

## Immunomodulatory effects of alcoholic and hydroalcoholic extracts of *Moringa olifera* Lam leaves

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Effects of 50, 100 and 200 mg/kg body weight of the alcoholic and hydro-alcoholic extract of leaves of *M. olifera* were studied on various immune paradigms like delayed type hypersensitivity reaction using SRBC as an antigen, determination of antibody titer, neutrophil adhesion test as an indicator for neutrophil index, total leucocyte count in cyclophosphamide induced immunosuppressed animals and carbon clearance assay as a measure of phagocytic activity. Hydro-alcoholic extract of *M. olifera* substantially enhanced cellular immune response, humoral immune response, neutrophil index and phagocytic activity in doses of 100 and 200 mg/kg body weight. The ethanolic extract (200 mg/kg body weight) was efficient in improving immune response. The results suggest that *M. olifera* has a significant role to play as an immune stimulator.

**Keywords:** Immunomodulator, Humoral response, *Moringa olifera*, Nutrient, Phagocytic activity

The primary goal of the immune system is to provide protection to the host when exposed to noxious stimuli. Under normal conditions, the integrity of the immune system is unquestionable as it salvages the host from precarious conditions. Immune system is bestowed with a rich arsenal to rectify, confront and overcome diseases. Very often the immune system may be exposed to repeated insult and injury causing impairment in its ability to fend off danger culminating in immunodeficiency. Immunodeficiency could be both inherited and acquired. Susceptibility to microbial, allergic and other disorders is higher in the presence of a compromised immune system. Competency of the immune system is enhanced by advocating the use of immunostimulants however the cost of these medications often limits their access. The Indian system of medicine is a versatile field providing wonder herbs like Triphala<sup>1</sup>, liquorice<sup>2</sup>, *Andrographis paniculata*<sup>3</sup>, *Echinacea*, ginseng, and *Astragalus*<sup>4</sup> which could potentially impact the immune system positively. In the Indian system of medicine, *Tinospora cordifolia* is referred to as a rasayana which is capable of boosting immune function. Several studies have shown that it to possess immunomodulatory action<sup>5</sup>; therefore it was used as a standard in this study.

*Moringa olifera* Lam (synonym: *Moringa pterygosperma* Gaertn.), belonging to the family Moringaceae, commonly called as drumstick or horesradish tree is a native to India. *Moringa olifera* is undoubtedly referred to as a 'miracle tree' as it is rich in vitamin A, B, C, E, minerals particularly calcium and potassium, however magnesium, phosphorous, copper, iron and selenium also exist in the plant. Essential and non essential amino acids are present in *Moringa olifera*<sup>6,7</sup>. Phytochemically, the presence of quercetin-3-O-glucoside, quercetin-3-O-(6"-malonyl-glucoside), lower amounts of kaempferol-3-O-glucoside, kaempferol-3-O-(6"-malonyl-glucoside)<sup>8</sup>, 4-(4'-O-acetyl-L rhamno-pyranosyloxy) benzyl isothiocyanate, 4-(L-rhamnopyranosyloxy) benzyl isothiocyanate and 4-(L-rhamnopyranosyloxy) benzyl glucosinolate<sup>9</sup>, nitrile glycosides, niaziridin<sup>10</sup> and niazirin<sup>11</sup> are present in the leaves. In the indigenous system of medicine, *M. olifera* is used as a panacea for urinary tract infections, external sores, fever, tumors, anemia, hypertension, diabetes, hypocholestermia, thyroid, colitis, gastritis, rheumatism and headache. Protein, vitamin, mineral, carotenoid and iron deficiency can be effectively handled by utilizing various parts of this tree<sup>12</sup>. The hepatoprotective<sup>13</sup>, hypercholesterolemia<sup>14</sup>, antioxidant<sup>15</sup>, anti-inflammatory, antispasmodic and diuretic<sup>16</sup> potential have also been established.

Keeping the abundance of nutrients in *M. olifera* in perspective, its potential as an immunomodulator has been evaluated in the present study.

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## Materials and Methods

**Drugs**—Cyclophosphamide was procured from Khandelwal Industries Limited, Mumbai and tragacanth from Loba Chemie, Mumbai. All other reagents and chemicals used were of analytical grade.

**Collection, identification of plant**—The leaves of *M. olifera* and *Tinospora cordifolia* were collected locally and authenticated by botanist Prof. Sidappa; Siddaganga Degree College, Tumkur; the voucher specimen have been preserved in the herbarium at Nalanda College of Pharmacy for future reference.

### Extract preparation

**Preparation of ethanolic extract:** Ethanolic extract of leaves of *M. olifera* (EEMO) was prepared by shade drying the leaves and reducing the biomass to a coarse powder Leaf powder. (200 g) was macerated with 500 mL of 95 % (w/v) ethanol for 24 h at room temperature. The mixture was subjected to filtration using a Whatman grade 1 filter paper. Concentration was done using a rotary evaporator at 50 °C yielding a thick brown extract with a yield of 20.15 %, (w/w). The same extraction procedure was adopted for the extraction of dried leaf powder of *T. cordifolia* (200 g) to obtain a yield of 20.15 %, (w/w).

**Hydro-alcoholic extract:** Hydro-alcoholic extract of the leaves of *M. olifera* (HAMO) was prepared by extracting the pulverized leaves (200 g) of the plant with ethanol: water mixture (1:1) at 33 °C. The solvent was removed under vacuum to obtain a dark brown residue with a yield of 22.16%, (w/w). All the extracts were refrigerated and the requisite quantity was suspended in an aqueous solution of tragacanth (1%, w/v) as vehicle and administered orally.

**Phytochemical analysis**—Preliminary phytochemical studies of the alcoholic and hydroalcoholic extracts of *M. olifera* was performed to identify major classes of constituents like alkaloids, saponins, glycosides, steroids, tannins and phenolic compounds according to standard methods<sup>17</sup>.

**Safety evaluation**—EEMO and HAMO were administered to Wistar rats and albino mice up to a dose of 2 g/kg, po as per the Organization for Economic Co-operation and Development (OECD) guidelines No. 425. Observations were made for gross behavioral changes such as locomotion, rearing, respiration, tremors, gait, passivity, righting reflex, lacrimation and mortality in the first 3 h and monitored continuously for 14 days<sup>18</sup>.

**Animals**—The protocol was approved by the Institutional Animal Ethical Committee bearing number IAEC Clear/39/09 and usage of animals was in compliance with the CPCSEA guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India). Wistar rats (180-200 g) and albino mice (25-30 g) of either sex were used. They were provided with water and food (Lipton India Ltd., Mumbai, India) *ad libitum*. Maintenance of the animals were maintained at 12.15:11.45 h L:D period at 23 ± 2°C.

**Antigen**—Fresh blood was collected in a sterile bottle containing Alsever's solution from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBC) were washed thrice in normal saline to adjust to a concentration of 5×10<sup>8</sup> cells/mL and 0.25×10<sup>8</sup> cells/mL for immunization and challenge respectively.

**Grouping of animals**—Wistar rats were randomized into 8 groups of 6 animals each for the delayed type hypersensitivity (DTH) reaction, cyclophosphamide induced immunosuppression and neutrophil adhesion test. Animals in the treated groups were administered with the EEMO and HAMO daily for 13 days in doses of 50, 100 and 200 mg/kg body weight respectively. Negative control animals received 1.0 % aqueous tragacanth and the standard group received 100 mg/kg of alcoholic extract of *T. cordifolia*. In the humoral immune response test, rats were divided into 9 groups of 6 each. Group-I was treated as control and received aqueous solution of tragacanth (1 mL), Group-II received cyclophosphamide (CP, 50 mg/kg) on 5<sup>th</sup> day, and Group III received 100 mg/kg of alcoholic extract of *T. cordifolia* which served as a standard. Groups IV-VI received 50, 100 and 200 mg/kg EEMO for 7 days and Groups VII-IX received 50, 100 and 200 mg/kg HAMO for 7 days. For the carbon clearance assay, mice were categorized into 8 groups of 6 animals each. Group-I received aqueous solution of tragacanth (1 mL) and served as a control. Group II received 100 mg/kg of alcoholic extract of *T. cordifolia* which served as a standard, Groups III-V received 50, 100 and 200 mg/kg of EEMO and Groups VI-VIII received HAMO respectively. All treatments were administered orally for 7 days.

### Pharmacological screening for immunomodulatory activity

**Delayed type hypersensitivity (DTH) reaction using SRBC as an antigen**—All the rats were immunized on

day 0 with 0.8 mL SRBC suspension in phosphate buffer containing  $5 \times 10^8$  cells/mL, ip. Wistar albino rats were treated with EEMO, HAMO and alcoholic extract of *T. cordifolia* (AETC) for 7 days. Edema was induced in the right hind paw of rats by challenging with 0.1 mL SRBC suspension ( $0.25 \times 10^8$  cells/mL) in the sub-plantar region on day 7. The contra-lateral paw received equal volume of phosphate buffer and served as a control. The increase in the paw volume at 0 and 48 h was assessed using a plethysmometer (UGO Basile, Comeiro, Italy). The difference in the thickness of the right hind paw and left hind paw was used as a measure of delayed type hypersensitivity reaction<sup>19</sup>.

**Carbon clearance assay in mice** — After treatment for 7 days, mice were injected with 0.1 mL of carbon suspension (Pellikan Tuschea Ink, Germany) intravenously through tail vein on the 8<sup>th</sup> day. Blood samples were collected from retro-orbital plexus at 10 and 20 min time intervals. Blood samples were lysed with 2 mL of 0.1% acetic acid and absorbance of samples was recorded at 675 nm with a spectrophotometer using pre-injected blood sample as a blank<sup>20,21</sup>. The graph for absorbance versus time was plotted for each animal in respective test groups and phagocytic index was calculated using the formula:

$$K = \frac{OD_1 - OD_2}{t_2 - t_1}$$

where,  $OD_1$  and  $OD_2$  are the optical densities at times  $t_1$  and  $t_2$  respectively and K represents the slope of regression line.

**Humoral immune response** — All rats were immunized (ip) on day 0 with 0.8 ml SRBC suspension containing  $5 \times 10^8$  cells/mL. In order to induce immunosuppression, cyclophosphamide (50 mg/kg) was administered orally on 5<sup>th</sup> day of the experiment to animals of all the groups except group-I. On 7<sup>th</sup> day, 2 h after the last dose, blood was withdrawn from the retro-orbital plexus under light anesthesia of all antigenically challenged rats. Serum (25 $\mu$ L) was serially diluted with 25 $\mu$ L of phosphate buffered saline. SRBC suspension (0.1 mL;  $0.25 \times 10^8$  cells/mL) was added to each of these dilutions and incubated at 37 °C for 1 h. The value of highest serum dilution carrying visible haemagglutination was taken as the antibody titre expressed in terms of number of wells<sup>22</sup>.

**Cyclophosphamide induced immunosuppression** — After an hour of the administration of extract all the animals except the negative control group were administered cyclophosphamide (50 mg/kg, po) orally on days 11, 12 and 13. Blood samples were collected on day 14 and the total white blood cell (WBC) count was determined<sup>23</sup>.

**Neutrophil adhesion test** — All the groups received the treatment for 14 days by the oral route. After 1 h of the last dose on the 14<sup>th</sup> day, blood samples were collected in heparinized vials by retro-orbital puncture and subjected to total as well as differential leukocyte count by fixing blood smears and staining with Leishman's stain. After initial counts, the blood samples were incubated with 80 mg/mL of nylon fibers at 37 °C for 15 min. The incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and % neutrophils known as neutrophil index was determined for each of the respective groups<sup>24</sup>. The % neutrophil adhesion for each of the test groups was determined as follows:

$$\text{Neutrophil adhesion (\%)} = \frac{NI_U - NI_t}{NI_U} \times 100$$

where  $NI_U$  = neutrophil count in untreated blood;  $NI_t$  = neutrophil count in fiber-treated blood

**Statistical analysis** — Assessment of statistical significance was done using one way analysis of variance (ANOVA) followed by Bonferrini's multiple comparison tests. All the values are expressed as mean $\pm$ SE. The level of significance was fixed at  $P < 0.05$ .

## Results

**Safety evaluation** — EEMO and HAMO up to a dose of 2000 mg/kg failed to exhibit any significant behavioural changes and did not produce mortality. Therefore 50, 100 and 200 mg/kg of the extracts were selected for the study.

**Preliminary phytochemical screening** — Screening of EEMO and HAMO revealed the presence of saponins, flavanoid, terpenoids and tannins.

**Delayed type hypersensitivity (DTH) reaction** — The impact of 50, 100 and 200 mg/kg of EEMO and HAMO on T-cell mediated DTH reaction is depicted in Table 1. HAMO (200 mg/kg) produced a significant increase in DTH response in rats at 48 h when compared with the control group ( $P < 0.05$ ). EEMO

(200 mg/kg) exhibited a significant enhancement of immune response to SRBC with increase in percentage edema as compared to control group ( $P<0.05$ ). The % paw edema was also significantly enhanced following treatment with *T. cordifolia*.

*Carbon clearance assay in mice* — Rate of carbon clearance determined as phagocytic index was significant with EEMO (200 mg/kg). HAMO dose dependently enhanced carbon clearance compared to control group ( $P<0.05$ ) (Table 1). Significant increase in phagocytic activity was also observed with AETC compared with the control ( $P<0.05$ ).

*Humoral immune response* — The influence of EEMO and HAMO on humoral immune response is presented in Table 2. AETC enhanced antibody titre compared with the negative control. HAMO (100 and 200 mg/kg) increased humoral antibody titre compared with the control ( $P<0.05$ ). The antibody

titre level was significant in animals treated with higher dose of EEMO (200 mg/kg) compared to the control ( $P<0.05$ ).

*Cyclophosphamide induced immunosuppression* — An improvement in the TLC count was observed with AETC. EEMO (100 and 200 mg/kg) produced an elevation in the TLC. HAMO in all three dose levels significantly countered the impact of cyclophosphamide compared with the control ( $P<0.05$ ).

*Neutrophil adhesion test* — A dose dependent increase in percentage of neutrophil adhesion was observed with HAMO which was significant compared to control ( $P<0.05$ ). Similarly EEMO (100 and 200 mg/kg) enhanced percentage neutrophil adhesion compared with the control ( $P<0.05$ ). The results produced by HAMO and EEMO were almost comparable to AETC (Table 3).

Table 1 — Effect of ethanolic and hydro-alcoholic extract of leaves of *M. olifera* on delayed type hypersensitivity reaction and phagocytic index

[Values are mean  $\pm$  SE from 6 observations]

Group	Treatment	DTH response (% increase in paw volume)	Phagocytic index
I	Control	10.45 $\pm$ 0.15	1.00 $\pm$ 0.01
II	AETC (200 mg/kg)	15.28 $\pm$ 1.02*	2.5 $\pm$ 0.62*
III	EEMO (50 mg/kg)	12.00 $\pm$ 0.14	1.05 $\pm$ 0.25
IV	EEMO(100 mg/kg)	12.72 $\pm$ 0.48*	1.076 $\pm$ 0.42
V	EEMO(200 mg/kg)	15.45 $\pm$ 0.71*	2.45 $\pm$ 0.18*
VI	HAMO(50 mg/kg)	11.63 $\pm$ 0.27	2.36 $\pm$ 0.32*
VII	HAMO(100 mg/kg)	13.48 $\pm$ 1.16*	2.78 $\pm$ 0.30*
VIII	HAMO(200 mg/kg)	17.29 $\pm$ 1.08*	3.15 $\pm$ 0.58*

EEMO: Ethanolic extract of leaves of *M. olifera*, HAMO: hydro-alcoholic extract of leaves of *M. olifera*, AETC : alcoholic extract of *T. cordifolia*, Significant at \* $P<0.05$  compared with control

Table 2 — Effect of ethanolic and hydro-alcoholic extract of leaves of *M. olifera* on humoral immune response and total leukocyte count

[Values are mean  $\pm$  SE from 6 observations]

Group	Treatment	Humoral immune response (mean antibody titre level)	WBC Count (cells/mm <sup>3</sup> )
I	Negative Control	6.52 $\pm$ 0.98*	5,560 $\pm$ 3.12*
II	Cyclophosphamide (50 mg/kg, positive control)	3.40 $\pm$ 0.45	3,420 $\pm$ 3.56
III	AETC (200 mg/kg)	19.36 $\pm$ 1.40	5,829 $\pm$ 2.56*
IV	EEMO (50 mg/kg)	3.60 $\pm$ 0.40	4,587 $\pm$ 3.48*
V	EEMO(100 mg/kg)	8.21 $\pm$ 0.67*	4,991 $\pm$ 4.01*
VI	EEMO (200 mg/kg)	14.50 $\pm$ 0.95*	5,965 $\pm$ 3.90*
VII	HAMO (50 mg/kg)	9.72 $\pm$ 0.56*	5,075 $\pm$ 2.80*
VIII	HAMO(100 mg/kg)	19.46 $\pm$ 0.50*	5,610 $\pm$ 2.89*
IX	HAMO (200 mg/kg)	23.22 $\pm$ 1.90*	6,275 $\pm$ 4.05*

EEMO: Ethanolic extract of leaves of *M. olifera*, HAMO: hydro-alcoholic extract of leaves of *M. olifera*, AETC : alcoholic extract of *T. cordifolia*, Significant at \* $P<0.05$  compared with positive control

Table 3 — Effect of ethanolic and hydro-alcoholic extract of leaves of *M. olifera* on neutrophil adhesion test[Values are mean  $\pm$  SE from 6 observations]

Group	Treatment	Neutrophil Index		
		Untreated blood sample	Fiber treated blood samples	Neutrophil adhesion (%)
I	Control	3.31 $\pm$ 0.08	2.79 $\pm$ 0.09	15.25 $\pm$ 0.91
II	AETC (200 mg/kg)	4.8 $\pm$ 0.35	3.4 $\pm$ 0.22	29.16 $\pm$ 0.68
III	EEMO(50 mg/kg)	3.63 $\pm$ 0.05	2.97 $\pm$ 0.07	17.90 $\pm$ 0.85
IV	EEMO(100 mg/kg)	3.80 $\pm$ 0.78	2.92 $\pm$ 0.60	23.15 $\pm$ 1.06*
V	EEMO(200 mg/kg)	5.50 $\pm$ 0.28*	4.17 $\pm$ 0.26*	24.23 $\pm$ 1.24*
VI	HAMO(50 mg/kg)	3.82 $\pm$ 0.98	2.85 $\pm$ 0.97	25.39 $\pm$ 0.86*
VII	HAMO(100 mg/kg)	5.80 $\pm$ 0.13*	4.20 $\pm$ 0.05*	27.58 $\pm$ 1.13*
VII	HAMO (200 mg/kg)	5.95 $\pm$ 0.10*	4.25 $\pm$ 0.04*	28.57 $\pm$ 1.14*

EEMO: Ethanolic extract of leaves of *M. olifera*, HAMO: hydro-alcoholic extract of leaves of *M. olifera*, AETC : alcoholic extract of *T. cordifolia*, Significant at \* $P$ <0.05 compared with control

## Discussion

The task of the immune system is to boost immunity and protect the physiological system from infection. The facets of specificity, adaptability and inducibility are striking features of the immune system. Macrophages play a pivotal role in humoral and cellular immunity as they orchestrate both cytotoxic and phagocytic response. Recruitment of leukocytes and natural killer cells occurs following the release of macrophage inflammatory proteins. Leukocytes play a vital role in the production of antibodies. An elevation in total leukocytes was observed following treatment with EEMO and HAMO. Neutrophils are considered as frontline cells in the immune system as they are capable of recognizing, phagocytosing and destroying foreign agents<sup>25</sup>. Polymorphonuclear lymphocytes could serve as modulators of immune function resulting in the elevation in neutrophil count. In the present study, a substantial elevation in neutrophil index and adhesion was observed with HAMO (100 and 200 mg/kg) and EEMO (200 mg/kg). This indicates that HAMO and EEMO facilitate the secretion of cytokines resulting in an increase in the neutrophil index.

Perturbations in immune milieu can arise due to cumulative pressure on the immune system. Cell-mediated immunity is modulated by thymus-derived lymphocytes (T lymphocytes) which are sensitized by the antigen and on subsequent contact they respond with a delayed-type hypersensitivity reaction. DTH reaction was measured by foot-pad thickness, after 48 h of antigenic challenge subsequent to immunization with SRBC. An evident rise in foot pad thickness was observed with 100 and 200 mg/kg of HAMO and EEMO which could be attributed to the

ability of the extract to activate lymphocytes and accessory cell types leading to enhanced production of antibodies thereby increasing cell mediated immunity.

B lymphocytes responsible for humoral immunity produce immunoglobulins which recognize and eliminate extra cellular antigens. Antigenic exposure could facilitate the proliferation and differentiation of B cells resulting in enhanced antibody titre<sup>26</sup>. Cyclophosphamide facilitates senescence of immune cells resulting in a decline in antibody titre. Challenge with SRBC produces rise in the haemagglutination antibody titre owing to sensitization of macrophages, T and B lymphocytes. A significant elevation in antibody titre was seen with 200 mg/kg of EEMO and HAMO in immunosuppressed animals.

Clearance of inoculated carbon particles from the circulation is reflective of the functional ability of macrophages of the liver, lung and spleen to undertake phagocytosis<sup>27</sup>. Blood monocytes and neutrophils are some of the other specialized cell partaking in this process. Enhanced phagocytic index was observed with HAMO in all doses and with 200 mg/kg of EEMO. This increased phagocytic index indicates an increase in immune response due to activation of the reticuloendothelial system.

A constant supply of nutrients is essential for functioning of the immune system, for the synthesis of effector molecules and new cells. Nutrients affect immune function by directly acting within the lymphoid system, or indirectly by affecting cellular material or other organ systems that act as immune regulators<sup>28</sup>. Minerals encompass one of the substrates which regulate metabolic sequences and physiologic processes. Minerals are involved in diverse roles as

modulating heart rhythm, oxygen transport, enzyme activation, immune functions and antioxidant activity to name a few. Calcium abundantly present in *M. olifera* could be responsible for enhancing immune function and IL-2 production<sup>29</sup>. The process of cell replication, formation of antibodies and cytokines which are proteins are expedited by the immune system during attack<sup>30</sup>. Therefore energy metabolism and protein requirements are enhanced under such circumstances which can be replenished from *M. olifera* which contains a cornucopia of vitamins, minerals and amino acids. The proportion of sulfur containing amino acids in *M. olifera* parallels that possessed by soy beans<sup>31</sup>. It has phenylalanine, lysine, arginine, methionine and histidine to name a few. Arginine is known to augment cellular immunity, enhance lymphocyte and macrophage proliferation<sup>32</sup>. Vitamin A present in *M. olifera* could improve the competence of the immune system as it has been documented that vitamin A facilitates lymphocyte proliferation and increases antibody production<sup>33</sup>. The presence of flavonoids in the extract of *M. olifera* leaves may be a contributing factor to its immune boosting qualities as they are particularly known to activate lymphocytes<sup>34</sup>. It was observed from the present study that both EEMO and HAMO exhibited an action in all the parameters evaluated in a dose of 200 mg/kg, however on priority basis HAMO exerted a better action. Hydro-alcoholic extract of *M. olifera* might cumulate a higher reserve of minerals, amino acids, vitamin B, vitamin C and fat soluble vitamins such as A, E and D. On the other hand, the ethanolic extract due to its polarity could possibly cumulate fat soluble vitamins.

In conclusion, both the extracts of *M. olifera* could play a plausible role in enhancing immunity and its impact may be attributed to the flavanoids, vitamins and minerals present in the extract.

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