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FLOW CYTOMETRY ANALYSIS OF CRUDE SAPONIN FROM THE LEAVES OF *MANGIFERA INDICA* AND *ANTHOCEPHALUS CADAMBA* FOR ITS ANTI-INFLAMMATORY ACTIVITY

Amit Gupta[1] and Sushama R Chaphalkar[2]*

[1]Assistant Professor, Department of Immunology, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India.

[2]Director, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India.

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***Correspondence for Author**
Dr Amit Gupta,
Assistant Professor, Department of Immunology, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India.

Dr. Sushama R Chaphalkar
Director, Vidya Pratishthan's School of Biotechnology (VSBT) Vidyanagari, Baramati, District Pune, India.

ABSTRACT

Crude saponin extracts of *Mangifera indica and Anthocephalus cadamba* were screened for anti-inflammatory activity using hepatitis vaccine as antigen on human whole blood and animal model studies. For the separation of saponin using thin-layer chromatography was used to identify the type of saponins present in the crude extracts. The immunological studies of the crude saponin extracts gave the following intraperitoneal injection of hepatitis vaccine (20 µg/ml) on day 0 and 7 and extract (10, 30 and 100 mg/ml) continuously given from day 0 to day 9 for the estimation of surface markers (CD3/CD4/CD8) and peritoneal macrophages activation. The anti-inflammatory study of the saponin crude extracts showed statistically significant decrease in the monocyte count as compared to control in human whole blood where as in case of animal model studies, there is decline in surface markers (CD3/CD4/CD8) and peritoneal macrophages activation. The study showed that the anti-inflammatory properties attributable to these plants may be due to their saponins contents.

KEYWORDS: Mangifera Indica, A. Cadamba, Anti-Inflammatory.

1. INTRODUCTION

Medicinal plants continue to play an important role in the health care system of large proportions of the world's population. The majority of the population in developing countries uses medicinal plants or plant preparations (leaf, stem and root) in their basic health care.[1, 2, 3] Medicinal plants are proposed to be new resources of drugs and helped to understand the plant toxicity and protect human and animals from natural poison as well as infections. So, continuous usage of medicinal plants by a large proportion of the population in the developing countries is largely due to the high cost of western pharmaceuticals and health care.[4, 5, 6] Based on current research needs, medicinal plants played an important role as a human health aid or disorders.[4, 5] Many of the modern medicines are produced directly or indirectly from the natural products or medicinal plants e.g. aspirin.[7] Among the human diseases treated with various medicinal plants showed number of activities such as anti-inflammatory, anti-viral, anti-diabetic etc.[8, 9, 10, 11] Out of these medicinal plants, mango (*Mangifera indica*, family *Anacardiaceae*) represents one of the most important medicinal plants in India. It is generally grown in the tropical and sub-tropical regions and commonly used as folk medicine for variety of diseases. Recently, it has been reported that extract of *Mangifera indica* leaf showed anti-fungal activity[12] and anti-ulcerogenic action.[13] The phytochemical constituents of *Mangifera indica* from different parts of the plant showed the presence of phenolic constituents, tritepenes, flavonoids, phytosterols and polypenols. The leaves of *Mangifera indica* yield an essential oil containing humulene, elemene, ocimene, linalool, nerol and many others.[14] This species is purported to possess numerous therapeutic uses including anti-amoebic[15], anti-diabetic[16], anti-hyperglycemic[14] etc.

In addition, *Anthocephalus cadamba* commonly known as Kadamba- vriksha belongs to the family *Rubiaceae*[17] which is used as folk medicine in the treatment of fever, anemia, leprosy, dysentery, blood and skin diseases. Recently, *Anthocephalus cadamba* has been reported to possess wound healing, antioxidant and hepatoprotective activity.[17, 18] Today, the medicinal purposes or properties of *Mangifera indica* and *Anthocephalus cadamba* leaf have been widely studied. However, anti-inflammatory effects of saponin from *Mangifera indica* and *Anthocephalus cadamba* leaf have not been reported so far. The present study is to examine the anti-inflammatory effect of saponin extracted from *Mangifera indica* and *Anthocephalus cadamba* leaf against hepatitis vaccine as antigen using human whole blood and animal model studies in mice.

2. MATERIALS AND METHODS

2.1. Plant Material and

The leaves of *Mangifera indica* and *Anthocephalus cadamba* were collected from the garden of Vidya Pratishthan's, Baramati, District Pune, Maharashtra in the month of December 2014.

2.2. Preparation of Extract

Dried powdered leaves of *Mangifera indica* and *Anthocephalus cadamba* (20 g) were prepared in phosphate buffered saline (40 ml). After the aqueous preparation, the aqueous extract was extracted thrice with diethyl ether (20 ml). After extraction, diethyl ether layer was discarded and retained aqueous layer extracted or settled at the bottom further with 60 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 < 75 °C in an oven and air dried at room temperature to yield 500 mg of crude saponin residue. The residue was screened for saponin using the foaming test.[19]

2.3. Estimation of blood counts in human whole blood

Samples of human blood were received for immunopharmacological 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.

For flow cytometric analysis, 50 µl of EDTA human whole blood is taken into the falcon tube and then add serial dilutions of test drug (1.25, 2.5 and 5 mg) candidates i.e. *Mangifera indica* and *Anthocephalus cadamba*. Incubate the human blood samples along with test drug for 2 h at 37°C, 5% carbon dioxide incubator for 2 hours. After incubation, lyse the cells with red cell lysis buffer (ammonium chloride, sodium bicarbonate and ethylene diamine tetraacetic acid) 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.[9, 10]

2.4. Animal model 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 9. 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 peritoneal macrophages.

For CD3 estimation in EDTA whole blood of mouse, 100 µl of whole blood were placed in falcon tube and then stained with CD3 FITC surface marker. Incubate the mouse whole blood 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.[11]

For peritoneal macrophages collection from the abdominal cavity, 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 peritoneal cells from the abdominal cavity and then transferred the cells into 6 well plates. Incubate the peritoneal cells for 24 h at 37°C. After 24h incubation, analyzed the cells using forward and side scatter through flow cytometric analysis.[11]

3. RESULTS

3.1. Effect of *Mangifera indica* and *A. cadamb* on human whole blood

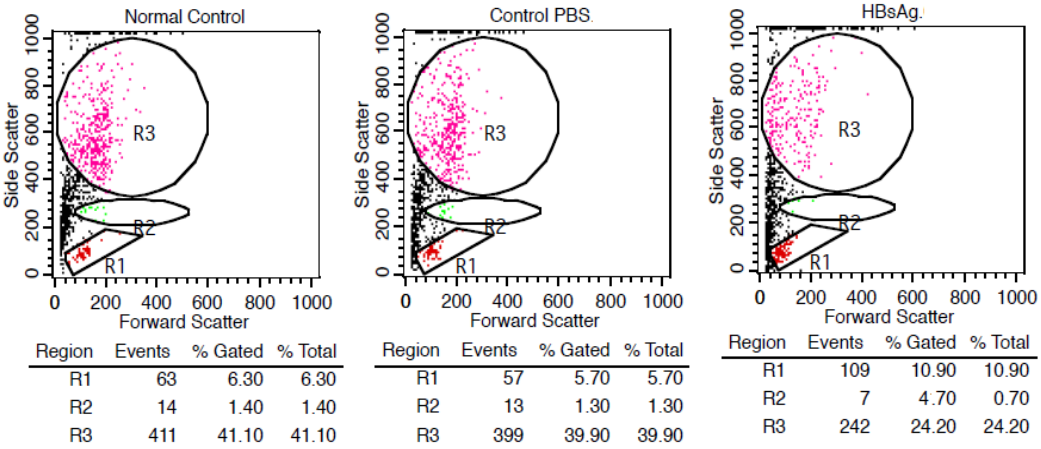
The effect of variable doses of saponin extracted from *Mangifera indica* and *Anthocephalus cadamba* on human whole blood as shown in **Fig.1.**The results showed that the saponin at higher doses with hepatitis B vaccine antigen showed inhibitory activity in monocytes count as compared to control. In 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, CD4 and CD8 in mice are shown in **Fig. 2 and 3.** The results showed that the saponin along with hepatitis vaccine antigen significantly decreased the T cell surface marker i.e. CD3, CD4 and CD8 as compared to the control group. Although the proportions of CD3, CD4 and CD8+ T cells in the whole blood from the mice treated with 10 mg/ml of *Mangifera indica* and *Anthocephalus cadamba* 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. 4.** 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 *Mangifera indica* and *Anthocephalus cadamba* showed immunosuppressive activity.

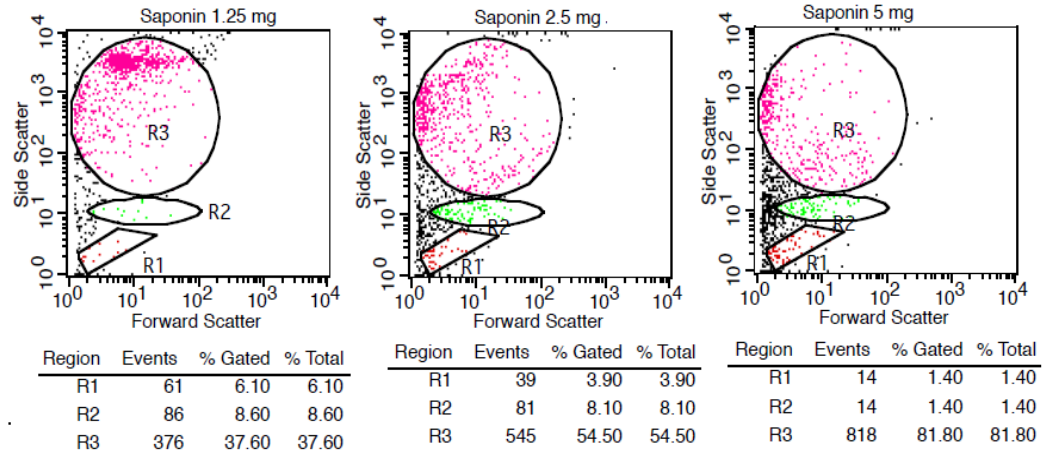


Region	Events	% Gated	% Total
R1	63	6.30	6.30
R2	14	1.40	1.40
R3	411	41.10	41.10

Region	Events	% Gated	% Total
R1	57	5.70	5.70
R2	13	1.30	1.30
R3	399	39.90	39.90

Region	Events	% Gated	% Total
R1	109	10.90	10.90
R2	7	4.70	0.70
R3	242	24.20	24.20

A) Mangifera indica

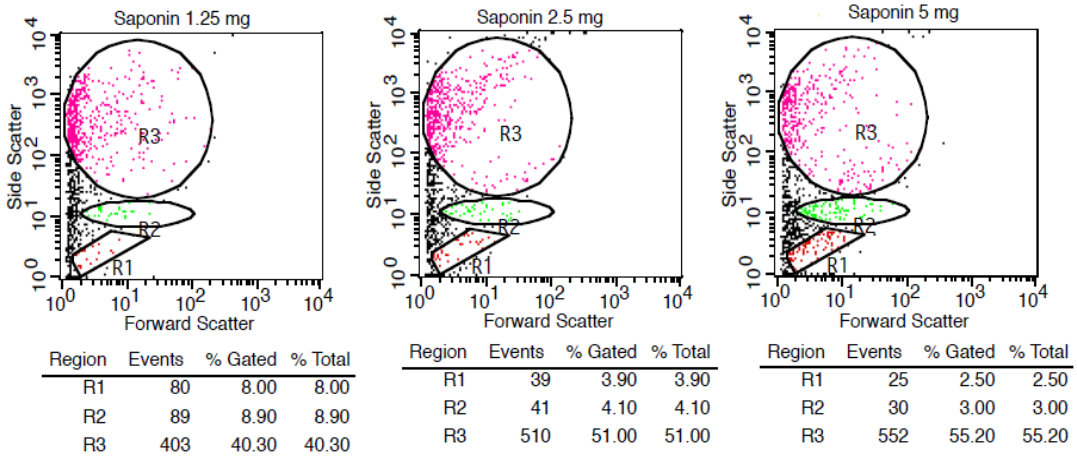


Region	Events	% Gated	% Total
R1	61	6.10	6.10
R2	86	8.60	8.60
R3	376	37.60	37.60

Region	Events	% Gated	% Total
R1	39	3.90	3.90
R2	81	8.10	8.10
R3	545	54.50	54.50

Region	Events	% Gated	% Total
R1	14	1.40	1.40
R2	14	1.40	1.40
R3	818	81.80	81.80

B) Anthocephalus cadamba



Region	Events	% Gated	% Total
R1	80	8.00	8.00
R2	89	8.90	8.90
R3	403	40.30	40.30

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	25	2.50	2.50
R2	30	3.00	3.00
R3	552	55.20	55.20

Fig.1. Effect of variable doses of saponin extracted from the leaves of *Mangifera indica and Anthocephalus cadamba* on human whole blood using flow cytometry. Human whole blood samples were incubated with serial dilutions of saponin and incubated the samples at 37°C, 5% carbon dioxide incubator for 2 h. After 2h, lysed the blood samples and wash the samples two times with phosphate buffered saline and then observed the cells in flow cytometer (FACS Calibur). Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software

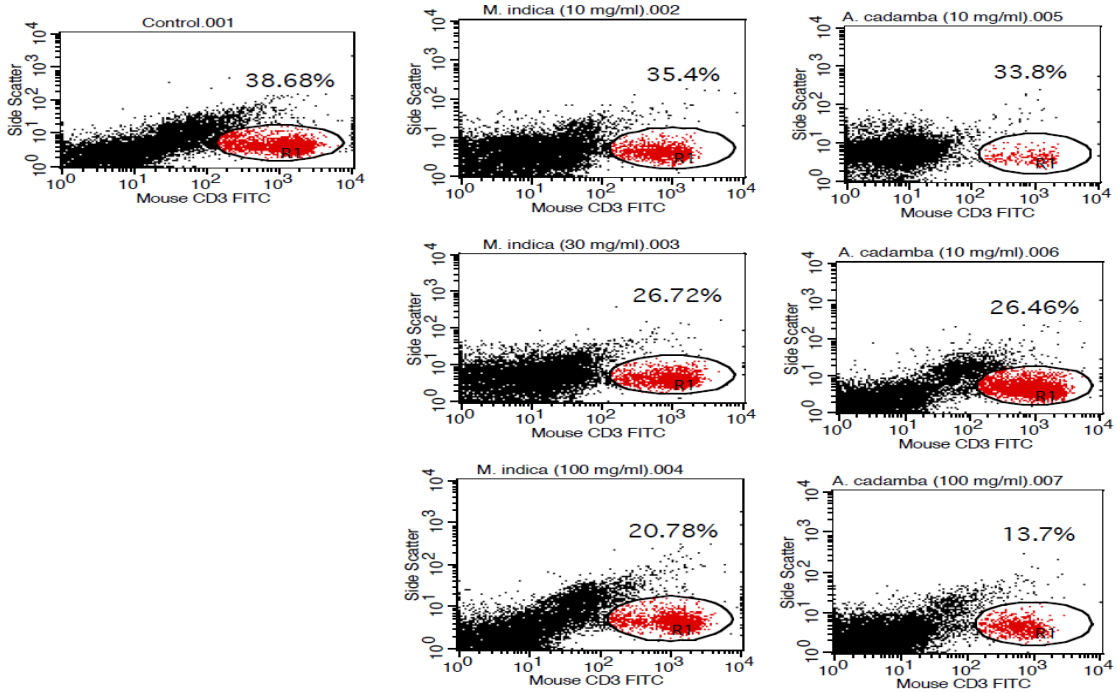


Fig.2. Effect of aqueous extract of *Mangifera indica* and *Anthocephalus cadamba* on CD3 (T cell) surface marker using flow cytometry. EDTA whole blood was collected on day 10 for the estimation of T cell surface marker i.e. CD3 FITC conjugated monoclonal antibody and then analyzed through flow cytometer (FACS Calibur).

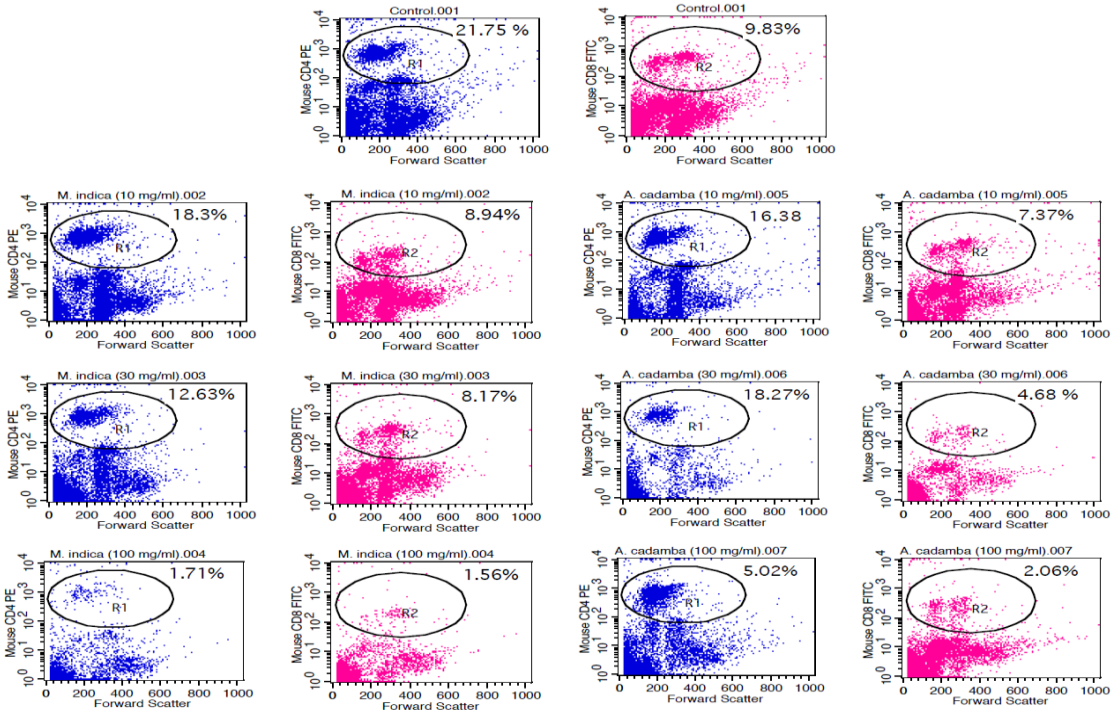


Fig.3. Effect of aqueous extract of *Mangifera indica* and *Anthocephalus cadamba* on T cell surface markers i.e., CD4 and CD8 using flow cytometry. EDTA whole blood was collected on day 10 for the estimation of T cell surface markers. Staining of whole blood with FITC conjugated monoclonal antibody i.e. CD8 and PE conjugated monoclonal antibody i.e. CD4.

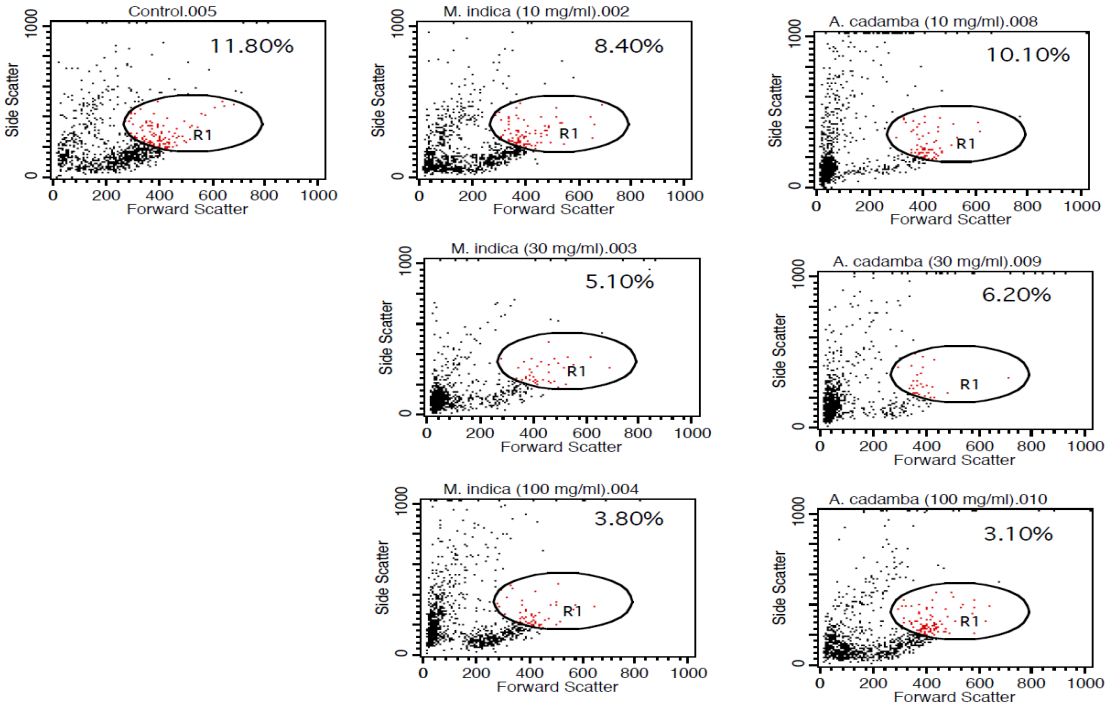


Fig.4. Effect of aqueous extract of *Mangifera indica and Anthocephalus cadamba* on macrophage function. Mouse peritoneal cells were collected on day 10. Mouse peritoneal cells (2 x 10^6 cells/ml) dissolved in phosphate buffered saline containing 10 % FCS (heat inactivated). 500 µl cell suspensions containing 2 x 10^6 cells/ml of immunized mice of variable doses of saponin (10, 30 and 100 mg/ml) were added in each 6 well plate. Samples were incubated for 24 h at 37°C in CO_2 incubator and then analyzed the forward and side scatter using flow cytometer.

4. DISCUSSION

The present study evaluated the anti-inflammatory activity of leaves aqueous extract of *Mangifera indica and Anthocephalus cadamba* on the following bioassays: estimation of blood counts in human whole count and determined the CD3, CD4 and CD8 surface marker in whole blood and peritoneal macrophages in mouse model studies. The aqueous extract derived from *Mangifera indica and Anthocephalus cadamba* were strongly active at higher doses in all the tested bioassays. It showed the inhibitory activity on the blood counts especially in monocyte and granulocytes profile of human and also decline in the cell surface markers (i.e. CD3, CD4, CD8) and peritoneal macrophages activation in mice.

During preliminary investigation of medicinal plants especially *Mangifera indica and Anthocephalus cadamba* contained number of primary and secondary metabolites which is already present in the aqueous extract. Out of these metabolites, saponins represents one of the member of secondary metabolites and showed wide range of biological activities such as haemolysis, pesticidal, molluscidal, antimicrobial, insecticidal, anthelmintic, analgesic, anti-

inflammatory, sedative and antitumor activities.[20, 21] Number of research articles which are already published or evidence related to these anti-inflammatory properties of saponins has been provided using different models of inflammation.[22, 23]

Saponins isolated from the leaves of *Mangifera indica and Anthocephalus cadamba* have been shown to inhibit the monocytes and granulocytes profile in a dose dependent manner in human whole blood which is determined through flow cytometry. Several studies related to immunopharmacology especially hematology have shown that the reduction of monocytes and granulocytes count contributes to the incidence of infections especially cardiovascular in our immune system.[11,22,23] Other mode of actions has been used to explain the anti-inflammatory activity of saponins in animal model studies using surface markers (CD3/CD4/CD8) and peritoneal macrophages activation. The anti-inflammatory activity of saponins isolated from the leaves of *Mangifera indica and Anthocephalus cadamba* have been shown to be due to the inhibition of surface markers (CD3/CD4/CD8) and peritoneal macrophages activation. The results indicated that saponins from the leaves of *Mangifera indica and Anthocephalus cadamba* could significantly decrease the activation potential of CD3, CD4 and CD8 count in hepatitis vaccine-immunized mice. Hepatitis vaccine along with the saponin from the leaves of *Mangifera indica and Anthocephalus cadamba* had a significant decline on CD3+, CD4+ and CD8+ T cells, thereby confirming its inhibitory effect on the cell-mediated immune response. It should be noted that the inhibitory effects observed in this study could be considered as the anti-inflammatory effect of these medicinal plants because in each case the viability of cells are decreased in case of monocytes as well as granulocytes profile and cell surface markers were observed. In addition, peritoneal macrophage activation after immunization with hepatitis vaccine as antigen immunized on different time intervals suggest that the immunopharmacological activity of these saponins could be mediated through the immunosuppressive effect on T lymphocytes and macrophages.[9, 10, 11] 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. Finally, the saponins from the leaves of *Mangifera indica and Anthocephalus cadamba* showed anti-inflammatory activity.

CONCLUSION

It may be concluded from this study that the saponin isolated from the leaves of *Mangifera indica and Anthocephalus cadamba* has anti-inflammatory activity against hepatitis vaccine antigen. However, further study on this saponin may help to elucidate on the specific principle associated with the observed activity of the fraction in this study.

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