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Potential immunosuppressive and anti-inflammatory activity of aqueous extract of *Mangifera indica*

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ABSTRACT

Background and aims: There is a worldwide interest in searching for potential and effective medicinal plant candidates against various diseases or disorders. Till now, there are number of anti-inflammatory drugs like NSAIDs are available which showed various adverse effects in our body. To reduce these adverse effects, some of medicinal plants have been experimentally validated. The aim of our immunopharmacological study was to investigate the immunosuppressive and anti-inflammatory activity of aqueous leaves extract of *Mangifera indica* in human peripheral blood mononuclear cells (PBMC) extracellular against hepatitis B vaccine antigen (HBsAg).

Methods: In this study, aqueous leaves extract was collected from *Mangifera indica* and determined its effect on total blood counts (using forward and side scatter) and monocyte CD14 FITC surface markers using flow cytometry. In addition, the effect of aqueous leaves extract on nitric oxide (NO) production from PBMC cell culture supernatant and also estimate its proliferation assay using Concanavalin A (Con A, 0.5 mg/ml, 50 µl). Data analysis was performed using BD cell Quest Pro software for flow cytometric analysis and one way ANOVA test (Boniferroni multiple comparison test).

Results: The results displayed that aqueous leaves extract of *Mangifera indica* showed dose dependent decline in blood counts (increased in side scatter and slightly decreased in forward scatter), monocyte CD14 FITC surface marker; Con A proliferation and nitric oxide production from cell culture supernatant in human PBMC.

Conclusion: Overall, the results claimed that aqueous leaves extract of *Mangifera indica* at higher doses showed immunosuppressive and anti-inflammatory activity.

Keywords: Immunosuppressive, Anti-inflammatory, *Mangifera indica*, Hepatitis B vaccine.

Original article

INTRODUCTION

The capability of these medicinal plants collected from various tropical and sub-tropical regions which have the ability to synthesize a large number of metabolites (i.e. primary and secondary). Those are used to perform important immune pharmacological functions and provide protection against various intracellular as well as extracellular micro-organisms.[1-3] Many of these phytochemicals or metabolites present in these medicinal plant products which provide beneficial as well as advantageous effects to

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human health only when consumed these plants forever.[4,5] Since there are number of scientific proofs as well as evidences which are supported that increasing in the world wide population depends on traditional medicinal plant product remedies for their health care.[6,7] The misuse of various medicinal plant products in human as well as animal health care is even much higher in particularly those areas with little or no access to modern health services.[5-7]

One of these medicinal plants, *Mangifera indica* (also called as Mango; family *Anacardiaceae*) commonly grown in Asian countries (India, Pakistan and Bangladesh) and displayed number of medicinal uses e.g. any part of the plant. *Mangifera indica* are used to treat number of diseases including viruses and bacteria e.g. abscesses, rabies, tumour (cancerous), datura poisoning, asthma and etc.[8-10] In contrast, *Mangifera indica* also used traditionally in India especially its leaves and fruit in weddings and religious ceremonies. Most of the times, fumes from the burning leaves of *Mangifera indica* are inhaled for relief from hiccups and infections of the throat.[9-11] There are number of polyphenolic compounds that are present in the leaves aqueous extract of *Mangifera indica*. Out of these, *Mangiferin* displayed a number of immune pharmacological activities such as wound healing properties; anti-diabetic[12]; immunomodulation;[13] anti-oxidant[14]and etc. In this study, our group focused on the leaves aqueous extract of *Mangifera indica* for determining its immunosuppressive and anti-inflammatory activity against specific (hepatitis B vaccine, HBsAg) antigen in human PBMC.

METHODS

Freshly harvested plant leaves of *Mangifera indica* were gathered from the garden of Vidya Pratishthan's School of Biotechnology, Baramati, District Pune, and Maharashtra. Firstly, harvested plant leaves of *Mangifera indica* were washed with tap water. Thereafter, leaves of *Mangifera indica* were air dried and cut into small pieces and macerated with liquid nitrogen (-196 °C) for one minutes to prepare fine powder. For aqueous extract preparation, weigh 8 g of leaves powder was macerated in 80 ml PBS (phosphate buffered saline) using mortar and pestle at room temperature for 2 minutes with occasional stirring. Thereafter, the aqueous extract of *Mangifera indica* was filtered and finally leaves aqueous extract was obtained or collected which was kept in refrigerator at 4 ºC.

The phytochemical screening of aqueous leaves extract of *Mangifera indica* was carried out to detect the presence of various secondary metabolites. The results exhibit that leaves aqueous extract of *Mangifera indica* showed qualitatively the existence of various secondary metabolites i.e. saponin (Foam test); flavonoids (alkaline reagent test); terpenoids (acetic anhydride test); phenolics (ferric chloride test) and glycosides (Borntranger test). For quantitative based assay using HPTLC (high performance thin layer chromatography) was performed [solvent system i.e. ethyl acetate: n Butanol in the ratio of 6:4 for the development of chromatogram]. Once, the chromatograms were scrutinized by densitometer at 220 nm after spraying with anisaldehyde with sulphuric acid for aqueous leaves extract of *Mangifera indica.* In HPTLC, relative factor (Rf) values were recorded by software (winCATS). The Rf value of phenolics is 0.83 (8.2 µg, 2.49%); terpenoids (0.92), and saponin (0.34 – 0.47).

Fresh heparinized or EDTA anti-coagulant whole blood samples (non-infected; age 18-25 years) of healthy people were collected with permission from *Mangal Pathology Laboratory*, Maharashtra, India.

For its preparation, human PBMC was separated from heparinized or EDTA anti-coagulant whole blood samples by means of Ficoll-Hypaque density gradient centrifugation. PBMC was counted (using haemocytometer) and adjusted to an appropriate/final concentration (2×10^6 cells/ml) in complete RPMI 1640 (supplemented with 100 U/ml penicillin G and 100 µg/ml streptomycin) containing 10% fetal bovine serum (FBS) for further assays.[15]

To evaluate the effect of variable doses (0.5 – 30 mg/ml, 50 µl) of aqueous leaves extract of *Mangifera indica* on PBMC (2×10^6 cells/ml, 100 µl) was plated into 96 well tissue culture plates. Incubated the samples (PBMC containing aqueous leaves extract) at 37 ℃ for 24 h. Hepatitis B vaccine (HBsAg) used as standard or positive control for these immunopharmacological studies. After incubation, lyses the PBMC with FACS lysing solution/red cell lysis buffer/ACK lysing solution and washed with phosphate buffered saline (PBS). Finally, the samples were analyzed on flow cytometer. The numbers of total blood counts and leukocytes especially monocytes i.e. CD14 FITC in PBMC were analyzed by flow cytometer (FACS Calibur).[16,17] Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software. CV represents coefficient of variation.

Briefly, purified PBMC (2×10^6 cells/ml, 100 µl) were cultured in triplicate in 96-well tissue culture plate along with variable doses of aqueous leaves extract of *Mangifera indica* at final concentrations (0.5-30 mg/ml, 50 µl) in complete RPMI 1640 containing 10% FBS in presence of Con A (0.5 mg/ml, 50 µl; T cell mitogen). PBMC cultures were incubated at 37 °C with 5% CO_2 incubator for 48 h. After incubation, centrifuge the 96 well plate (2500 rpm, 10 minutes at 4 °C) and collect the supernatant (100 µl) without disturbing the pellet for the estimation of nitric oxide production. Afterwards, again add fresh medium (100 µl) into 96 well tissue culture plates. Incubated the plate again for another 24 h. After incubation, MTT (5 mg/ml, 10 µl) is added into 96 well tissue culture plates. Again, incubate the plate for another 4 h. Centrifuge the 96 well plate (2500 rpm, 10 minutes at 4 °C) and discard the supernatant and dissolved the pellet (containing formazon crystals) in dimethyl sulphoxide (DMSO). The optical density (OD) was measured at 570 nm and the readings will be measured within 10-15 minutes.[18]

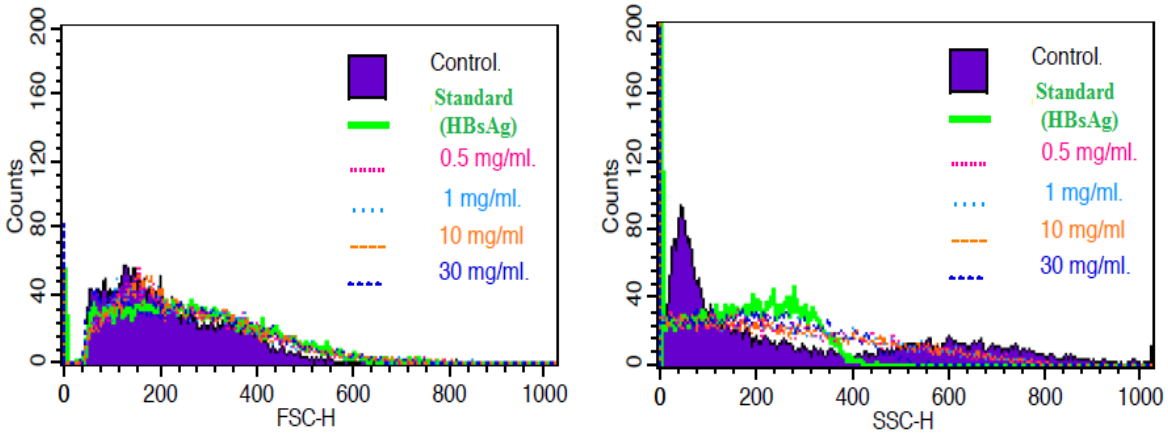
The quantity of nitrite accumulated in the cell culture supernatant of PBMC along with aqueous leaves extract of *Mangifera indica* was measured (as mentioned above); which is an indicator of nitric oxide production. Briefly, 50 µl of cell culture supernatant was mixed with 50 µl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2- 2.5% phosphoric acid) and incubated the 96 well plates at room temperature for 7-8 minutes and the absorbance or optical density was measured at 540 nm was measured in a microplate reader. The cell culture medium (PBS, phosphate buffered saline containing 10 % fetal bovine serum) was used as a blank. The nitrite quantity (µM) was determined from a sodium nitrite standard curve.[19]

Data are reported as means ± standard error (S.E). The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test).

RESULTS

In order to estimate the total blood counts in human PBMC using variable doses of leaves aqueous extract of *Mangifera indica* using flow cytometry as shown in Figure 1 and 2, CD14 is a marker present on

the surface of monocytes. The flow cytometric results showed that leaves aqueous extract at a dose range of 10 and 30 mg/ml showed sudden decline/decrease in the number of total blood counts (with respect to enhancement of side scatter and decrease in forward scatter) and CD14 monocyte surface marker as compared to control and standard. Hepatitis B vaccine (HBsAg) used as positive control for these studies and the results showed that there is a significant increase in blood counts and CD14 monocyte surface marker as compared to control.



File: Control

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	243.24	197.61	55.45

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	174.25	64.28	80.17

File: HBsAg (20 ug/ml, 10 ul)

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	256.56	214.32	52.08

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	156.97	66.48	72.61

File: 0.5 mg/ml, 50 ul

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	245.95	209.83	51.07

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	218.18	82.56	84.78

File: 1 mg/ml, 50 ul

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	243.97	206.68	51.57

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	220.02	84.07	84.45

File: 10 mg/ml, 50 ul

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	242.08	205.34	52.01

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	221.70	85.12	84.84

File: 30 mg/ml, 50 ul

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	203.84	175.79	52.63

Events	% Gated	% Total	Mean	Geo Mean	CV
10000	100.00	100.00	248.81	135.54	100.51

Figure 1: Effect of leaves aqueous extract of *Mangifera indica* on human whole blood using flow cytometry, i.e. Forward scatter (FSC) and side scatter (SSC)

Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software. CV represents coefficient of variation.

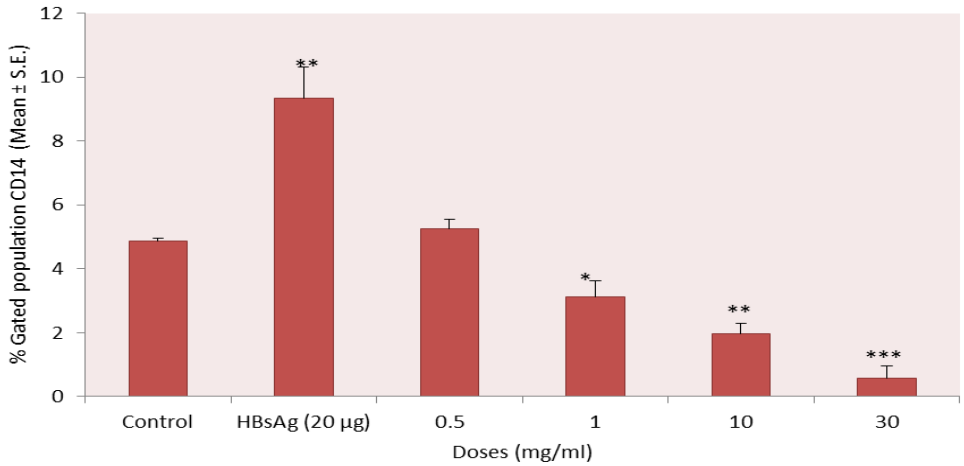


Figure 2: Estimation of CD14 surface marker on human PBMC using flow cytometry

PBMC's were stained with CD14 FITC surface marker and incubated in dark for 30 minutes and then lysed with red cell lysis buffer and washed the cells in PBS and then analyzed in a flow cytometer (FACS Calibur). Values are expressed as Mean ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

The effect of leaves aqueous extract of *Mangifera indica* on PBMC on Con A (T cell) proliferation and collection of cell culture supernatant for the estimation of nitric oxide production as shown in Figures 3 and 4. The results showed that leaves aqueous extract (at higher doses i.e. 10 and 30 mg/ml) of *Mangifera indica* decreased in Con A stimulated lymphocyte proliferation and nitric oxide production as compared to control. Overall, the results suggest that leaves aqueous extract of *Mangifera indica* showed immune suppressive and anti-inflammatory activity at higher doses as compared to control.

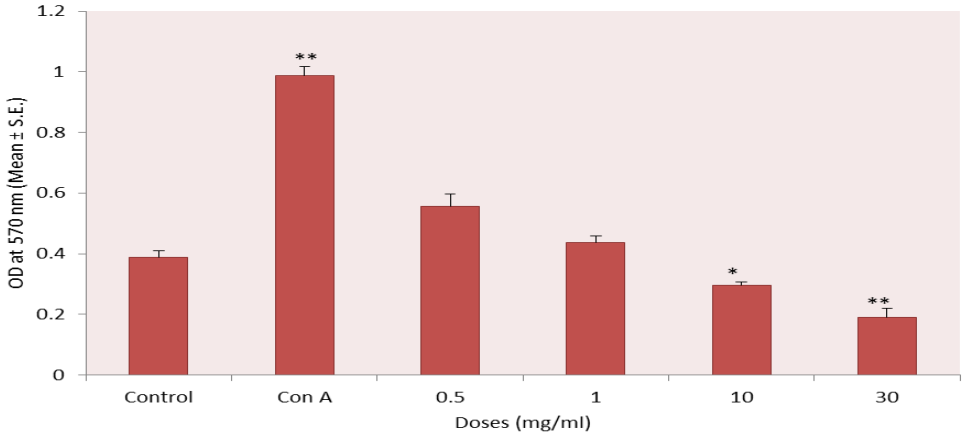


Figure 3: Effect of leaves aqueous extract of *Mangifera indica* on human PBMC using T cell mitogen Con A

PBMC (2 x10^{6} cells/ml, 100 µl) were cultured in triplicates in 96-well tissue culture plate along with variable doses of leaves aqueous extract in complete RPMI 1640 medium in presence of Con A (0.5 mg/ml, 50 µl). Proliferation was measured by MTT assay. Values are expressed as Mean ± S.E. The absorbance was evaluated in an ELISA reader at 570 nm.

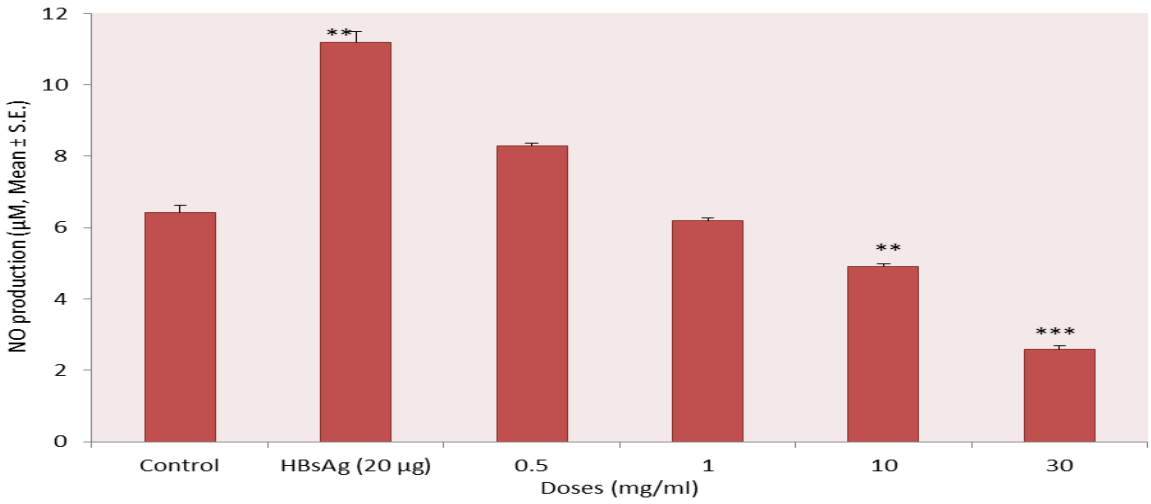


Figure 4: Estimation of Nitric Oxide (NO) production

The supernatant nitrite concentration was determined by Griess reagent after 24 h culture of PBMC in the presence of leaves aqueous extract of Mangifera indica. Values are expressed as Means ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

DISCUSSION

Inflammation (complex biological process) means tissue injury induces a complex cascade of non- specific events and is characterized into 2 types i.e. acute (characterized by classical signs such as edema, erythrema, pain, heat and loss of function) and chronic (destruction and healing of injured tissue) inflammation.[20,21] As per the literature survey, there was a number of medicinal plant products and its active constituents (secondary metabolites i.e. alkaloids, flavonoids, terpenoids, and etc)[2,3] showed anti-inflammatory activity.[3,19] The aim of our present study was to further understand the mechanism of immune suppressive and anti-inflammatory activity of *Mangifera indica* in human PBMC extracellular against hepatitis B vaccine antigen (HBsAg) using flow cytometer (FACS Calibur). The main advantage of this instrument is to measure live and dead cells in the form of forward and side scatter. As per the literature survey of flow cytometer, it will mention that dead cells have high side scatter and low forward scatter.[15-17] Generally, flow cytometer correlates with the cell size in terms of forward scatter and the density of the particle/cell (i.e. number of cytoplasmic granules, membrane size) can be distinguished based on differences in their size and density.[16,17] In the present study, similar observations were observed in case of human PBMC after incubation with variable doses of aqueous leaves extract of *Mangifera indica* for 24 h incubation. The results showed that there was a significant increase in side scatter and decrease in forward scatter in the form of total blood counts where hepatitis B vaccine (HBsAg) used as positive control for these studies and it showed a significantly increase in forward scatter and a decline in side scatter in case of total blood counts in human PBMC. Over all, the results displayed that aqueous leaves extract at higher doses showed immunosuppressive and anti-inflammatory activity.

To further confirm its activity, human PBMC exposed to aqueous leaves extract of *Mangifera indica* for 24 h and 48 h for determining its CD14 monocyte surface

marker population, mitogen (Con A) induced lymphocyte proliferation and nitric oxide production. It may be suggested that human PBMC provides reliable information related to immunosuppressive and anti-inflammatory activity of treated as well as control samples. The main advantage of this initial screening is its sensitivity, general feasibility, low cost and possibility of large-scale performance. Since the production of nitric oxide (NO) from antigen presenting cells i.e. dendritic cells macrophages (including PBMC) may depend on the immune cell types and its species origin, different immune cells of our system have different requirements for signal transduction pathways.[19] In contrast, CD14 surface marker is normally present on human PBMC (mononuclear cells i.e. monocyte/ macrophages) and served as homing receptor for the lipopolysaccharide (LPS) of gram negative bacteria. On the other hand, it is generally known that Con A stimulates T cell proliferation, when human PBMC were exposed to aqueous leaves extract, it is possible that human PBMC recognize and receive second signals and test candidate viz. aqueous leaves extract of *Mangifera indica* could significantly declining in Con A for inhibiting non-specific T lymphocyte proliferation response by helper T cells. Over all, the results displayed that aqueous leaves extract showed immunosuppressive as well as anti-inflammatory activity with respect to significant decrease in CD14 surface marker, Con A stimulated PBMC population and nitric oxide production at higher doses as compared to control.

CONCLUSION

In the present study, we found that the aqueous leaves extract of *Mangifera indica* significantly inhibited the production of total blood counts, CD14 population, nitric oxide and mitogen (Con A) stimulated PBMC population. Further investigations will focus on the *in vivo* assessment of the immunopharmacological activity of these aqueous leaves extract and identify the major active components responsible for immunosuppressive and anti-inflammatory activity in the efficacious extracts.

CONFLICT OF INTEREST

Authors have declared that no conflicts of interest exist.

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