Prosopis spicigera* showed the presence of Cu (0.46), Fe (0.55), Mn (3.18), Mg (0.1), Ca (1.01) and Zn (0.1). Further studies are taken, HPLC analysis of the aqueous extract it was observed that it's totally endotoxin free fraction.

2.3. Cyclophosphamide-induced immunosuppression

Briefly, Swiss mice (female, 5–6 weeks old) were distributed into five groups consisting of five animals. The animals were properly maintained as per ethical guidelines. Immune system suppression using cyclophosphamide (250 mg/kg) two days prior to immunization with specific antigen bovine serum albumin (BSA, 1 mg/ml) was given to all mice by a single intraperitoneal injection. Animals were divided into five groups of five animals each: (Group I) control, received normal saline; (Group II) cyclophosphamide, 250 mg/kg body weight; (Group III) 50 mg/kg body weight, (Group IV) 100 mg/kg body weight and (Group V) 300 mg/kg body weight. The leaves of *Prosopis spicigera* was dissolved in phosphate buffered saline and was administered per oral for 14 days. The dose volume was 0.2 ml. On day fourteen, EDTA blood samples were collected from retro-orbital plexus of all animals for the estimation of cell surface marker CD3, CD4 and CD8 using flow cytometry and also estimates the antibody titre

and Th1 type of cytokines by Elisa. All these studies are done according to ethical regulations on animal research; all animals used in experimental work received humane care.

2.4. Estimation of IgG titre by Elisa

In brief, Elisa plate wells were coated with 100 µl BSA solution (1 mg/ml) for IgG antibodies in 50 mM carbonate-bicarbonate buffer, pH 9.6) for 24 h at 4 °C. The wells were washed three times with PBS containing Tween 20 and then blocked with 5 % FCS/PBS at 37 °C for 1 h. After three washings, 100 µl of diluted serum sample (IgG, 1: 100) was added to triplicate wells of 96 well plate. Again, the plates were incubated for 1 h at 37 °C, followed by three times of washing. Aliquots of 100 µl of goat anti-mouse IgG horse radish peroxidase conjugate diluted 1 : 1000 with 0.5 % FCS/PBS were added and then further incubated for 1 h at 37 °C. After washing, the TMB substrate solution was added and the plate was incubated for 10 min at 37 °C and enzyme reaction was terminated by adding 50 µl/well of stop solution i.e.2 N H_2SO_4. The optical density (OD) was measured in an ELISA reader at 450 nm.

2.5. Flow cytometric analysis in immunocompromised mice using

a) Whole blood

Flow cytometry analysis of whole blood of Swiss mice for counting and examine the cells count suspended in a stream of fluid. On day 14, for the estimation of variable doses of aqueous extract of *Prosopis spicigera* (50 – 300 mg/kg, body weight) in immunized mice on whole blood using forward and side scatter gating applied for data acquisition of 10000 events of cell populations representing different phenotypes analyzed using cell quest software. In this experiment, 100 µl of whole blood was taken in each tube. FITC labeled CD8+, CD3+ and PE labeled CD4+ monoclonal antibody were added directly to 100 µl of whole blood. Falcon tubes were incubated in dark for 30 min at room temperature. Subsequently, 2 ml of 1× FACS lysis solution was added at room temperature with gentle mixing followed by incubation for 10 min. The samples were spinned (300 – 400 × *g*) and the supernatant was aspirated and washed two times with phosphate buffered saline. After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer [14].

b) Peritoneal macrophages

Swiss mice were injected intraperitoneally with 10 ml of phosphate buffered saline. The abdomen was gently massaged and peritoneal cells were lavaged out in tubes. The peritoneal cells were washed thrice with phosphate buffered saline by centrifugation at 1000 rpm, 10 min in cold and finally suspended at 2 x 10^6 cells/ml in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions of immunized mice of variable doses of aqueous extract (50 – 300 mg/kg, body weight) were added in each 6 well plate and then add again exposure of aqueous extract. Samples were incubated for 24 h at 37^{o}C in CO2 incubator and then analyzed the forward and side scatter using flow cytometer.

2.6. Estimation of Th1 (IFN-gamma) and Th2 (IL-4) cytokines in serum by Elisa

Cytokine concentrations in the serum were determined by ELISA kits that were specific against murine cytokines. Levels of Th1 (IFN-gamma) and Th2 (IL-4) cytokines were measured using ELISA (BD optia, ELISA kit). Assays were performed according to the manufacturer's instructions [15, 16].

3. Results and Discussion

3.1. Effect of aqueous extract of Prosopis spicigera on

3.1.1 Antibody titre

Oral administration of aqueous extract of *Prosopis spicigera* (50 - 300 mg/kg) for 14 days showed the following reaction in Swiss mice. The control animals immunized with antigen bovine serum albumin did not show any characteristic humoral antibody titer. The results showed that animals treated with dose of aqueous extract of *Prosopis spicigera* (50 mg/kg) showed significant decrease in humoral antibody titer at higher doses as shown in **Fig.1** when compared with the control group. Cyclophosphamide used as standard and also showed a significant decrease in antibody titre as compared to control.

3.1.2 T (CD3, CD4 and CD8 surface markers)

In this study, the higher dose of the aqueous extract showed significant result. The higher dose i.e. 300 mg/kg showed significant decrease in T cell surface markers i.e. CD3, CD4 and CD8 when compared with control group as shown in **Fig. 2**. The standard drug cyclophosphamide also showed the significant decrease in T cell surface markers as compared to control.

3.1.3 Peritoneal macrophages

The results showed that animals treated with higher dose (300 mg/kg) of aqueous extract of *Prosopis spicigera* and standard drug cyclophosphamide (250 mg/kg) showed significant decrease in the activation of macrophages as shown in **Fig. 3** which is confirmed through flow cytometry when compared with the control group.

3.1.4 Th1 (IFN-gamma and TNF alpha) cytokines in serum

The results obtained from the animals that received higher dose of aqueous extract showed that there was a highly significant decrease in the Th1 cytokines when compared to control group **(Fig. 4**). The effect of this extract were comparable to the standards drug cyclophosphamide all the data represents the immunosuppressive activity of aqueous extract of *Prosopis spicigera.*

Discussion

The present study evaluated the anti-inflammatory activity of aqueous extract of *Prosopis spicigera* on the following bioassays: IgG titre; CD3, CD4 and CD8 surface marker in whole blood, peritoneal macrophages activation and also determined the Th1 type of cytokines in serum. The aqueous extract derived from *Prosopis spicigera* was strongly active at higher doses in all the tested bioassays. It showed the inhibitory activity on the antibody titre, cell surface markers (i.e. CD3, CD4, CD8), peritoneal macrophages and also decreases the level of Th1 level of cytokines in serum. In the present study, it showed the inhibitory effect at higher doses on the immune functions in mice. Inhibitory effects were observed on the humoral immune response which is confirmed through Elisa it showed that the plant showed a decrease at all doses in antibody titre as compared to control. When the effect of

aqueous extract of *Prosopis spicigera* on humoral immune response to BSA was examined, it was found that oral administration of aqueous extract into mice markedly augmented the antibody response to BSA.

The result indicated a inhibitory effect of aqueous extract on the ability of mice to produce antibodies against a T dependent antigen. The data showed that aqueous extract had potent anti-inflammatory activity *in vivo*. This type of finding was consistent with other reports or published and demonstrated the active substances isolated from several traditional Chinese medicinal herbs [17] for anti-inflammatory activities. The inhibitory effect may be due to the release of mediators of hypersensitivity reactions and tissue responses to these mediators in the target organs by *Prosopis spicigera.*

Cell mediated immunity, mediated by T lymphocytes which played an important role to fight against intracellular infections. Among the T lymphocytes, helper T cells induce B lymphocytes to secrete antibodies and cytotoxic T lymphocytes help phagocytes to destroy infection induced by pathogen and to kill intracellular pathogens or microbes. Humoral response, however mediated by antibodies which are produced by B lymphocytes and functions as to neutralizing and eliminating the extracellular microbes or pathogens [18, 19, 20]. The capacity to elicit an effective T cell immunity can be shown by the stimulation of lymphocyte proliferation response and cytokine estimation. The results indicated that shami could significantly inhibit the potential of T cells in BSA-immunized mice. The results of cell mediated response and status of IFN-TNF and phagocytic activity after immunization with T-dependent antigen suggest that the activity of *Prosopis spicigera* could be mediated through the anti-inflammatory effect on T lymphocytes and macrophages [18, 19, 20]. Macrophages are cells reside within the tissues that originate from specific white blood cells called monocytes which are present in the blood. Monocytes and macrophages are phagocytes, acting in either non-specific defense (or innate immunity) as well as specific defense (or cell-mediated immunity) of vertebrate animals. The results showed that there is significant decrease in level of macrophages at higher doses as compared to control.

Furthermore, it has been demonstrated that the Th1 cytokine IFN-gamma is an important B cell switch factor for the induction of antigen-specific IgG2a-secreting B cells and that many viral infections induce an antibody-mediated response characterized by a predominance of IgG2a (Coutelier *et al*, 1988). Indeed, our data showed that mice immunized with BSA, aqueous extract had a significant decrease in the Th1 cytokine IFN-gamma and TNF alpha in serum [18, 19, 20].We speculated that aqueous extract might mediate their function *in vivo* in mice by reducing the induction of proinflammatory cytokines, which resulted in inhibiting CD4+ and CD8+ T-cell response. The inhibitory effect on CD4+ and CD8+ T cells, thereby confirming its general effect on the cell-mediated immune response.

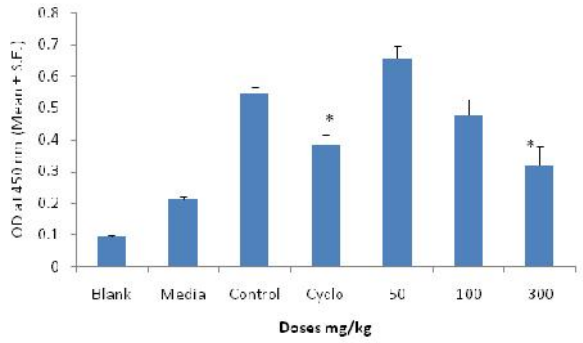
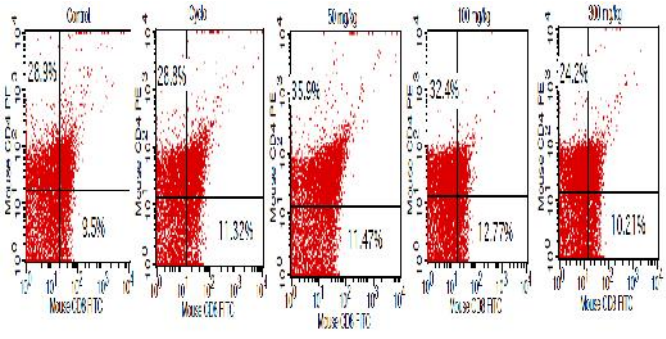


Figure1: ELISA assay. On day 14, blood samples were collected from retro-orbital plexus for the estimation of antibody titre. The results are presented as Mean ± S.E. P values: *P < 0.05, **P < 0.01, ***P < 0.001 as compared to control.



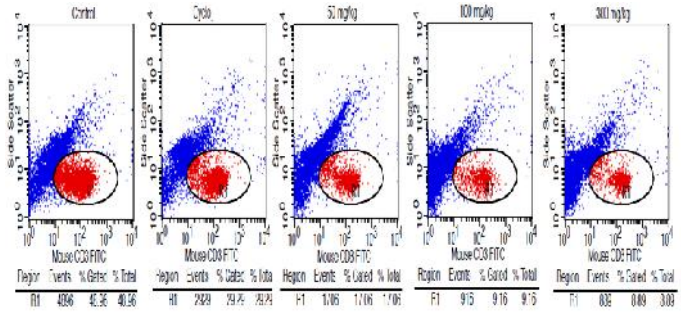
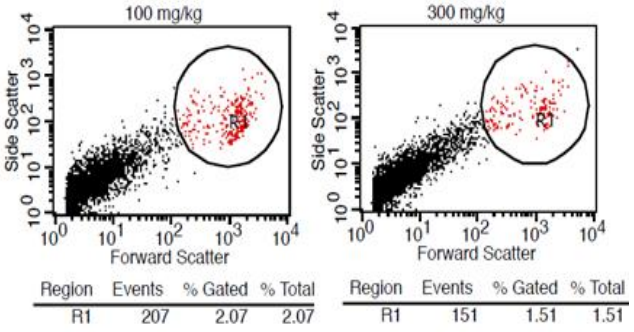


Figure 2: Effect of aqueous extract of Prosopis spicigera on T cell surface markers i.e. CD3, CD4 and CD8 using flow cytometry. EDTA whole blood was collected on day 14 for the estimation of T cell surface markers. Staining of whole blood with FITC conjugated monoclonal antibody i.e. CD3 and CD8 and PE conjugated monoclonal antibody i.e. CD4.



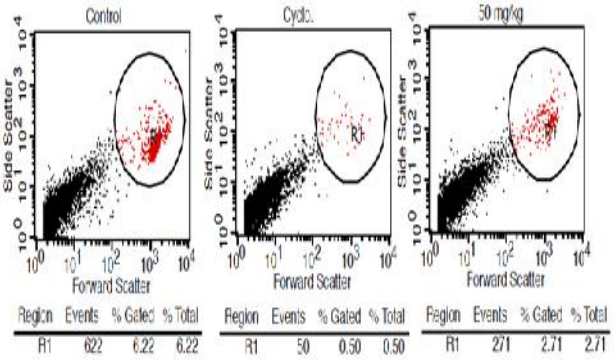


Figure 3: Effect of aqueous extract of *Prosopis spicigera* on macrophage function. Mouse peritoneal cells were collected on day 14. Mouse peritoneal cells (2 x 10_6 cells/ml) dissolved in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions containing 2 x 10_6 cells/ml of immunized mice of variable doses of aqueous extract (50 – 300 mg/kg, body weight) were added in each 6 well plate and then add again exposure of aqueous extract. Samples were incubated for 24 h at 37_oC in CO2 incubator and then analyzed the forward and side scatter using flow cytometer.

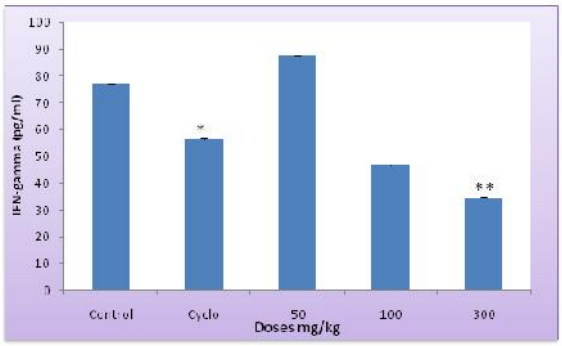
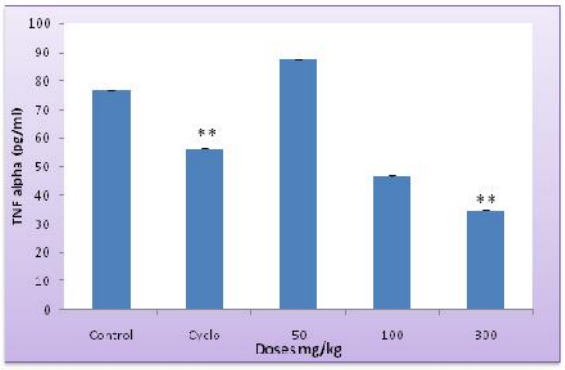


Figure 4: Effect of variable doses of aqueous extract of leaf of Prosopis spicigera on Th1 (IFN-gamma and TNF alpha) cytokines in mouse serum. Groups received variable doses of leaf aqueous extract (50,100 and 300 mg/kg) from day 0 to 14. Animals were bled on day 14 after the secondary immunization and the sera were evaluated for the estimation of Th1 (IFN-gamma, TNF alpha) cytokines. Each bar represents the group mean (n=5). Value for the concentration of cytokine expressed in pg/ml. P values: *P < 0.05, **P < 0.01 and ***P < 0.001 when compared to the value of control.



4. Conclusion

Oral administration of aqueous extract of *Prosopis spicigera* leaves displayed an anti-inflammatory effect on antibody titre, cell surface markers (i.e. CD3, CD4 and CD8) and macrophages activation and also restored the suppressive effects induced by cyclophosphamide in mice. Moreover, leaves aqueous extract (300 mg/kg) showed anti-inflammatory effect. Therefore, the present investigation indicates that aqueous extracts from leaves of *Prosopis spicigera* have a significant dose dependent anti-inflammatory effect in mice. Thus, support the traditional use of *Prosopis spicigera* as immunoprotective and could be a promising alternative for cancer therapy.

5. References

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