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IMMUNOMODULATORY ACTIVITY OF METHANOLIC LEAF EXTRACT OF *MORINGA OLEIFERA* IN ANIMALS

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Abstract : Aim: The aim of the present study was to investigate the immunomodulatory action of methanolic extract of *Moringa oleifera* (MEMO) in an experimental model of immunity.

The cellular immunity was evaluated using neutrophil adhesion test, cyclophosphamide induced neutropenia and carbon clearance assay, whereas, humoral immunity was tested by mice lethality test, serum immunoglobulin estimation and indirect haemagglutination assay in animals.

Administration of MEMO (250 and 750 mg/kg, po) and *Ocimum sanctum* (100 mg/kg, po) significantly increased the levels of serum immunoglobulins and also prevented the mortality induced by bovine *Pasteurella multocida* in mice. They also increased significantly the circulating antibody titre in indirect haemagglunation test. Moreover, MEMO produced significant increase in adhesion of neutrophils, attenuation of cyclophosphamide-induced neutropenia and an increase in phagocytic index in carbon clearance assay.

From the above results, it can be concluded that MEMO stimulate both cellular and humoral immune response. However, low dose of MEMO was found to be more effective than the high dose.

Key words : cellular immunity humoral immunity *Moringa oleifera*

INTRODUCTION

An increasing number of people are adopting alternative systems of medicine owing to the irreversible effects of modern drugs and therapies (1, 2). Use of medicinal plant products for treatment of various acute and chronic diseases is gaining increasing importance around the globe. Many plant products have been exploited for modulation

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of immune system in number of Ayurvedic formulation either alone or in groups (1, 3–5).

The leaves and fruits of *Moringa oleifera* (Moringaceae) commonly known as drumstick or horseradish tree in English are used as vegetable in different parts of south Asia and Africa (6). Traditionally, the leaves of the plant are used for the treatment of a variety of disorders. The plant is reported to possess wide range of pharmacological effects that includes antifertility (7), antitumor (8), antipyretic (9), antiepileptic (9), antispasmodic (10), anti-inflammatory (10), diuretic (10), antiulcer (11), hypotensive (12), hypolipidemic (13), hypoglycemic (14), hepatoprotective (15), antifungal (16) and antibacterial activities (17).

The immune system is affected by the environmental and dietary habits and it is believed that diet rich in antioxidants and micronutrients can boost the immune system (18). An earlier study carried out in Africa revealed that Moringa powder supplementation might act as an immune stimulant for patients suffering from HIV infection (19). As the plant is widely used in folklore for the treatment of suppressive conditions of immune system and so far no study has been carried out to prove the stimulatory actions of *Moringa oleifera* on immune system, therefore present work was undertaken using modern scientific techniques in experimental models of cellular and humoral immunity in animals.

MATERIALS AND METHODS

Experimental animals – Wistar rats weighing between 200–250 g and Swiss albino mice weighing between 25–35 g were used. Institutional Animal Ethics Committee approved the experimental protocol; animals were maintained under standard conditions in an animal house approved by Committee for the Purpose of Control, and Supervision on Experiments on Animals (CPCSEA). The animals were given pellet food (Lipton India Ltd., Mumbai, India) and water *ad libitum*.

Procurement of plant material and extraction – Moringa oleifera leaves was collected from Vellore district of Tamil Nadu (India) during the month of May and June. The plant were identified and authenticated by Regional Research Institute (Bangalore, India) (authentication number: GNG/SMP-PROG/800). The leaves were shade dried and extracted using methanol in a soxhlet. The ethanolic extract of *Ocimum sanctum* was used as standard immunomodulatory agent.

Chemicals – Leishmann's stain, Indian ink and gluteraldehyde were purchased from Merck (Mumbai, India). WBC diluting fluid, zinc sulphate and barium chloride were from Nice Chemicals (Cochin, India). Cyclophosphamide (Endoxan Injection) was from German Remedies (Mumbai, India). *Pasteurella multocida* of bovine origin and its vaccine were obtained from Institute of Animal Health and Veterinary Biologicals (Bangalore, India).

Acute toxicity studies (20) – The acute toxicity study was carried out according to the limit test described in the OPPTS guidelines. Briefly, a test dose of 2 g/kg and 5 g/kg were given orally to the mice. No mortality was found even at 5 g/kg; po. However, methanolic extract of *Moringa oleifera* caused significant changes in the total RBC, packed cell volume (PCV), haemoglobin percentage (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), total and

differential WBC when evaluated at 1.5 g/kg and 2 g/kg. Hence we opted for half of 1.5 g/kg as high dose and 1/6th of 1.5 g/kg as low dose corresponding to 750 mg/kg and 250 mg/kg respectively.

Experimental Protocol – The drug solutions were prepared as suspension using sodium carboxymethyl cellulose (0.5% w/v) in distilled water for oral administration. Immunomodulatory activity was evaluated using following models of cellular and humoral immunity. All the experiments had four groups consisting of six animals each. The first group served as control (vehicle 1 ml/100 g, po), the second group received the ethanolic extract of *Ocimum sanctum* (OSE) at a dose of (100 mg/kg, po) (21). The third and fourth groups were given low (250 mg/kg, po) and high dose (750 mg/kg, po) of MEMO respectively. However, in mice lethality, an additional negative control group was also present.

Neutrophil Adhesion test (22, 23) – The rats were treated orally with vehicle or extracts for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinised vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The percentage of neutrophils in the treated and untreated blood was determined and the difference was taken as index of neutrophil adhesion.

Mice lethality test (24) – Swiss albino mice were subjected to MEMO and OSE treatment orally for 21 days in their respective groups. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine) through subcutaneous route. On the 21st day, the animals were challenged subcutaneously with 0.2 ml of lethal dose ($25 \times LD_{50}$) of *Pasteurella multocida* (bovine origin) containing 10^7 cells per ml. The animals were observed for a period of 72 hr and the mortality percentage was determined using the formula.

Mortality Percentage : 100 × 'Number of animals dead'/total number of animals

Cyclophosphamide induced neutropenia (25) – Swiss albino mice received the drug or vehicle orally for 10 days. On 10th day, neutropenic dose of cyclophosphamide (200 mg/kg, *s.c*) was injected and this day was labelled as day zero. Blood was collected; the total leukocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and neutrophil counts (%) in treated groups were compared with the values of the control group.

Carbon clearance test (26, 27) – Swiss albino mice were treated with the drug or vehicle orally for 10 days. After 48 hr of the last dose of the drug, animals were injected 0.1 ml of Indian ink via the tail vein. Blood samples were withdrawn at 0 and 15 min after injection. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following equation:

$$K = (Log_e OD1 - Log_e OD2)/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively.

Serum immunoglobulin (28) – The drugs were administered to Wistar rats orally for

21 days. Six hours after the last dose of drug, blood was collected and the serum was used for immunoglobulin level estimation following a method described by Mullen (1975). Briefly, for every sample of serum to be analyzed, a control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution were prepared. To each, 0.1 ml of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard $BaSO_4$ solution was prepared by adding 3 ml of barium chloride solution (1.15% w/v) to 97 ml of 0.2 N sulphuric acids. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units.

Indirect haemagglutination test (22) – Rats were pretreated with the drugs for 14 days and each rat was immunized with 0.5×10^9 sheep red blood cells (SRBCs) intraperitoneally, including control rats. The day of immunization was referred to as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions with SRBC (0.025×10^9 cells) in microtitre plates. The plates were incubated at room temperature for 2 hr and examined visually for agglutination. The minimum volume of serum showing heamagglutination was expressed as heamagglutination (HA) titre.

Statistical analysis – The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's comparison test. The values are expressed as mean±SEM and $P<0.05$ was considered significant.

RESULTS

Effect on neutrophil adhesion test – Incubation of neutrophils with nylon fibres (NF) produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. Both doses of MEMO and OSE showed significant increase in the neutrophil adhesion when compared to control. The low dose of MEMO was found to be more effective than high dose of MEMO. There was also rise in neutrophil count in untreated blood of all treatment groups (Table I).

TABLE I: Effect on neutrophil adhesion in rats.

Treatment	*Differential leucocytes count(% neutrophils)*		*Difference*
	Untreated Blood	*Nylon fibres treated blood*	
Control	19.00±1.41	13.66±0.84	5.33±0.80
OSE (100 mg/kg, po)	30.66±0.71	17.50±0.80	13.50±0.56**
MEMO (250 mg/kg, po)	25.16±0.60	15.83±0.47	9.34±0.55**
MEMO (750 mg/kg, po)	22.66±0.49	14.16±0.54	8.50±0.84**

All values are expressed as mean±SEM of six observations. **P<0.01 when compared to control.

Effect on mice lethality test – Mortality was found to be 100% within 72 hr in control group upon administration of *Pasteurella multocida*. There was 83.33% mortality in vaccinated group without any prior treatment of drug. The low and high doses of MEMO reduced the mortality percentage to 66.66% and 83.33% respectively, whereas, OSE

showed 33.33% decrease in the mortality with survival of four animals out of six (Table II).

Effect on cyclophosphamide induced neutropenia – Administration of cyclophosphamide reduced the TLC in control animals by 54.52%. Pretreatment of animals with MEMO for 10 days before cyclophosphamide administration produced 48.91% and 52.81% reduction in TLC with low and high doses respectively. The pretreatment of animals with OSE showed 45.93% fall in TLC when compared to initial values. The percentage reduction in neutrophil count was found to be 57.01 and 38.53 in control and OSE groups respectively. The low and high doses of MEMO demonstrated 40.00% and 48.23% reduction in neutrophil count compared to initial values (Table III).

TABLE II: Effect on mice lethality test.

Treatment	*Number of animals died*			*Mortality percentage*
	Day 1	*Day 2*	*Day 3*	
No Drug, No vaccination	1	5	–	100%
No Drug, Vaccination	1	4	–	83.33%
OSE (100 mg/kg, po) + Vaccination	1	1	–	33.33%
MEMO (250 mg/kg, po) + Vaccination	1	1	2	66.66%
MEMO (750 mg/kg, po) + Vaccination	2	2	1	83.33%

Effect on carbon clearance test – Both the doses of *Moringa oleifera* extract and OSE showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best with low dose of MEMO and OSE (Table IV).

Effect on serum immunoglobulins – The low dose of MEMO and OSE showed a significant increase in the serum immunoglobulin levels when compared to

TABLE III: Effect on cyclophosphamide induced neutropenia.

Treatment	*Total leucocytes count (cells/mm³)*		*% Reduction*	*% neutrophils*		*% Reduction*
	Before	*After*		*Before*	*After*	
Control	5516.67±165.67	2441.52±65.54	54.52	14.33±1.05	6.16±0.66	57.01
OSE (100 mg/kg, po)	5241.65±154.07	2825.00±101.45	45.93**	13.83±0.65	9.00±0.57	38.53**
MEMO (250 mg/kg, po)	5366.68±186.93	2741.67±86.03	48.91*	12.50±0.88	8.60±0.61	40.00**
MEMO (750 mg/kg, po)	5458.34±142.84	2575.00±88.270	52.81*	14.16±1.07	6.83±0.59	48.23*

All values are expressed as mean±SEM of six observations. **P<0.01, *P<0.05 when compared to control.

TABLE IV: Effect on phagocytic index, serum immunoglobulin levels and HA titre.

Treatment	*Phagocytic index in carbon clearance assay*	*Serum immunoglobulin level (ZST units)*	*Haemagglutination (HA) titre*
Control	0.0090±0.0025	15.605±0.1668	0.150±0.223
OSE (100 mg/kg, po)	0.0390±0.0074**	27.610±0.7363**	0.054±0.017**
MEMO (250 mg/kg, po)	0.0385±0.0046**	22.318±0.5803**	0.044±0.010**
MEMO (750 mg/kg, po)	0.0638±0.0049**	17.837±0.3513	0.075±0.167*

All values are expressed as mean±SEM of six observations. **P<0.01, *P<0.05 when compared to control.

control. The high dose of MEMO was not able to significantly increase immunoglobulin levels compared to control (Table IV).

Effect on indirect haemagglutination test – The haemmagglutinating antibody (HA) titre value was significantly increased in animals that received vaccination along with low or high dose of MEMO or OSE compared to animals that received vaccination alone (Table IV).

DISCUSSION

In this paper we report for the first time the immunomodulatory activity of methanolic extract of *Moringa oleifera* (MEMO) in experimental models of cellular and humoral immunity in animals. The extract was found to be most effective at low dose (250 mg/kg, po), whereas, high dose (750 mg/kg, po) of MEMO was moderately effective in modulating immune system. The study was carried out using six different methods, each of which provides information about effect on different components of the immune system.

The neutrophil adhesion to nylon fibres describes the margination of polymorphonuclear lymphocyte in the blood vessels and the number of microphages reaching the site of inflammation (23). Both low and high doses of MEMO (250 & 750 mg/kg, po) showed a substantial rise in the neutrophil adhesion to nylon fibres. This might be due to the upregulation of the β2 integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres (29). Hence, it was inferred that MEMO causes stimulation of neutrophils towards the site of inflammation.

The mouse lethality test is one of the commonly employed tests to assess serological responses in animals immunized with vaccines. *Pasteurella multocida* is pathogenic to mice. The mouse lethality test involves injecting mice with the vaccine before administration of the bacterial culture and determining the mortality percentage (30). The vaccination induces humoral immunization. The survival of animals is dependent on the ability of drug to produce adequate number of antibodies which can counter the pathogen. The low and high dose of MEMO prevented the death of 33.33% and 16.67% of animals at the end of 72 hrs, whereas, OSE showed only 33.33% mortality. Hence it is speculated that MEMO in low dose is better effective in increasing the number of survival, while the OSE is remarkably effective in preventing the mortality.

The cyclophosphamide induced neutropenia model concentrates on the protective effects against cyclophosphamide induced myelo-suppression in the experimental animals (31). Both low and high doses of MEMO caused decrease in the cyclophosphamide induced neutropenia suggesting that it attenuates the effect of cyclophosphamide on the haemopoetic system.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells. Cells of the RES play a vital role in the clearance of particles from the bloodstream. When colloidal carbon particles in the form of ink are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (27). Both doses of MEMO as well as OSE showed remarkable augmentation in the phagocytic index due to their ability to increase the activity of the reticuloendothelial system.

The serum immunoglobulin levels suggest the amount of antibodies present in the serum. The zinc sulphate turbidity test is used to gain a rough estimation of the amount of immunoglobulins present in the serum. A small amount of serum was added to a zinc sulfate solution and allowed to incubate at room temperature for 1 h. Zinc sulfate will cause precipitation of the immunoglobulins, which makes the solution cloudy instead of clear. A lack of cloudiness signifies lack of immunoglobulins (32). The turbidity is expressed as ZST units, which in turn indicate the amount of immunoglobulin present in the sample. *Moringa oleifera* extract at both the doses showed a significant increase in the serum immunoglobulin levels.

The indirect haemagglutination test was performed to confirm the effect of *Moringa oleifera* extract on the humoral immune system. It is composed of interacting B cell with antigens and subsequently proliferating and differentiating into antibody producing cells. Antibody works by binding with antigens and neutralizing it or facilitating its elimination by cross linking to form latex that is more readily ingested by phagocytic cells (27). The results showed that levels of circulating antibodies are increased if the test animals are pretreated with *Moringa oleifera* extract or OSE.

The main constituents of *Moringa oleifera* include two nitrile glycerides: niazirin and niazirinin and three mustard oil glycosides isothiocynate, niaziminin A and niazimin B. The mustard oil glycosides were attributed for hypotensive activity of *Moringa oleifera*. As discussed earlier, *Moringa oleifera* is reported to possess antioxidant property with immunostimulatant behavior (18) as per traditional claim. The moringa powder was also exploited as anti-AIDS agent (19). Anti-HIV activity could be due to it immunostimulatory effect. It is also known that polysaccharide isolated from the hot aqueous extract of mature pods of *Moringa oleifera* showed significant macrophage activity through the release of nitric oxide on mouse monocyte cell line (33). Hence, it speculated that the immunostimulatory activity of MEMO is due to presence of its active constituent in methanolic extract of *Moringa oleifera*. However, further studies should be carried for evaluating the immunomodulatory activity of isolated pure active constituent.

In conclusion, both low dose (250 mg/kg, po) as well as high dose (750 mg/kg, po) of *Moringa Oleifera* stimulates immune system by acting through cellular and humoral immunity in experimental models of immunity in animals. However, low dose was found to be most effective than the high dose. This could be due to the presence of toxicant such as isothiocyanate and glycoside cyanides that may pose stress at high concentration and hence reducing the antioxidant potential of *Moringa oleifera* (34).

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