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IMMUNOSUPPRESSIVE ACTIVITY OF SAPONIN FROM THE LEAVES OF *ADHATODA VASICA* USING FLOW CYTOMETRY

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ABSTRACT

Immunosuppressive activity of saponin extracted from the leaves of *Adhatoda vasica* were observed in human whole blood using hepatitis vaccine antigen to estimate the monocytes, lymphocytes and granulocytes count. In addition, the effect of saponin along with hepatitis vaccine antigen was observed in Swiss mice and evaluated the peritoneal macrophages activation and estimates the T cell surface marker i.e. CD3. The results showed that the saponin showed a significant decrease in the number of monocytes and granulocytes count in human whole blood and also showed the sudden decline in the level of peritoneal macrophage activation and T cell surface marker i.e. CD3 as compared to control. No mortality was occurred in all the tested drug samples. Overall, the saponin showed immunosuppressive effect on the cell mediated immune (T cell surface markers) response and macrophage activation in mice.

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1. INTRODUCTION

Saponins are the glycosides of twenty seven carbon atom steroids or thirty carbon atom triterpenes in plants. These saponin are found in various parts of the plant especially leaves, stems, roots, bulbs, flowers and fruits [1, 2]. These are characterized through their bitter taste and their ability to showed hemolytic activity in human red blood cells. These saponins are commonly used in Ayurvedic medicine as a treatment for eczema, psoriasis and for removing freckles. Saponins are believed to be useful in the human diet for controlling cholesterol [1, 2, 3, 4]. Digitalis - type saponins strengthen the heart muscle causing the heart to pump more efficiently [5, 6]. Saponins also inhibit cancer tumor growth in animal model studies, particularly, lung and blood cancers, without killing normal cells. Saponins are the plant's immune system acting as an antibiotic to protect the plant against microbes and fungus [7, 8, 9, 10]. To achieve this objective, our group focused on saponins isolated from the medicinal plants for determining its various immunopharmacological activities. According to Ayurveda, one of the highly reputed medicinal plant i.e. *Adhatoda vasica* (adulsa, or malabar nut tree, family Acanthaceae) is small evergreen, sub-herbaceous bush which grows commonly in India, Sri Lanka, Burma and Malaysia [11]. This plant is generally used for the treatment of various diseases e.g. bronchitis, asthma, malaria, dysentery and diarrhea [12, 13]. In addition, it also showed potent anti-inflammatory activity, analgesic, antioxidant, hepatoprotective, sedative, antispasmodic, antihelmintic properties, antimicrobial activity, antidiabetic activity, wound healing effect, Infertility, anti-ulcer, antibacterial and anti-fungal activity [11, 12, 13, 14]. So far, the immunomodulatory activities of saponin extracted from *Adhatoda vasica* have not been studied *in vivo*. Crude saponin fractions isolated from the leaves of various medicinal plants and these were subjected to an immunological screening. In this article, our group focused on the immunomodulatory effect of saponin extracted from *Adhatoda vasica* using flow cytometry.

2. MATERIALS AND METHODS

2.1 Plant collection

Leaves of *Adhatoda vasica* were collected in January 2015 from the garden of Vidya Pratishthan's, Baramati, Maharashtra, India. The leaves were sun dried and make a powder for the isolation of crude saponin was analyzed.

2.2. Phytochemical Screening and extraction of saponin

The procedure was carried out for the separation of saponins e.g. foaming assay from the aqueous extract of *Adhatoda vasica*. To prepare the aqueous extract, the dried plant materials (10 g) of *Adhatoda vasica* were prepared in phosphate buffered saline (20 ml). After preparing the aqueous extract, this was extracted thrice with diethyl ether (10 ml). The diethyl ether layer was discarded and the retained aqueous layer extracted at the bottom further with 30 ml n-butanol (four times). The n-butanol extracts were bulked together and washed four times using 5 ml of five percent NaCl. The washed extract was concentrated at < 70 °C in an oven and air dried at room temperature to yield 500 mg of crude saponin residue. Residue was screened for saponin using the foaming test. Powder is dissolved in phosphate buffered saline, filtered through a Whatman filter paper [15].

2.3. Human blood samples and estimation of blood counts through flow cytometry

Human Blood samples were received for immunological studies from Mangal Pathology lab, Baramati region, District Pune Maharashtra especially for the estimation of blood counts i.e. lymphocytes, monocytes and granulocytes count using flow cytometry. Briefly, 100 µl of human whole blood is taken into the falcon tube and add serial dilutions of test candidate i.e. *Adhatoda vasica*. Incubate the samples for 2h at 37°C, 5% carbon dioxide incubator for 2 h. After incubation, lyse the cells with red cell lysis buffer and then washed the samples 2-3 times with phosphate buffered saline and then analyzed the cells using forward and side scatter through flow cytometer [16, 17].

2.4. Animal studies

The animal experiment i.e. mouse model based studies will be done as per the ethical guidelines. Animals were immunized on day 0 and 7 with hepatitis vaccine antigen (20 µg) and drugs continuously given from day 0 to day 10. On day 10, collect the whole blood from retro-orbital plexus and peritoneal macrophages from the abdominal cavity of mouse for the estimation of CD3 surface marker and macrophages activation.

For CD3 estimation, mice whole blood (100 µl) were placed in falcon tube and then stained with CD3 surface marker. Incubate the samples for 30 minutes in dark. After incubation, lysed and washed the cells with phosphate buffered saline and then analyzed the cells through flow cytometer [18].

For peritoneal macrophages, mice were injected with 10 ml of ice cold phosphate buffered saline containing fetal bovine serum. The abdomen was gently massaged and collects all these cells from the peritoneal cavity and then transferred the cells into 6 well plates. Incubate the cells for 24 h at 37°C. After 24h incubation, analyzed the cells using forward and side scatter through flow cytometric analysis [18].

2.5. Statistical analysis

Values are expressed as Mean ± S.E. The difference between the control and treated is determined through Bonferroni multiple comparison test.

3. RESULTS

3.1. Effect of *Adhatoda vasica* on human whole blood

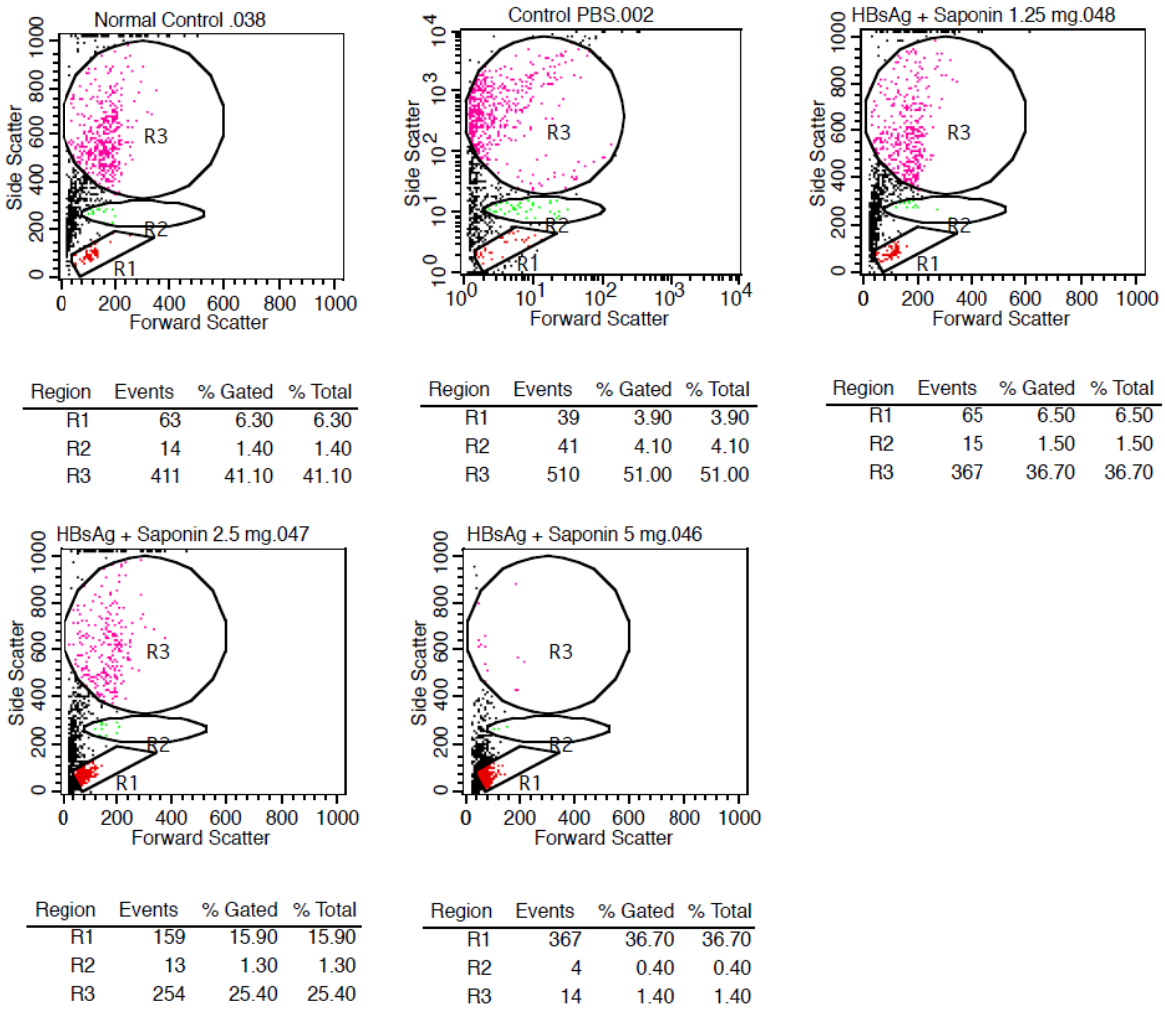
The effect of variable doses of saponin extracted from *Adhatoda vasica* on human whole blood as shown in **Fig.1.**The results showed that the saponin at higher doses with hepatitis B vaccine antigen showed inhibitory activity in monocytes and granulocytes count as compared to control. In addition, lymphocyte count increases at higher doses.

3.2. Effect of saponins on T cell surface marker (CD3, CD4 and CD8)

The effects of variable doses of saponin on T cell surface marker i.e. CD3. in mice are shown in **Fig. 2.** The results showed that the saponin along with hepatitis vaccine antigen significantly decreased the T cell surface marker i.e. CD3 as compared to the control group. Although the proportions of CD3+ T cells in the whole blood from the mice treated with 10 mg/ml of *Adhatoda vasica* were higher than those from the control group.

3.3. Effect of saponins on peritoneal macrophages activation

The effects of variable doses of saponin along with hepatitis vaccine antigen are shown in **Fig. 3.** At higher doses, there is decline in peritoneal macrophages activation as compared to control group. Although, the proportion of peritoneal macrophages in mice at lower dose i.e. 10 mg/ml is still higher as compared to control group using forward and side scatter. In this case, both forward and side scatter count increased in the same fashion. It means that saponin extracted from *Adhatoda vasica* showed immunosuppressive activity.



Region	Events	% Gated	% Total
R1	63	6.30	6.30
R2	14	1.40	1.40
R3	411	41.10	41.10

Region	Events	% Gated	% Total
R1	39	3.90	3.90
R2	41	4.10	4.10
R3	510	51.00	51.00

Region	Events	% Gated	% Total
R1	65	6.50	6.50
R2	15	1.50	1.50
R3	367	36.70	36.70

Region	Events	% Gated	% Total
R1	159	15.90	15.90
R2	13	1.30	1.30
R3	254	25.40	25.40

Region	Events	% Gated	% Total
R1	367	36.70	36.70
R2	4	0.40	0.40
R3	14	1.40	1.40

Fig.1. Flow cytometric analysis of saponins extracted from *Adhatoda vasica* on lymphocytes, monocytes and granulocytes count. EDTA human whole blood were treated with variable doses of saponin and then lysed and wash the cells with phosphate buffered saline and analyzed through flow cytometer. Values are expressed in Mean ± S.E. of fifty four human whole blood samples.

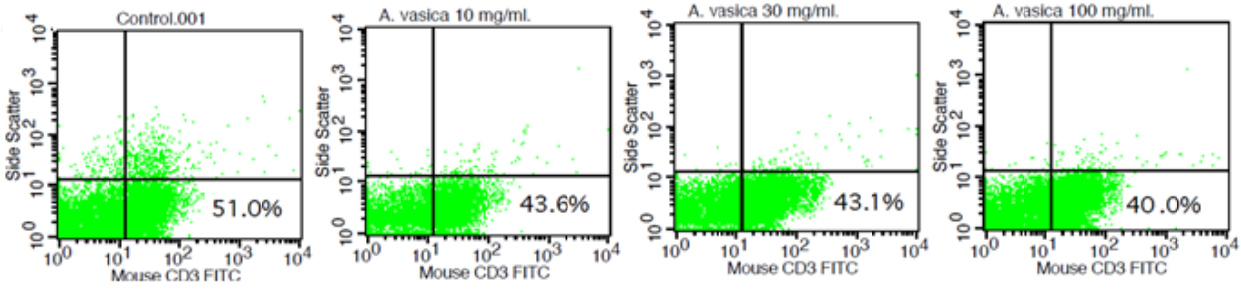


Fig.2. Flow cytometric analysis of saponins on CD3 T cell surface marker. Swiss mice were treated orally with variable concentration of saponins with hepatitis B surface antigen from day 0 and day 7. On day 10, whole blood were collected from retro-orbital plexus of mice and stained with CD3 FITC surface marker. After 30 minutes incubation with monoclonal antibody, cells were lysed and washed the cells with phosphate buffered saline and analyzed through flow cytometer (FACS Calibur).

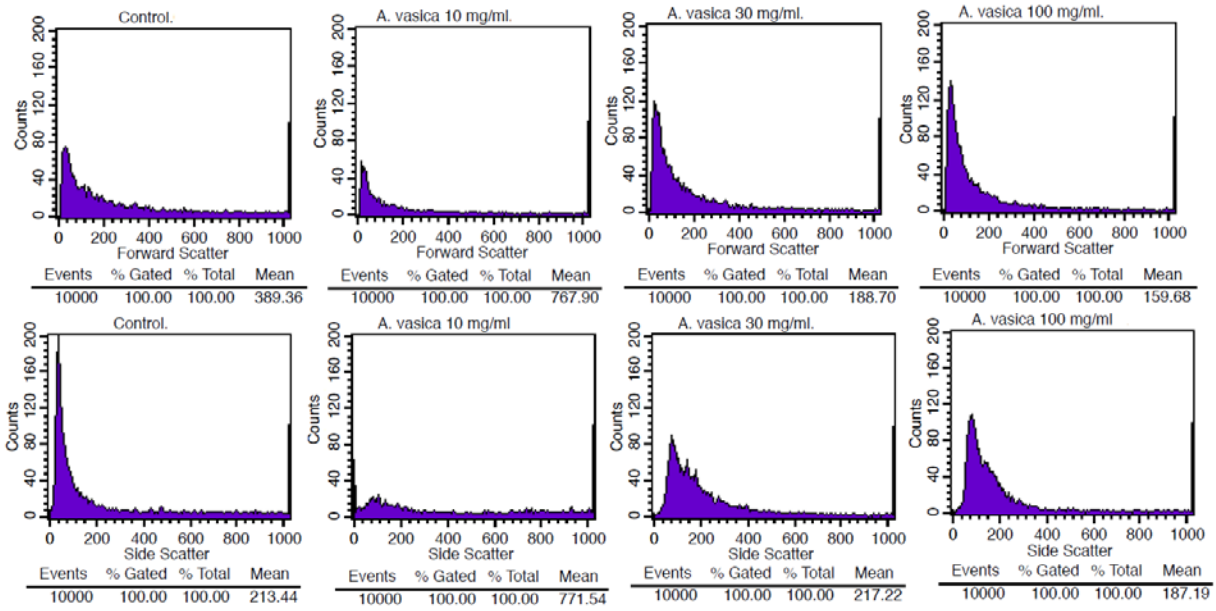


Fig.3. Flow cytometric analysis of saponins extracted from *Adhatoda vasica* on peritoneal macrophages. Mouse peritoneal cells were collected on day 16. Mouse peritoneal cells (2 x 10^6 cells/ml) dissolved in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions containing 2 x 10^6 cells/ml of treated mice of variable doses of saponins (10 – 100 mg/ml) were added in each 6 well plate and then add again exposure of saponins. Samples were incubated for 24 h at 37°C in CO2 incubator and then analyzed the forward and side scatter using flow cytometer. Experiment repeatedly three times.

4. DISCUSSION

Suppression or decline in the level of immune response through medicinal plant products as a possible therapeutic measure has become a subject of scientific investigation. Due to these synthetic based immunosuppressants which are available in the market showed serious adverse effects among which nephrotoxicity, hepatotoxicity, induction of diabetes, induction of hypertension and neurotoxicity are most notorious for cyclosporine and tacrolimus [19, 20, 21]. As a consequence, there continues to be a high demand for new immunosuppressants. In an effort to search for new immunosuppressants from medicinal plants which is clinically useful and safe product that could suppress immune response and may have future use for the local people. This study reported the effect of saponins extracted from *Adhatoda vasica* on human whole blood and also observed the T cell marker i.e. CD3 and peritoneal macrophages activation in mice intraperitoneally immunized with hepatitis B vaccine antigen.

The study of the immunomodulatory effects of various medicinal plants on animal and human model based studies is a matter of interest for many researchers. Several studies have previously been published and demonstrated the immunomodulating either immunostimulatory or immunosuppressive effects of medicinal plants on humoral and cell mediated immune response. This study focused on the influence of medicinal plant that has shown inhibitory activity of monocytes on human whole blood and also showed the decline in the level of T cell count i.e. CD3 marker and peritoneal macrophages activation in mice. The results obtained from this study which is indicated that saponins extracted from *Adhatoda vasica* showed immunosuppressive effect on human whole blood and animal model studies with a dosage-dependent relationship. In the experiments undertaken to study the effect of saponin on human whole blood along with hepatitis vaccine antigen were observed and it showed that with the administration of increasing doses of saponins, there is decline in the level of monocytes and granulocytes count as compared to control. The capacity to elicit a decline in T cell immunity can be shown by the CD3 (total T cell count) surface marker. The results indicated that saponin could significantly inhibit the potential of T cells in hepatitis vaccine immunized mice. The results of cell mediated response (decline in monocytes level and CD3 count) and macrophage activation after immunization with T-dependent antigen suggest that the activity of saponin could be mediated through the immunosuppressive effect on T lymphocytes and macrophages [23, 24]. Macrophages reside within the peritoneal cavity of mice and these were originated from specific white blood cells called monocytes which are present in the blood. Monocytes and macrophages are phagocytes, acting in either or innate as well as 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.

CONCLUSION

In the present study, the immunosuppressant activity of *Adhatoda vasica*, an important plant in indigenous medicinal practice was explored. Administration of *Adhatoda vasica* was found to decrease in the level of monocytes in human whole blood and also confirmed through in mice where there is decline in CD3 count and macrophage activation.

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