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Immunostimulant phytoconstituents from *Mangifera indica* L. bark oil

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Abstract

The present study was undertaken to isolate oil from *Mangifera indica* bark, Identification of various phytoconstituents and screening of immunostimulant activity in experimental animals.

Methods: Immunostimulant activity was evaluated in rats as well as in mice by determining neutrophil adhesion to nylon threads and phagocytic index. Levamisole at a dose of 50 mg/kg, b.w was used as standard drug. Acute toxicity studies were carried out and the test drug doses were selected. *Mangifera indica* bark oil in the dose of 150 & 300 mg/kg b.w, p.o, were used.

Results: Results indicate a significant increase in percent neutrophil adhesion ($P < 0.01$) for test and standard groups compared to control group and haemagglutination antibody (HA) titer ($P < 0.01$) and phagocytic index ($P < 0.001$) values are also significantly increased in test groups.

Conclusion: The results obtained in the present study indicate that *Mangifera indica* Linn bark oil possesses potent immunomodulatory activity and have therapeutic efficacy for the prevention of autoimmune and infectious diseases.

Keywords: Haemagglutination antibody (HA) titer, Immunomodulatory, *Mangifera indica*, Neutrophil adhesion test, Phagocytic activity.

Introduction

Recently, much attention has given to diseases alleviated by the modulation of the immune responses such as autoimmune diseases. In, Immunomodulation the immune system of an organism is altered, if it results in an increase of immune reactions it is called as an immunostimulative drug, which primarily implies stimulation of non specific system, i.e., macrophages, granulocytes complement like T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors.¹

Researchers have identified 80-100 different autoimmune disorders and suspect at least 40 additional disorders of having an autoimmune basis. These disorders are chronic and can be life-threatening.²

A large population of the world uses plants for its healing, preventive, curative and much therapeutic property together with immunostimulatory property. Certain medicinal plants promote positive health and maintain organic resistance against infection by re-establishing body equilibrium. Many polysaccharides isolated from higher plants are considered to be a biological response modifier and enhance various

immune responses, like complement activation, proliferation of lymphocytes and stimulation of macrophages. Though various synthetic drugs are available as immunostimulative agent such as levamisole, but there are various side effects of these agents such as nephrotoxicity, hepatotoxicity, bone marrow depression, gastrointestinal disturbance, etc., hence drugs of plant origin are used more, because these are safer, much more effective and cheaper.³

Now a day's alternative method of medicines was successfully used as adjuvant therapy for various diseases including autoimmune disease. It has been reported that *Hypericum connatum* Lam, *Dunbaria bella* Prain, *Aglaia odorata*, *Moringa oleifera* medicinal plants have been used in the treatment against herpes simplex virus.⁴ Herpes simplex is a type of virus causing immune disease in which watery blisters on the skin or mucous membranes of the mouth, lips and genital organs are affected. For the treatment of herpes simplex immune boosters are used.⁵

Mangifera indica Linn belongs to family Anacardiaceae a plant widely used in the traditional medicinal systems of India has been reported to possess antiviral, antibacterial and anti-inflammatory activities.⁶ Oil of mango tree bark is an effective home remedy for sore throat and can be used to cure throat infections.⁷ The alcoholic extract of the stem bark of *Mangifera indica* has been also reported to possess immunomodulatory activity with immunostimulant properties. In present study *Mangifera indica* bark oil is extracted and subjected to Gas chromatography-Mass spectroscopy (GC-MS) for identification of various constituents and neutrophil adhesion test, carbon clearance test were performed to evaluate immunomodulatory activity.

Materials and Methods

Plant material and extraction

Mangifera indica stem barks were collected from the Nigadi region of Dharwad (Karnataka, India) in the month of August. The stem barks were inspected to be healthy, collected and were washed with water thrice in the bucket and dried overnight over a cotton cloth. The stem barks were grounded into fine powder using a domestic electric grinder. Powdered stem barks were passed through the sieve no.20. Around 100 g of powder was collected and extracted with Petroleum ether 600-800 (8 x 300 ml; 8 hr each). Finally the fatty residue was collected and

evaporated to dryness. Spectral characterization of the extract was carried out with the help of TLC and GCMS.

Experimental animals

Experiments were carried out using either sex of Albino Wister rats weighing 200-250 g and albino mice weighing 20-30 g, which were supplied by Venkateshwara Enterprises, Bangalore. They were housed in polypropylene cages (47 cm x34m x20 cm) lined with husk, renewed every 24 h under a 12:12 h light dark cycle at around 22°C. The animals had free access to water and food, ad libitum. The animals were fed on a standard pellet diet (Venkateshwara Enterprises, Bangalore). The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Animal Ethical Committee of SET's College of Pharmacy S.R. Nagar, Dharwad Karnataka, India (Approval No. SETCP/IAEC/2010-2011/449; and Date-11-12-2010).

Chemicals

Levamisole (Khandelwal laboratories Pvt Ltd., Mumbai), Heparin (Neon Labs, Mumbai), Leishman's Stain (Span Diagnostics Ltd., Surat), WBC Diluting Fluid (S D Fine-Chem. Ltd., Mumbai).

Thin Layer Chromatography (TLC)

TLC plate was prepared by using silica gel (GF 254), air dried and kept for activation at 110°C for 30 min. n-hexane and ethyl acetate were used as mobile phase in a ratio of 9:1. TLC chamber was kept for saturation. MBO was dissolved in chloroform and sample spot was put 1 cm from the end of the plate. Further plate was placed in chamber for development. After development, plate was air dried and kept in an iodine chamber for spot visualization. Rf value was calculated.

Spectral analysis

Gas Chromatography – Mass Spectroscopy (GCMS)

The *Mangifera indica* Bark oil was given for GC-MS analysis in Karnataka university, Science Instrument Center, Dharwad. The bark oil was dissolved in Dimethyl Sulfoxide (DMSO) and subjected to GC-MS (Shimadzu).

Acute toxicity studies

The acute oral toxicity study was carried out as per the guidelines set by OECD 423. Animals (n=3) were overnight fasted prior to dosing. The dose was selected from one of four fixed levels, i.e. 5, 50, 300 and 2000 mg/kg b.w. The test substance was administered in a single dose by gavage using intubation canula. Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 h, with special attention given during the first 4 h.

Animals were observed for following profiles.

1. *Behavioral profile*: Alertness, restlessness, irritability and fearfulness
2. *Neurological profile*: Spontaneous activity, reactivity, touch and pain response
3. *Autonomic profile*: Defecation and urination.⁸

After a period of 24 and 72 hrs the animals were observed for any lethality.

Evaluation of Immunomodulatory activity

Neutrophil Adhesion Test

Group-I received normal saline for 14 days
 Group-II received MBO (300 mg/kg) for 14 days
 Group-III received MBO (150 mg/kg) for 14 days
 Group-III received Levamisole (50 mg/kg) for 14 days

Percent (%) neutrophil adhesion was calculated as per the method reported by Fulzele SV et al. On the 14th day of drug treatment, blood samples were collected by puncturing the retro-orbital plexus into heparanized vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophils gives Neutrophil index (NI) of a blood sample.⁹

Percent Neutrophil adhesion was calculated as shown below:

$$\text{Neutrophil adhesion (\%)} = (\text{NIu} - \text{NI}t) / \text{NIu} \times 100$$

Where, NIu = Neutrophils index of the untreated blood sample
 NI_t = Neutrophil index of treated blood sample

Carbon clearance test

Group-I received normal saline for 7 days
 Group-II received MI (150 mg/kg) for 7 days
 Group-III received MI (300 mg/kg) for 7 days
 Group-IV received Levamisole (50 mg/kg) for 7 days
 Group-V received Cyclophosphamide (25 mg/kg) for 7 days

Mice were divided into above mentioned groups. At the end of treatment with the test drug for 7 days, i.v injection of 0.3 ml per 30 g body weight of dilute Indian ink was given to albino mice, Blood samples were collected at 0 and 15 min, 50 µl of blood was diluted with 4 ml of 0.1% sodium carbonate and absorbance of the diluted blood was measured spectrophotometrically at 660 nm.

The phagocytic index (K) was calculated using the following equation:

$$K = (\text{Loge OD}_1 - \text{Loge OD}_2) / 15$$

Where OD₁ and OD₂ were the optical densities at 0 min and 15 min, respectively.¹⁰

Indirect Hameagglutination Test

Group-I received normal saline for 28 days
 Group-II received MI (300 mg/kg) for 28 days
 Group-III received MI (150 mg/kg) for 28 days
 Group-IV received Levamisole (50 mg/kg) for 28 days
 Group-V received Cyclophosphamide (50 mg/kg) for 3 days

Rats were divided into above mentioned groups and animals were pretreated with the test drug for 14 days and each rat were immunized with 0.5×10⁹ sheep red blood cell (SRBCs) intraperitoneally including control rats. The day of immunization was referred to as day 0. The drug treatment was continued for another 14 more days, cyclophosphamide was administered to last 3 days and blood samples were collected from each rat at the end of the drug treatment and the titer value was determined by titrating serum dilution with SRBC (0.025×10⁹ cells) in microtitre plates. The plates were incubated at room temperature for 2 hrs and examined visually for agglutination. The highest dilution of serum showing heamagglutination was expressed as heamagglutination titer.¹¹

Statistical analysis

All the experimental results were expressed as mean ± SEM. One-way ANOVA followed by Tukey's test using

GraphPad InStat, version 5.0. The intergroup difference was considered significant when $p < 0.05$.

Results

Analytical Profile

Oil was extracted from dried coarse powder of *Mangifera indica* bark in soxhlet apparatus using petroleum ether and analytical properties observed are given in Table 1.

Table 1: Analytical Properties of MBO

Analytical Properties	Values
Color	Pale yellow
Melting point	55-58 °C
Boling point	232-237 °C
Refractive index	1.535

Thin Layer Chromatography (TLC)

Chromatography techniques were adopted to identify the purity of oil. Totally 5 spots were seen (Figure 1) with different R_f values (Table 2).

Table 2: Spots and their R_f values

Spots	R _f Value
Spot 1	0.12
Spot 2	0.16
Spot 3	0.23

Spot 4	0.35
Spot 5	0.67



Figure 1: Thin Layer Chromatography of MBO

Spectral Analysis

Gas Chromatography – Mass Spectroscopy (GC-MS)

GC-MS study was undertaken to identify the individual components of the mango bark oil. Results are shown in Figure 2 [a-e].

Totally there are 13 different peaks observed in the chromatogram. The R_t values found are as follows, 4.734, 5.115, 5.539, 5.809, 6.609, 6.723, 6.850, 7.142, 7.241, 7.524, 7.726, 7.808, 8.016 respectively. The main peak observed at R_t value 6.723, this corresponds to the 26.92% composition of the oil.

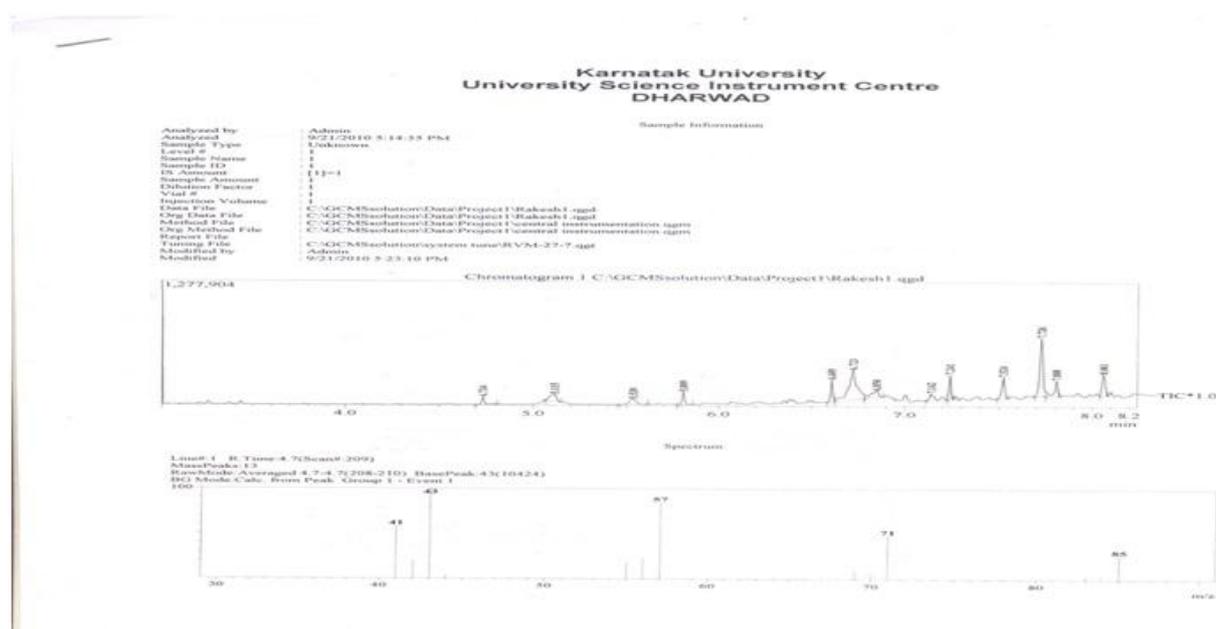


Figure 2(a): GC-MS of *Mangifera indica* Bark oil

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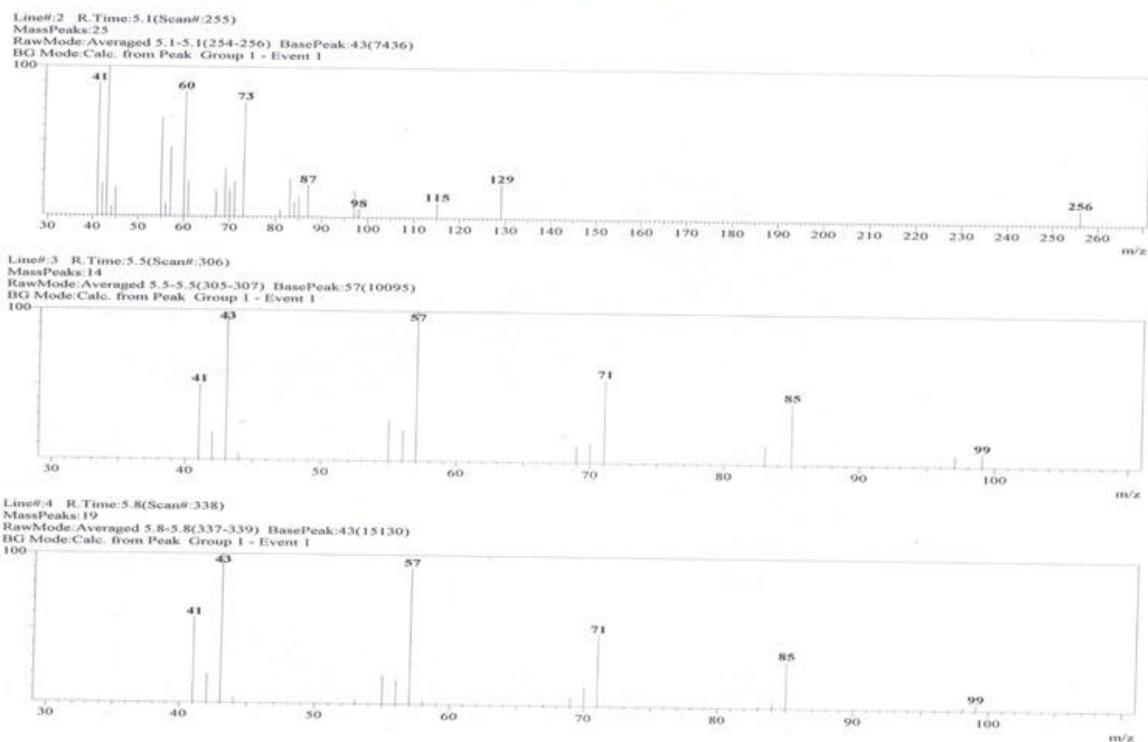


Figure 2 (b): GCMS of *Mangifera indica* Bark oil

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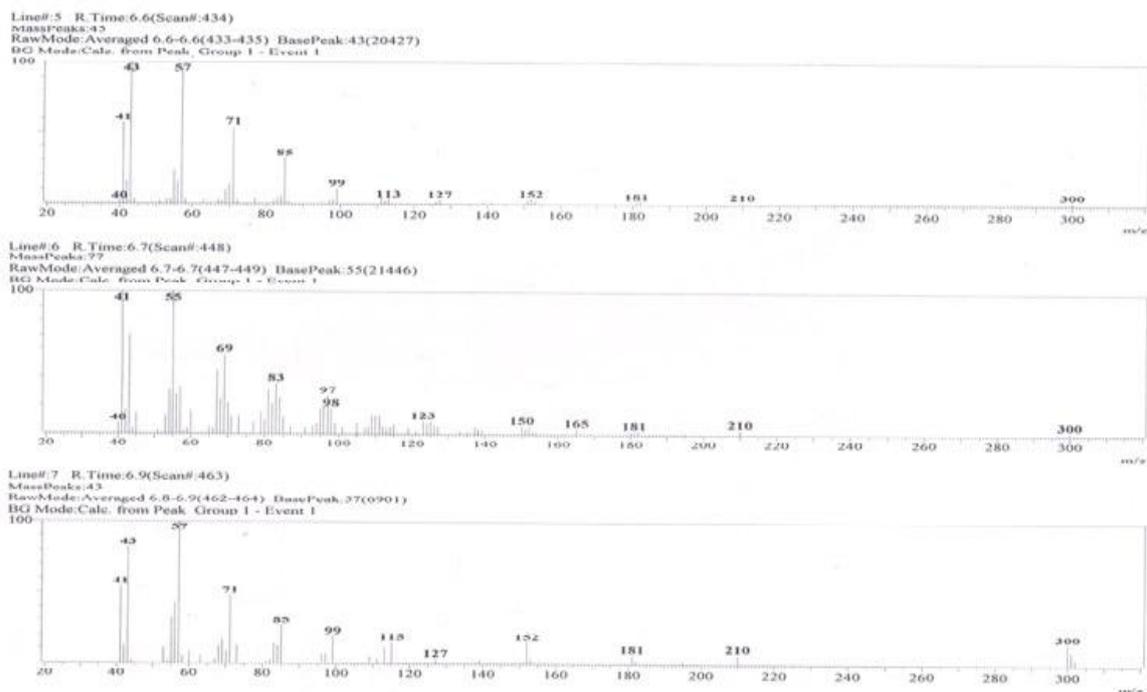


Figure 2 (c): GCMS of *Mangifera indica* Bark oil

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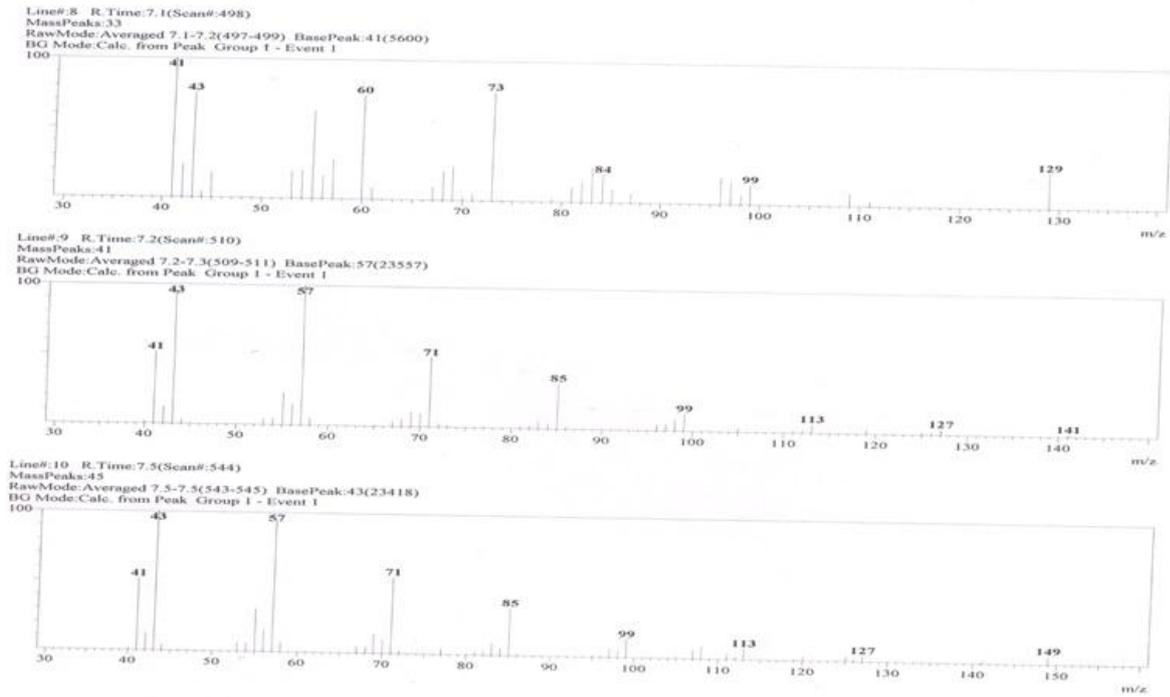


Figure 2 (d): GCMS of *Mangifera indica* Bark oil

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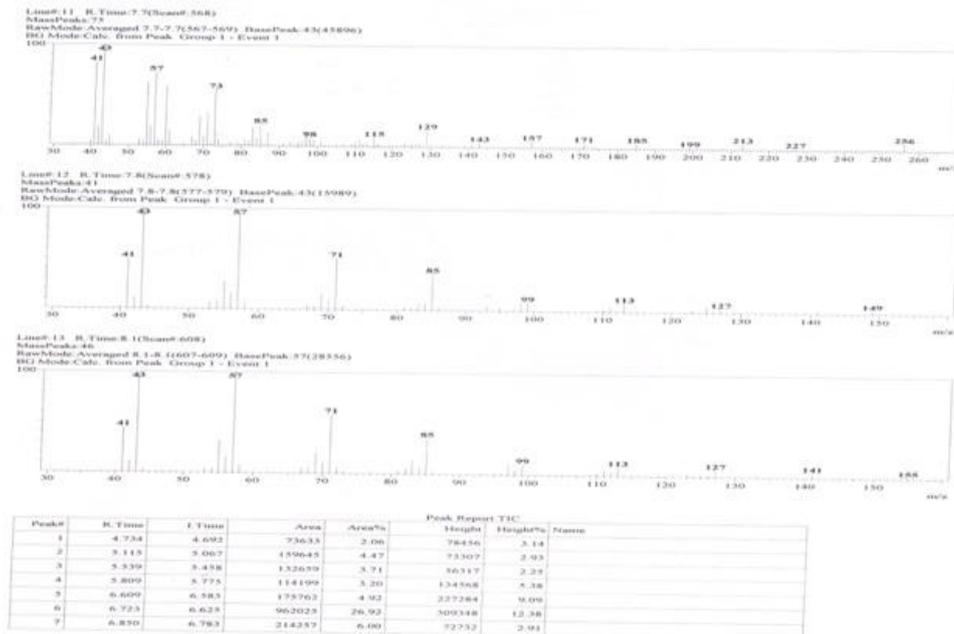


Figure 2 (e): GCMS of *Mangifera indica* Bark oil

Corresponding molecular weight m/z values of the 13 peaks observed in GCMS are 85, 256, 99, 99, 300, 300, 300, 129, 141, 149, 256, 149, and 155 respectively. According to the literature values, following compounds which may be present are protocatechic acid (154.12), Epicatechin (290.27), Oxyresveratrol (244.24) and n-Heneicosane (269.59) palmitic acid (256.42).

Acute toxicity study

An acute toxicity study revealed that the animals showed good tolerance (up to a dosage of 3000 mg/kg b.w) to single doses of MBO. It did not produce any noticeable effect on general behavior or appearance of the animals and all mice survived during and after the test period. There were no signs and symptoms of restlessness, irritability, fearfulness, pain response, convulsions, defecation, urination or coma. Therefore, two non-lethal doses (150 and 300 mg/kg b.w) of MI were selected for screening of immunomodulatory activity in experimental animals.

Immunomodulatory Activity

Neutrophil adhesion test

Incubation of neutrophils with nylon fibers produced a decreased in the neutrophil counts due to adhesion of neutrophils to the fibers. The percent neutrophil adhesion in control group animals was noted to be 15.09 ± 0.90 , whereas, in MBO (300 mg/kg) treated group it was found with increased pattern of 20.96 ± 1.08 as compared to their respective control group and at the dose 150 mg/kg of MBO (14.64 ± 0.84) has not significantly increased in neutrophil adhesion as compared to control group. However, the dose of 300 mg/kg revealed significant increase ($P < 0.01$) in neutrophil adhesion as compared to control suggesting possible immunostimulant action MBO (Table 3). Levamisole, an immunostimulant drug, increases percent neutrophil adhesion value to 23.98 ± 0.75 , which was significant ($P < 0.001$) to control group.

Table 3: Neutrophil adhesion test for various treatments

Treatment	TLC ($10^3/\text{mm}^3$) (A)		Neutrophil % (B)		Neutrophil index (A \times B)		Neutrophil adhesion (%)
	NFTB	UB	NFTB	UB	NFTB	UB	
Control	5.840 ± 0.16	6.300 ± 0.15	23.90 ± 0.58	25.78 ± 0.47	139.7 ± 5.54	164.4 ± 5.93	15.09 ± 0.90
Lev 50 mg/kg	7.960 ± 0.28	8.920 ± 0.27	32.85 ± 0.74	38.56 ± 0.69	261.0 ± 5.43	343.6 ± 9.14	$23.98 \pm 0.75^{***}$
MI 300 mg/kg	6.960 ± 0.10	8.000 ± 0.08	28.76 ± 0.80	31.50 ± 0.85	200.5 ± 8.44	253.4 ± 8.20	$20.96 \pm 1.08^{**}$
MI150 mg/kg	7.140 ± 0.11	7.940 ± 0.11	28.60 ± 0.60	30.14 ± 0.71	204.5 ± 7.58	239.6 ± 8.58	14.64 ± 0.84

Each group consists of n=6 animals. Values are Mean \pm S.E.M. $P < 0.05$ is considered as significant. $***P < 0.001$, $**P < 0.01$ as compared to control group.

Carbon Clearance Test

The faster removal of carbon particles has been correlated with the enhanced phagocytic activity. The phagocytic activity of the reticular-endothelial system was measured by the removal of carbon particles from the blood circulation. The phagocytic index of control group was 0.0057 ± 0.00055 (Figure 3). Oral administration of MBO 150 and 300 mg/kg for 10 days and 15 min prior to carbon injection exhibited a dose-related increase in the clearance

rate of carbon by the cells of the RES. MBO showed significant increase in the phagocytic index as 0.0108 ± 0.0006 and 0.0128 ± 0.0014 , with doses of 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.01$) respectively. Cyclophosphamide, an immunosuppressant agent shows the phagocytic index of 0.0048 ± 0.0014 . Levamisole, an immunostimulant drug, shows phagocytic index of 0.0130 ± 0.0004 which shows a significant increase ($P < 0.01$) compared to control group.

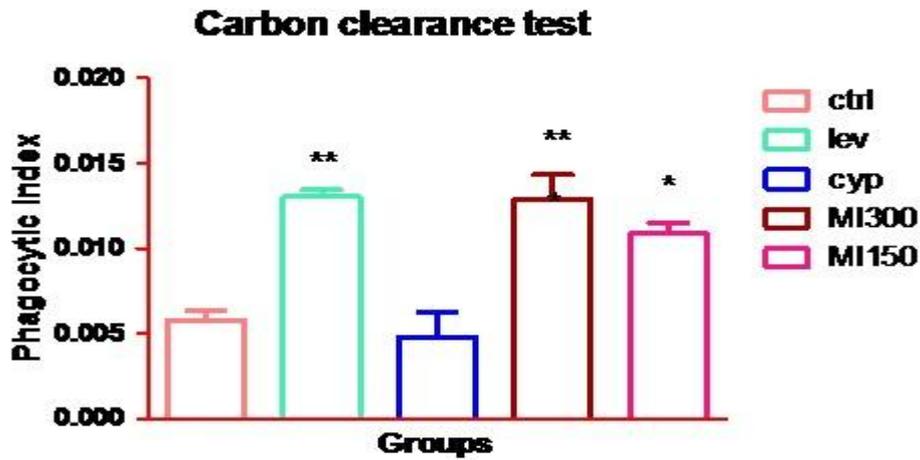


Figure 3: Carbon clearance test for various treatments. Each group consists of n=6 animals. Values are Mean ± S.E.M. P<0.05 is considered as significant. **P<0.01, *P<0.05 as compared to control group.

Haemagglutinating antibody (HA) titer

The HA titer was used to assess humoral immune response. The humoral antibody titer value of control group was found to be 22.40±3.91. Cyclophosphamide, an immunosuppressant agent decreases the HA titer value to 4.00±1.09. Levamisole, an immunostimulant drug, increases HA titer value to 179.2±31.35, which was

significant as compared to control group (P<0.001). Administration of test doses 300 mg/kg and 150 mg/kg produced a significant increase in HA titer value to 140.8±31.35 (P<0.01) and 115.2±12.80 (P<0.05) respectively, which were significant to control group, as an evidence from haemagglutination after incubation of serum with SRBCs (figure 4).

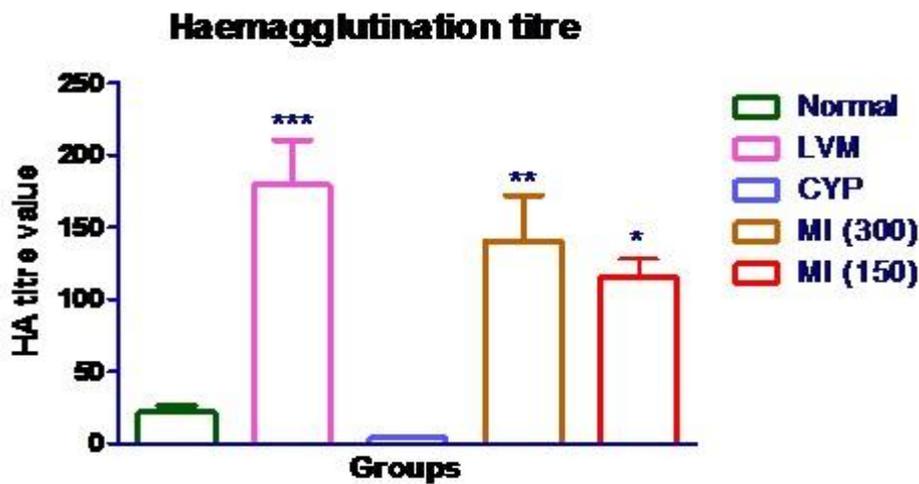


Figure 4: Haemagglutinating antibody (HA) titer for various treatments. Each group consists of n=6 animals. Values are Mean ± S.E.M. P<0.05 is considered as significant. ***P<0.001, **P<0.01, *P<0.05 as compared to control group.

Discussion

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune

reactions it is named as an immunostimulation by stimulation of specific and nonspecific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factor. The results obtained in the present study indicate that of MBO is a potent immunostimulant, stimulating specific and nonspecific immune mechanisms.¹²

There were significant decreases in neutrophils, lymphocytes, eosinophils and monocytes in animals treated with cyclophosphamide as compared to control group because cyclophosphamide showed that in rat lymphocytes decrease due to immunotoxic effect as well as a decrease in the activity of lymphoid cells especially the CD4+ lymphocytes.¹³

In neutrophil adhesion test, cytokines are secreted by activated immune cells for the imagination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. A significant increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels. This might due to the upregulation of the $\beta 2$ integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres.¹² In the present study, MBO 300 mg/kg treated group, evoked a significant increase in percent of neutrophil adhesion. This may help in increasing immunity of body against microbial infections.

Phagocytosis represents an important innate defense mechanism against ingested particulates, including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils and tissue macrophages. Once particulate material is ingested, the phagosomes fuse with lysosomes and the ingested material is then digested.¹⁴ Enhanced uptake of particulate matter with the treatment of MBO is evident from the carbon clearance test. Indian ink, which is nothing but colloidal, carbon preparation, when injected intravenously, the phagocytes in the liver and spleen eliminate the carbon particles. The rate of removal of these carbon particles from the blood stream is the measure of phagocytic activity. MBO showed stimulation of macrophage as evidenced by an increase in phagocytic index in carbon clearance test.

Antibody molecules which are secreted by plasma cells mediate the humoral immune response. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells.^{15, 16} Antibody production to T dependent antigen SRBC requires co-operation of T and B-lymphocytes and macrophages. Cyclophosphamide has a particularly intense effect on short-lived lymphocytes known to include a great proportion of B-cells.¹⁷ MBO 300 mg/kg treated groups, showed an increase in HA titer value. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness to macrophages and T and B lymphocyte subsets involved in antibody synthesis.

Catechin and epicatechin possess strong antimicrobial activity against various bacteria and virus and antioxidant activity which contributes to their immunomodulatory effect.^{18, 19} Epicatechin present in diet rich in flavonoids might have protective effects against impairment of structure and properties of Fg, a key plasma protein in homeostasis, caused by strong biologic oxidant/nitration and inflammatory mediators.²⁰ Oxyresveratrol possess strong inhibitory action against African swine fever virus replication which can suppress the immune system.²¹

Petroleum ether extract of *Mangifera indica* extract showed the presence of Catechin, Epicatechin and Oxyresveratrol in GC-MS report, and these phytoconstituents possess potent immunostimulants because of their strong antimicrobial and antiviral activity. Thus above study suggests potent immunomodulatory effect of *Mangifera indica* extract.

Immunomodulatory agents of plant origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. However, these agents and the herbal formulations should be subjected to systematic studies to substantiate the therapeutic claims to their clinical utility. The studies reveal that MBO is effective in first line defense.

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