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Immunomodulatory activity for methanolic extract of *Moringa oleifera* leaves in albino rats

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Abstract
The aim of the present study was to investigate the immunomodulatory action of methanolic extract of *Moringa oleifera* 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 hemagglutination assay in animals. 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.

Keywords: *Moringa oleifera*, albino rats, households, immunity

Introduction
Indian medicinal plants are rich source of substances that are claimed to induce immunity [1]. The immune system is involved in the etiology, as well as pathophysiology mechanism of many diseases. Modulation of immune responses to alleviate various diseases has been of interest for many years.
Medicinal plants are rich sources of substances which are non-specific immunomodulation of essentially granulocytes macrophages, natural killer cells and complement functions 1. Because of the concerns about the side effect of conventional drugs, the use of natural products as an alternative to conventional treatment in healing and treatment of various diseases has been on the rise in the last few decades [2]. Present study is an attempt to find out the immunomodulatory activity of *Moringa oleifera* L. leaves.

Material and Methods
The Plant *Moringa oleifera* L. was collected from Career college Gandhi Nagar Campus Bhopal in the month of December-January, 2020. The plant was then identified and authenticated by Dr. Jagrati Tripathi, Asst. Prof. of Botany Govt. College Khemlasha (M.P.) and Specimen Voucher No. 397 was put in the P.G. Department of Zoology Career College Bhopal.

Extraction
The plant material cleaned of adulterants and ground to powder or cut into small pieces was soaked in first with petroleum ether solvent for defatting with occasional shaking at room temperature for about 4-5 days. It was then filtered through a muslin cloth and then through Whatman qualitative grade 1 filter paper. After filtration the mesh obtained was then further soaked with methanol in closed vessel with occasional shaking at room temperature. It was then filtered through a muslin cloth and then through Whatman qualitative grade 1 filter paper. The filtrate was combined and concentrated on vacuum rotary evaporator (RE-111, Buchi, Flawil, Switzerland) accompanied with B-700 recirculation chiller and a water bath model 461 at 40 °C to thick pasty mass Phytochemical Screening of different extracts of *Moringa oleifera* L. and Fractions Biologically active Petroleum ether extract, Methanolic extracts and crude fractions of successive methanolic extract (ethyl acetate crude fraction and methanol crude fraction) were evaluated for preliminary phytochemical screening to know the presence of different primary and secondary metabolites present in them *i.e.*, Alkaloids, Terpenoids, Glycosides, Steroids, Triterpenoids, Flavonoids, Carbohydrates, Saponins, Tannins and proteins etc.

Chromatographic purification of crude

TLC study has shown the presence of different components present in methanolic extract of *Moringa oleifera* Lam when the corresponding fractions were run in specific solvent system.

Table 1: Showing *Rf* values of methanolic extract of *Moringa oleifera* L.

Solvent System	Spot No.	R*f* Values	Colors of Peaks
Toulene: Aceteic acid: Formic acid (5: 3.5: 0.5)	1	0.08	Blue
	2	0.39	Yellow
	3	0.44	Brown
	4	0.7	Green
	5	0.73	Green

The methanolic extract of *Moringa oleifera* Lam crude fractions were further purified by using silica gel 'G' glass column and elution was carried out from non-polar to polar solvents by gradient elution method. Different fractions like 0, 4, 3, 7, 3, 5 and 5 were eluted for *Moringa oleifera* Lam Fraction 0, 4, 3, 7, 3, 5 and 5 eluted yielded no residue or less quantity after evaporation and hence these fractions were discarded. The fractions (7) eluted with chloroform: ethyl acetate (50:50) showed single spot-on TLC, afforded a compound (F-I) (6.1% w/w, 2.275 gm).

Table 2: Separation of constituents from methanolic extract of *Moringa oleifera* L.

S. No.	Eluting Solvent	Ratio (%)	Fractions Collected	% Yield of Isolated Compounds
1.	n-Hexane	100%	0	No Residue after evaporation
2.	Chloroform	100%	4	Very less quantity was isolated
3.	Chloroform and ethyl acetate	75%/25%	3	Very less quantity was isolated
4.	Chloroform and ethyl acetate	50%/50%	7	Fr-I(R_f=0.37) (6.1% w/w, 2.275 gm)
5.	Chloroform and ethyl acetate	40%/50%	3	Very less quantity was isolated
6.	Chloroform and methanol	50%/50%	5	Very less quantity was isolated
7.	Methanol	100%	5	No Residue after evaporation

Acute oral Toxicity

As per OECD 423 guidelines three animals are used for each step. The dose level to be used as the starting dose was selected as 5 mg/kg of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals. It was observed that none of the animals were dead throughout the observation. Thus, as per the study 2000 mg/kg was considered as NOAEL and the quantitative availability of drug the dose was selected accordingly as $1/40^{th}$ and $1/20^{th}$ of NOAEL (*i.e.* 2000 mg/kg). Hence selected doses were 50 mg/kg and 100 mg/kg respectively.

Immunomodulatory activity of crude extract of methanolic extract of *Moringa oleifera* L.

Selection of doses

From the acute oral toxicity studies as per the OECD guidelines 423, no mortality or other behavioral and morphological changes were observed at all doses up to level of 2000 mg/kg b.w. of methanolic extract *Moringa oleifera* L. in female albino rats. Thus, the two different doses selected for test groups were $1/10^{th}$ (200 mg/kg BW) and $1/5^{th}$ (400 mg/kg BW) of the safe dose. Levamisole has showed good immunomodulatory activity at a dose of 2.5 mg/kg BW in albino rats, the same dose was selected for study as a standard drug. Cyclophosphamide was used as an immunosuppressant drug at a dose of 200 mg/kg b.w. (a neutropenia dose).

Preparation of the test extract

Methanol fraction was suspended in dimethyl sulphoxide (DMSO) to prepare different doses (200 and 400 mg/kg body weight) and administered orally with the help of gastric canula. The control animals were given an equivalent volume of Phosphate buffer Saline (PBS pH 7.4) vehicle.

Antigen

Fresh blood was collected from sheep sacrificed in local slaughterhouse in Alsever's solution (formula is mentioned ahead). During the experiment, adequate amount of stock solution (sheep red blood cells (SRBC) stored in Alsever's solution) was taken and allowed to stand at room temperature. It was washed three times with normal saline. The settled SRBCs were then suspended in normal saline and RBCs of this suspension was adjusted to a concentration of 5×10^9 SRBC/ml for immunization and challenge [16].

Preparation of Alsever's solution

Formula:
Citric acid 0.055 gm
Sodium citrate 0.8 gm
Glucose 2.05 gm
Sodium chloride 0.42 gm
Distilled water to make volume up to 100 ml
All the above solids were weighed and dissolved in distilled water in a conical flask and made the volume up to 100 ml. It was then stored in refrigerator.

Blood withdrawal

For withdrawing the blood samples, the animals were lightly anaesthetized using ethyl ether. A fine capillary was gently inserted into the lower angle of eye at 45 ^oC and blood was withdrawn from retro orbital plexus into micro centrifuge tubes.

Immunomodulatory protocols

A) SRBC-induced humoral antibody (HA) titre

Groups of six rats per treatment were immunized by injecting 20µl of SRBC suspension (5×10^9 SRBC /ml) subcutaneously into right hind foot pad. Seven days later they were challenged by injecting 20µl of SRBC suspension (5×10^9SRBC /ml) intradermally into the left hind foot pad. The day of immunization was referred to as day 0. Blood

samples were collected from all the animals separately by retro orbital puncture on day +7 (before challenge) for primary antibody titre and on day +14 for secondary antibody tire. Antibody levels were determined by the method described [6]Briefly 25µl aliquot of serum of each animal was taken in microtiter plates. To serial two –fold dilutions of pooled serum (made in 25µl normal saline), 25µl of 1% v/v SRBC suspension (in normal saline) was added. The microtiter plates were kept at room temperature for 1 hour and then observed for hemagglutination (until control wells showed unequivocally negative pattern). The value of the highest serum dilution showing haemagglutination was taken as the antibody titre. Methanolic extract/ isolated compound were fed orally once daily, starting with 7 days prior to sensitization till the challenge.

Result and Discussion

Immunomodulatory activity

Searching of substances with immunostimulative or immunorestorative effects could contribute to the maintenance of the immune system. Immune modulation helps in maintain disease Free State of body. Many plants have been evaluated for immunostimulant and immunosuppressive properties using simple techniques. An attempt has been made to evaluate the immunomodulatory activity of the isolated bioactive compounds that showed potent antioxidant activity using different by investigating its effect on both humoral as well as cell mediated immunity using different models as: Haemagglutination antibody titre, cyclophosphamide induced myelosuppression, Delayed type hypersensitivity and phagocytic index.

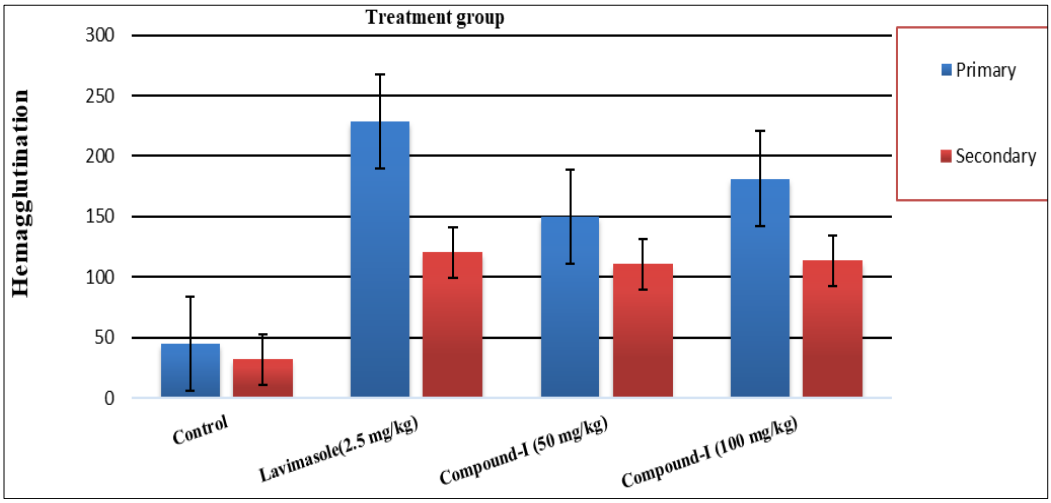
Haemagglutination antibody titre

The effect of methanolic extract of *Moringa oleifera* L. administration as such is shown in Hemagglutination antibody was determined to establish the humoral response against SRBC. The purified isolated compounds showed significant increase ($p<0.05$) in HA titer value compared to control at a dose of 100 mg/kg b.w.

Table 3: Effect of Isolated compound-I on Hemagglutination antibody titer

Group [n]	Treatment	Hemagglutination antibody titer	
		Primary (1^0)	Secondary (2^0)
I	Control (PBS pH 7.4)	44.66 ± 6.74	32.0 ± 0.0
II	Levamisole (2.5 mg/kg b.w.)	228.23 ±28.62***	120.12 ± 12.11***
III	Compound-I (50 mg/kg b.w.)	149.59 ± 23.34*	110.31 ± 32.01*
IV	Compound-I (100 mg/kg b.w.)	181.43 ± 34.72**	113.21 ± 13.71**

Values are expressed as Mean ± SEM; * $p<0.05$ as compared to control.



Graph 1: Showing the effect of Compound-I on Hemagglutination antibody titre (Primary and Secondary).

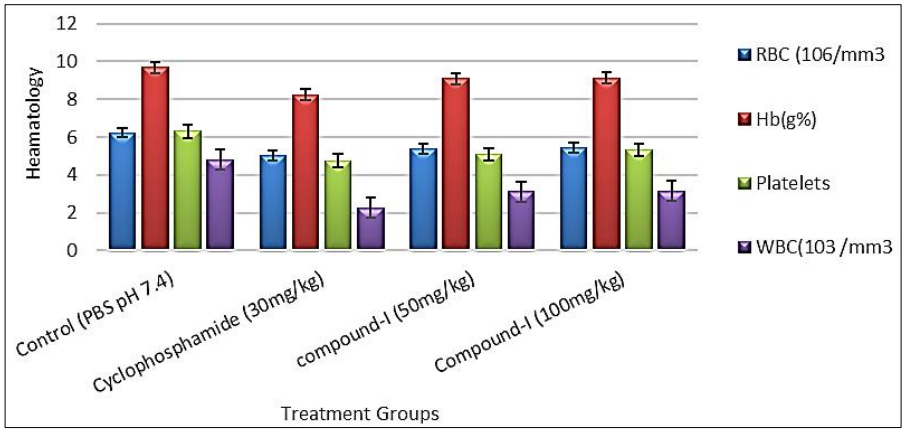
Cyclophosphamide induced myelosuppression

Cyclophosphamide at the dose of 30 mg/kg (intraperitonial) caused a significant reduction in total WBC count, differential leukocyte counts and platelets and marginal reduction in RBC and Hb% as compared to control group (Group-I), the results are represented in (Table 4).

Table 4: Effect of isolated Compound-I on Cyclophosphamide induced myelosupression (*Hematology*)

Group [n]	Treatment	RBC (10^6/mm^3)	Hb (g%)	Platelets	WBC (10^3/mm^3)
I	Control (PBS pH 7.4)	6.232 ± 0.070	9.683 ± 0.101	6.300 ± 0.057	4.800 ± 0.096
II	Cyclophosphamide (30 mg/kg)	5.023 ± 0.056	8.250 ± 0.136	4.767 ± 0.088	2.267 ± 0.244
III	Compound-I (50 mg/kg)	5.390 ± 0.183	9.100 ± 0.068	5.083 ± 0.068	3.117 ± 0.075
IV	Compound-I (100 mg/kg)	5.433 ± 0.169	9.117 ± 0.070	5.317 ± 0.124	3.150 ± 0.076

Values are expressed as Mean ± SEM; * $p<0.05$ as compared to control.

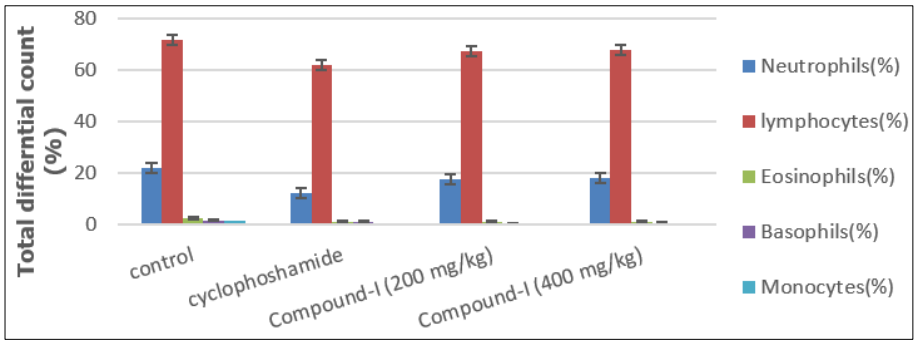


Graph 2: Showing the effect of Compound-I on Cyclophosphamide induced myelosupression (*Hematology*)

Table 5: Effect of isolated Compound-I on Cyclophosphamide induced myelosupression (Total differential count %)

Group n	Treatment Group	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Basophils (%)	Monocytes (%)
I	Control (PBS pH 7.4)	22.00 ± 0.577	71.83 ± 0.654	2.667 ± 0.210	1.500 ± 0.223	1.667 ± 0.210
II	Cyclophosphamide (30 mg/kg)	12.17 ± 0.703	62.17 ± 0.477	0.666 ± 0.210	0.166 ± 0.166	0.167 ± 0.167
III	Compound-I (50 mg/kg)	17.67 ± 1.085	67.50 ± 0.845	1.167 ± 0.401	0.333 ± 0.211	0.333 ± 0.211
IV	Compound-I (100 mg/kg)	18.33 ± 1.054	68.00 ± 0.365	1.267 ± 0.401	0.500 ± 0.223	0.661 ± 0.210

Values are expressed as Mean ± SEM; * $p<0.05$ as compared to control.



Graph 3: Showing the effect of Compound-I on Cyclophosphamide induced myelosupression (Total differential count %).

The results obtained in the present study conclude that *Moringa oleifera* L. leave is a potent immunostimulant, stimulating specific and non-specific immune mechanisms. It may be due to the presence of various phytoconstituents present in *Moringa oleifera* like phenolics, flavonoids, tannins, and alkaloids, are already reported to possess immunomodulatory activity. In present study, antioxidant potential of varied crude extracts of *Moringa oleifera* L. was investigated by using DPPH assay. The antioxidant activity of the samples was noted as IC_{50} values. The IC_{50} value was described as the concentration (in µg/ml) of the drug or standard which hinders the formulation of DPPH radicals by 50%. The minimum IC_{50}hints the robust capability of the extracts to serve as DPPH radical scavengers. IC_{50} values were computed from the percentage inhibition versus concentration plot, employing a non-linear regression algorithm. Methanol crude extract showed strongest ability to act as DPPH radical scavengers by showing lower IC_{50} values of 384.35µg/ml for methanol extract On the basis of above results, it was concluded that MK2 has lower IC_{50} value and hence has highest antioxidant potential because the minimal IC_{50}announces the vigorous capability to serve as DPPH radical scavenger. 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*.

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