Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Suppl 5, 2011

Research Article

MORINGA OLEIFERA LEAVES: IMMUNOMODULATION IN WISTAR ALBINO RATS

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Received: 1 Aug 2011, Revised and Accepted: 11 Nov 2011

ABSTRACT

This work has been carried out to find out immunomodulatory potential of leaves of *Moringa oleifera* (Family Moringaceae). The different extracts of leaves *viz*. Petroleum Ether (PEM), Chloroform (CHM), and Methanol (MEM) were administered at doses of 100, 200, and 400 mg/kg b.w, p.o. The measurement of immunomodulation was carried out in Wistar albino rats by Delayed Type Hypersensitivity (DTH), Humoral antibody (HA) titre, Cyclophosphamide induced myelosuppression, and T cell population tests. Results were compared with standard immunosuppressant drug Cyclophosphamide (30 mg/kg b.w, p.o) and standard immunomodulatory drug Levamisole (50 mg/kg b.w, p.o). Results of present study clearly indicate that PEM400 mg/kg and MEM at all doses shown potentiation of DTH response ($p<0.01$) after 24 hrs challenge. The increasing doses of methanolic extract have shown the augmentation of antibody titre ($p<0.05$). Cyclophosphamide induced immunosuppression was counteracted by MEM indicating restoration of hematological parameters ($p<0.01$). Groups treated with other extracts have shown restoration of hematological parameters as compared to Cyclophosphamide treated group but not regained the normal values. Above reports suggests that methanolic extract of *Moringa oleifera* leaves can stimulate both cellular and humoral immunity.

Keywords: *Moringa oleifera*, Immunomodulation, DTH, HA titre, Myelosuppression, T cell population.

INTRODUCTION

Traditional Indian systems of medicines like Siddha and Ayurveda have suggested means to increase the body's natural resistance to disease. A number of Indian medicinal plants and various 'Rasayanas' have been claimed to possess immunomodulatory activity[1-4]. Herbal drugs possess immunomodulatory property and generally act by stimulating both specific and non specific immunity[5]. Immunology is a branch of microbiology and is defined as the study of defence mechanism of the body against harmful invading causes[6]. Immunology is one of the most developing and crucial area of biomedical research. It also opens the doors of new hopes and major advances in the prevention and treatment in wide range of disorders. Arthritis, ulcerative colitis, asthma, allergy, parasitic and infectious diseases are primarily considered as immunologic disorders[7,8]. Severe side effects and cost of the allopathic drugs have attracted most of the researchers to find out the drugs which are without side effects especially belonging to the traditional systems of medicines. Herbal medicines have been the foundation of treatment and cure for various ailments. Natural products provide an excellent material for the discovery and development of novel immunomodulatory compounds.

M. oleifera, belongs to family Moringaceae, is also known as 'Horse radish' or 'Drumstick'. It is widely cultivated throughout Tropical countries and Sub-Himalayan tracts of India. Its leaves, flowers, and fruits are used as vegetables. As all the parts of plant are very nutritious so it has also called as 'Multipurpose Tree' or 'The Miracle Tree of Life'[9,10]. Leaves contain high level of proteins (27%) and are rich source of vitamin A and C, calcium, iron, and phosphorus[11]. Leaves, roots, and flowers of the plant were used in the treatment of rheumatism, cardiac and circulatory disorders in folk medicines and also possess antitumor, anti-inflammatory, antihypertensive, antidiabetic, hepatoprotective, cholesterol lowering, antioxidant, antibacterial and antifungal properties. Plant is also reported for its liver tonic activity[12-14]. Seeds of this plant are reported for immunosuppressant activity by Mahajan *et al*[15]. Important chemical constituents identified in *M. oleifera* are alkaloids, moringine, vitamin C, flavonoids like quercetin and kaempferol, tocopherols, benzylisothiocyanate derivatives and glycosides[14,16,17].

Present study is an attempt to find out the immunomodulatory potential of *M. oleifera* leaves.

MATERIAL AND METHODS

Plant Material Collection and Extract Preparation

Fresh leaves of *M. oleifera* were collected from Ahmednagar district of Maharashtra. Authentication of plant was done at Botanical Survey of India, Pune, India (Voucher No. SWITMOO3). Shade dried leaves were crushed and successively extracted with petroleum ether, chloroform, and methanol. Extracts were concentrated by vacuum distillation. Extracts were dissolved in 1% Gum acacia in saline and used for further study.

Animals Used

Wistar albino rats (Approx 150 to 180 gm) were procured from Gentox Bioservices, Hyderabad. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India (Reg. No.1175/ac/08/CPCSEA).

Antigens

Sheep Red Blood Cells (SRBC) were collected in Alsever's solution from animal house of National Institute of Nutrition, Hyderabad, India. SRBC were washed 3-4 times with large quantity of sterile and pyrogen free saline[18].

Acute Toxicity Studies

All the three extracts (PEM, CHM, MEM) were tested for acute toxicity studies as per procedure given in OECD guidelines. Rats (n=6) were starved for overnight and fed orally with the extracts doses (10, 40, 100, 400, 1000 and 2000 mg/kg .b.w). Animals were observed for next 14 days for behavioral changes and mortality.

Delayed Type Hypersensitivity

Antigen Challenge

On 0th day, all groups were sensitized with 0.1 ml of SRBC containing 1×10^8 cells, i.p.

Experimental Design

Animals were divided into different groups each containing 6 animals.

Group I - Control, 1% Gum acacia suspension in saline

Group II - Standard, Levamisole, 50 mg/kg b.w, p.o (1st to 7th day)

Group III - Negative control, Cyclophosphamide, 30 mg/kg b.w, p.o (4th, 5th, 6th day)

Group IV, V, VI - PEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 7th day)

Group VII, VIII, IX - CHM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 7th day)

Group, X, XI, XII - MEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 7th day)

On 7th day prior to injection, right hind footpad thickness was measured with Micrometer screw gauge (Mitutoyo Digimatic). Then animals were challenged by injecting 1% SRBC (20 µl) into the right hind footpad. On 8th and 9th day footpad thickness was again measured. Difference between prior and post challenge footpad thickness was reported as DTH response[19-22].

Humoral Antibody (HA) Titre Response to SRBC

Experimental design was done same as mentioned in Delayed type hypersensitivity model. On 7th day before challenge, blood was withdrawn from retro-orbital plexus of each animal. Blood was centrifuged, and serum was separated. Serial two fold dilutions were made i.e. 50 µl of serum was added to 1st well of 96-well micro titre plate containing 50 µl normal saline. To this 1% SRBC (50 µl) dissolved in normal saline was mixed. From 1st well 50 µl of diluted serum was added to 2nd well containing 50 µl normal saline and 50µl 1% SRBC. Such dilutions were done till 24th well. Plates were incubated at 37ºC for 1 hr. Highest dilution that has shown visible agglutination was considered as haemagglutination antibody titre[19,21].

Cyclophosphamide Induced Myelosuppression

Experimental Design

Animals were divided into different groups each containing 6 animals.

Group I - Control, 1% Gum acacia suspension in saline

Group II - Standard, Levamisole, 50 mg/kg b.w, p.o (1st to 13th day)

Group III- Negative control, Cyclophosphamide, 30 mg/kg b.w p.o (11th, 12th, 13th day)

Group IV, V, VI - PEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 13th day)

Group VII, VIII, IX - CHM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 13th day)

Group, X, XI, XII - MEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 13th day)

On 0th day, blood was withdrawn from retro-orbital plexus of animals of each group and subjected to haematological parameter determination. Drugs were fed as per the schedule from 1st to 13th day. Cyclophosphamide (30 mg/kg, p.o) was given to all animals on 11th, 12th and 13th day, 1 hr after extracts administration except control and standard group. On day 14th, blood was again withdrawn from retro- orbital plexus of animals of each group and subjected to haematological parameter determination and restoration of parameters were observed[18,22,23].

T - Cell population

Experimental Design

The rats were divided into eleven groups

Group I - Control, 1% Gum acacia suspension in saline

Group II - Standard, Levamisole, 50 mg/kg b.w, p.o (1st to 10th day)

Group III, IV, V - PEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 10th day)

Group VI, VII, VIII - CHM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 10th day)

Group IX, X, XI - MEM 100, 200, and 400 mg/kg b.w, p.o respectively (1st to 10th day)

On 11th day, blood was collected from the retro-orbital plexus and anticoagulated with Alsever's solution in separate test tubes. Test tubes containing blood were kept in sloping position (45º) at 37ºc for 1 hour. RBCs were allowed to settle at bottom and supernatant was collected from each test tube by using micropipette which contains lymphocytes. An amount of 50 µl of this lymphocyte suspension & 50 µl SRBC were mixed in test tube and incubated. Resultant suspension was centrifuged at 200 rpm for 5 min and kept in a refrigerator at 4ºC for 2 hr. The supernatant fluid was removed and one drop of cell suspension was placed on a glass slide. Total lymphocytes were counted and a lymphocyte binding with three or more erythrocytes was considered as rosette[24,25].

Statistical Analysis

Values are expressed as Mean ± SEM, n=6 rats in each group. Results obtained were statistically analyzed by using one-way ANOVA followed by Dunnett's multiple comparison test. $p < 0.05$ was considered a significant value.

RESULTS AND DISCUSSION

In chemotherapy, immunomodulators are used as adjuvant to control and prevent infections[26]. Compounds like phenols, alkaloids, glycosides, polysaccharides, saponins have been tested for their efficacy as both chemical and biological markers[27]. The main objective of this study was to explore the immunomodulatory potential of leaves of *M. oleifera* in wistar albino rats. Previous reports and phytochemical screening of extracts has shown the presence of flavonoids, alkaloids, proteins, glycosides, vitamins and traces of polyphenolic contents.

Acute Toxicity Studies

No behavioral changes or mortality were observed after 14 days of all extracts administration. So 1/10th of the dose i.e 200 mg/kg, b.w has been selected for the present study.

Delayed Type Hypersensitivity

In the present investigation, SRBC induced DTH reaction was used to study the effect of extracts on cell mediated immunity. DTH is an antigen specific and mediated by T cells rather than antibody. T cells are required to initiate the reaction. Activation of T cells releases lymphokines, which lead to activation and accumulation of macrophages, increases vascular permeability, induce vasodilatation, and produce inflammation. It also boosts phagocytic activity and increases concentration of lytic enzymes for more effective killing, ultimately results in increase footpad thickness in immunized animals. General characteristics of DTH are an invasion of immune cells at site of injection and induction became apparent within 24 to 72 hrs[6,28-30]. Pretreatment of the MEM has shown significant increase in paw thickness ($p<0.01$) after 24 hrs of challenge at all doses. DTH response was lowered after 48 hrs in all extract treated groups and significant for MEM at all doses as compared to control animals. Potentiation of DTH response was observed in Cyclophosphamide treated animals ($p<0.01$) because it has damaged short lived suppressor T cells in immune system. Levamisole, a standard immunomodulatory drug, has shown maximum potentiation of DTH response ($p<0.001$). After 72 hrs footpad thickness was observed normal. Increase in paw edema after 24 hrs of challenge was observed in all extract treated groups when compared to control. PEM400 has shown mild increase in DTH response ($p<0.01$) after 24 hrs and response has subsided upto 48 hrs. CHM has shown mild potentiation of DTH response at a dose of 200 mg/kg b.w. PEM100, PEM200, CHM100, and CHM400 have not shown any significant increase in DTH response. It may be concluded that these extracts are unable to stimulate the macrophages function to stimulate T cell for the hypersensitivity reaction in the immunized animals. Increase in DTH response of animals revealed the stimulatory effect of MEM on T lymphocytes i.e. cell mediated immunity or specific immunity. The results are mentioned in the Table 1.

Table 1: Effect of different extracts on DTH response using SRBC as an antigen in rats

Groups	Drug & Dose	Mean footpad thickness (mm)	
		24 hrs	48 hrs
I.	Control	0.0211±0.120	0.02±0.123
II.	Levamisole	1.5764±0.084***	0.893±0.230**
III.	Cyclophosphamide	0.8902±0.066**	0.4242±0.04**
IV.	PEM100	0.3883±0.046	0.1030±0.08
V.	PEM200	0.3102±0.023	0.1364±0.155
VI.	PEM400	0.4083±0.011**	0.1362±0.120
VII.	CHM100	0.3901±0.029	0.2432±0.082
VIII.	CHM200	0.4650±0.020**	0.2781±0.230
IX.	CHM400	0.3436±0.170	0.2105±0.136
X.	MEM100	0.5750±0.011**	0.4316±0.132**
XI.	MEM200	0.5266±0.023 **	0.4120±0.216**
XII.	MEM400	0.6270±0.176**	0.4503±0.02**

Values are expressed as Mean ± SEM, (n=6), Comparison of Group I (Control) with all groups. ***p<0.001 Extremely Significant, **p<0.01 Very Significant, *p<0.05 Significant

Humoral antibody (HA) titre response to SRBC

The reaction of an antibody and antigen can be easily detected by agglutination (clumping) of the antigen. If the antigen is an erythrocyte the term haemagglutination is used. Agglutination tests can also be used to measure the level of antibodies to particulate antigens. In this test, serum containing antibodies was collected from animals of each group and serial dilutions were done in microtiter plate. Fixed number of SRBC (50 µl) were added in each well. The maximum serum dilution that shows visible agglutination was considered as antibody titre. Sometimes agglutination is not visible to naked eyes so it can be observed under microscope[6]. Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody screening plasma cells. Antibody functions as the effectors 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[31,32]. To evaluate the effect of *M. oleifera* leaves on humoral response, its influence was tested on sheep erythrocyte specific haemagglutination antibody titre in rats (Table 2). Low dose of all the three extracts i.e. 100 mg/kg has not shown haemagglutination. While immunosuppressant group i.e. Cyclophosphamide treated group (30 mg/kg p.o), showed significant inhibition of haemagglutination titre (2.4±0.4) as compared to control group (8.8±1.96). Immunostimulation of humoral response by standard immunomodulatory drug Levamisole has resulted in higher antibody titre (p<0.01). Mild potentiation of humoral immunity was observed in the PEM200, PEM400, CHM200, and CHM400, while increase humoral immunity was observed in MEM200 and MEM400 treated animals (p<0.05). Dose dependant increase in HA titre value was observed only with methanolic extracts. Studies suggest that 7 days pretreatment of methanolic extract of the leaves at a higher dose level was capable to enhance responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis.

Table 2: Effect of different extracts on HA tire level using SRBC as an antigen in rats

Group	Drug & Dose	Titre Level
I.	Control	8.8±1.96
II.	Levamisole	819.2±125.41**
III.	Cyclophosphamide	2.4±0.4^{ns}
IV.	PEM100	No haemagglutination
V.	PEM200	9.6±1.96 ^{ns}
VI.	PEM400	10±2.02 ^{ns}
VII.	CHM100	No haemagglutination
VIII.	CHM200	3.5±1.1 ^{ns}
IX.	CHM400	5.0±1.25 ^{ns}
X.	MEM100	No haemagglutination
XI.	MEM200	36.4± 4.3*
XII.	MEM400	46.7±8.6*

Values are expressed as Mean ± SEM, (n=6), Group II to XII were compared with Group I.

^{ns}p>0.05 not significant,*p<0.05 Significant

Cyclophosphamide Induced Myelosuppression

Myelosuppression is a decrease in the production of blood cells. Cyclophosphamide is a most potent cytotoxic and immunosuppressive agent which act at various levels on cells involved in defense mechanism against various invaders by inhibiting both cell mediated and humoral immunity. It also causes dose dependant bone marrow suppression means it significantly decreases the Hb, RBC, and WBC counts[33,34]. Cyclophosphamide treatment for the period of 3 days showed significant reduction in Hb (p<0.05) and WBC count (3.22± 0.48, p<0.001) and thereby exerted immunosuppressant effect when compared to control animals. Combined treatment of extracts and myelosuppressive drug at all doses showed restoration of WBC count when compared to Cyclophosphamide treated groups. Levamisole, a standard immunomodulatory drug, has not shown marked difference in Hb and RBC count, but WBC count was increased markedly but not abnormally (p<0.001). Total WBC count was regained normal values only with MEM100 mg/kg on 14th day of study (p<0.01). Hb and RBC counts were increased at a dose of 200 and 400mg/kg of methanolic extract. PEM was not found to be significant to increase the Hb and RBC counts. Suppressive effect of Cyclophosphamide was protected by animals pretreated with Pet. ether extract and has shown restoration of WBC count on 14th day of study. Increasing doses of extract have not shown much restoration of hematological parameters. Results shown in Table 3 have revealed that administration of MEM of leaves could stimulate the haemopoetic system.

Table 3: Effect of different extracts on Cyclophosphamide induced myelosuppression in rats.

Groups	Drug & Dose	HB (g/dL)		RBC ($\times 10^6$/mm^3)		WBC ($\times 10^3$/mm^3)	
		0 day	14 day	0 day	14 day	0 day	14 day
I.	Control	11.21±0.29	11±0.51	7.27±0.06	7.69±0.21	9.55 ± 0.73	9.3± 0.45
I.	CP	11.68±0.71	6.5±0.57$^{\#\#}$	7.83± 0.20	6.84± 0.12	10.1 ±0.28	3.22± 0.48$^{\#\#}$
I.	Levamisole	11.25±0.96	11.60±1.4 ^{ns}	7.34±0.12	7.95±0.28^{ns}	9.73 ± 0.42	10.68±0.22***
V.	PEM100+ CP	11.56±0.21	10.95±0.33^{ns}	7.58±0.12	8.43±0.227 **	5.32±0.17	4.24±0.19*
V.	PEM200+ CP	12.41±0.32	12.75±0.54*	7.72±0.17	8.00±0.09 ^{ns}	5.81±0.24	3.65±0.82 ^{ns}
I.	PEM400+ CP	11.56±0.23	12.06±0.58*	7.23±0.06	7.98±0.07^{ns}	5.67±0.32	4.94±0.73**
I.	CHM100+ CP	11.70±0.33	12.56±0.21*	7.12 ±0.05	7.45± 0.18 ^{ns}	5.77±0.10	4.31±0.88*
I.	CHM200+ CP	12.13±0.34	13.16±0.19*	7.23±0.09	8.77±0.17**	5.56±0.59	4.40±0.46*
X.	CHM400+ CP	10.90±0.17	10.95±0.27 ^{ns}	7.60±0.08	7.85±0.01^{ns}	5.67±0.36	4.55±0.43*
X.	MEM100+ CP	10.66±0.13	13.48±0.19**	7.39±0.09	7.24±0.06 ^{ns}	5.38±0.17	5.41±0.16**
I.	MEM200+ CP	11.3±0.29	13.63±0.14**	7.69±0.01	8.37±0.08 **	5.41±0.16	4.49±0.31*
I.	MEM400+ CP	12.76±0.17	12.55±0.27*	7.48±0.09	8.98±0.02**	6.49±0.17	5.40±0.16**

CP- Cyclophosphamide.

Values are expressed as Mean ± SEM, (n=6),

##p<0.01: Group II was compared with Group I.

Group III to XII were compared with Group II, *p< 0.05 Significant, **p<0.01 Very Significant, ***p<0.001 Extremely Significant, nsp>0.05 not significant.

T cell population

T lymphocytes are involved in both the cellular and humoral immune response and T cell formation is a very important factor. These cells do not secrete the antibody but attack the tissue cells that have been transplanted from one host to other. Therefore, only T cells come into close contact with foreign or infected cell in order to destroy them and to provide cell mediated immunity. Attachment of lymphocytes to foreign or infected cell is called as rosette. If the foreign cell (Here, SRBC) is erythrocyte then called as E- rosette[6]. Increased lymphocyte formation was found to be more in Levamisole treated group while binding of lymphocytes to the SRBC also increased (p<0.01). As compared to control group, lymphocyte formation was found to be more in PEM400 but not significant. MEM pretreated animals specifically MEM200 shown significant increased population of lymphocyte and E- rosette. Results are given in Table 4.

Table 4: Effect of different extracts on Lymphocyte and E-Rosette formation

Group	Drug &Dose	Lymphocyte	E-Rosette
I.	Control	125.5±2.1	10±1.3
II.	Levamisole	198.23±3.5**	23.05±2.7**
III.	PEM100	126.54±1.7ns	13.98±3.5ns
IV.	PEM200	128.43±4.9ns	13.65±7.2ns
V.	PEM400	121.87±9.8ns	14.17±3.1ns
VI.	CHM100	98.74±5.9ns	13.12±01ns
VII.	CHM200	106.34±3.3ns	11.27±3.2ns
VIII.	CHM400	143.76±5.2ns	11.09±1.6ns
IX.	MEM100	130.90±1.2ns	15.74±2.6*
X.	MEM200	165.98±5.6*	19.75±1.4**
XI.	MEM400	172.95±10.6**	15.61±4.2*

Values are expressed as Mean ± SEM, (n=6), Group II to XI were compared with Group I,*p< 0.05 Significant, **p<0.01 Very Significant, nsp>0.05 not significant.

CONCLUSION

Extensive literature survey revealed the presence of flavonoids, glycosides, polyphenols, and alkaloids in *Moringa oleifera* and these constituents are well established for their antioxidant, anti-inflammatory, neuroprotective, and hepatoprotective properties. Methanolic extract of leaves of *M. oleifera* was found to be more significant than other extracts during the study of immunomodulation. Methanol extract stimulates both cellular and humoral immune systems. Methanol extract also showed potential effect on haemopoetic system. Immunomodulatory potential of *M. oleifera* leaves could be attributed for the presence of flavonoids, polyphenols and terpenoids which may modulate one of the above mentioned immune-mechanisms. Separation and characterization of active principles, different types of T cells and their mechanisms responsible for immunomodulation is the future scope of the study.

ACKNOWLEDGEMENT

The authors are very thankful to The Principal, Dr. C.V.S Subramanyam and M. Gangaraju, Associate Professor, Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India for providing animal house facility. Authors express their gratitude to JNTUH, Hyderabad for financial support.

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