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Research Article

IMMUNOMODULATORY ACTIVITY OF METHANOLIC EXTRACT OF *MURRAYA KOENIGII* LEAVES AGAINST AZATHIOPRINE INDUCED IMMUNOSUPPRESSION IN LABORATORY ANIMALS

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ABSTRACT

The present work has been carried out to annotate the ameliorative effect of methanolic extract of leaves of *Murraya koenigii* in Azathioprine induced immunosuppression in mice. Healthy Swiss albino mice of either sex were divided into six groups (n=6). The control group of animals received 1% CMC solution as vehicle. AZP 50mg/kg b.w i.p was administered to animals in second group that served as disease control group. Levamisole was used as standard drug that was administered to group III at a dose of 50mg/kg b.w p.o. Groups IV, V, VI were given with 250, 500 & 750 mg/kg b.w of the extracts p.o. after the treatment period, Immunomodulatory activity of Murraya koenigii was evaluated by Delayed Type Hypersensitivity reaction, hematological parameters, Phagocytic index and determination of organ weights. Intraperitoneal administration of Azathioprine caused myelosupression and compromised immune responses. Methanolic extract of *Murraya koenigii* significantly increased DTH response when compared to control and disease control group. The extract also increased the Phagocytic index, WBC count and % neutrophills in a dose dependent manner. The obtained results were comparable with that of the standard drug Levamisole. The findings demonstrate that MK is a promising drug with a potent therapeutic value in stimulating the suppressed or weakened immune responses in laboratory animals and hence can act as a potent immunomodulator.

Keywords: immunosuppression, hematological parameters, Delayed Type Hypersensitivity reaction, Phagocytic index, Immunomodulation.

INTRODUCTION

When the host defense mechanism has to be activated under the conditions of impaired immune response, Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy. Indigenous system of medicine provides the use of several herbs that may modulate the body's immune system. The usage of medicinal plants as immunomodulators is still in its infancy in the days of modern medicine. Use of some plants like *Emblica officinalis*[1], *Ocimum sanctum*[2], *Viscum album*[3], *Moringa oleifera*[4], *Piper longum Linn*[5], *Panax ginseng*[6] etc have been reported earlier. Different varieties of plant derived materials such as lectins, polysaccharides, peptides, flavanoids and tannins have been reported to modulate the immune system.

One such plant used from ancient times in traditional medicine is *Murraya koenigii* commonly known as curry leaf tree belonging to the family Rutaceae native to India. The plant is extensively used in many indigenous preparations. It is reported to have anti-inflammatory, anti-arthritic, anti tumor, anti bacterial, anti diabetic[7], anti obesity and many other such activities. Murraya koenigii administration was found to increase Hb level, RBC and WBC levels; reduces the serum cholesterol, ESR etc. administration of extracts of MK could decrease leucopenia, enhanced bone marrow cellularity and the ratio of normochromatic to polychromatic erythrocytes in mice. Enhancement of WBC count, bone marrow cellularity as well as esterase positive cells with the administration of MK extracts was also observed in mice treated with AZP. This herb is used for several medicinal purposes and is reported to exhibit chemomodulatory and antioxidant properties.
The present investigation has been carried out to evaluate the Immunomodulatory activity of methanolic extracts of leaves of *Murraya koenigii*.

MATERIALS AND METHODS

Collection of plant materials

The leaves of *Murraya koenigii* were collected from the rural areas of Guntur district, Andhra Pradesh in the month of December 2014. The plant was authenticated by Dr. J. Sunitha, Department of Botany, Government College, Rajahmundry and the plant sample was deposited under the Herbarium voucher number 002.

Preparation of extract

The leaves of the plant were shade dried and made into coarse powder with the help of a grinder. The powder was then extracted by successive extraction method using ethyl acetate and methanol as solvents. The collected material was then dried and evaporated to dryness with the help of rotavapor. The yield was found to be 18% w/w. solution of M. koenigii extract was prepared by suspending the extract in 1% CMC solution.

Preliminary phytochemical screening

The extract of *M. koenigii* was subjected to preliminary phytochemical screening according to the method described by Khandelwal et al and Patil[8]. Phytochemical screening of the extract was carried out for the identification of phytochemicals like alkaloids, sesqueterpenes, monoterpenes, coumarins etc.

Animals

Healthy Swiss albino mice (25g) of either sex obtained from the animal house of GIET School of Pharmacy-Rajahmundry were used. The animals were housed in groups of six in propylene cages. They were fed with standard pellet diet, water ad libitum and maintained

under standard environmental conditions in pyrogens free conditions. All experimental studies were performed in accordance with the guidelines on regulation of scientific experiments on animals as approved by the Institutional Animal Ethics Committee Clearance number GSP/IAEC/2015/03/01.

Chemicals and reagents

The drug used was Azathioprine (AZP). AZP was dissolved in normal saline and was administered orally by oral feeding tube to mice at a dose of 50mg/kg body wt. all other chemicals and solvents used for experimental work were of analytical grade. Sheep red blood cells were collected from the local slaughter house in Alsevers solution.

Acute toxicity studies

Acute oral toxicity studies of extract in swiss albino mice were carried out according to the OECD guidelines 423. Graded doses of methanolic extract of *M. koenigii* from 50mg/kg body weight upto 5000mg/kg body weight were administered orally and the animals were observed for weeks following administration. Parameters such as body weight, food consumption, fluid intake and psychomotor activities were recorded daily.

Antigen

Fresh sheep blood was collected from the slaughter house and collected in sterile Alsevars solution (1:1 proportion). Pyrogens free normal saline was used to wash the sheep red blood cells and was centrifuged at 2500-3000 rpm for 10 min. the clear supernatant liquid was removed and suspended in normal saline. A volume of 0.1ml with $1x10^8/mm^3$ cells concentration was adjusted using improved Neubaur Chamber for immunization and challenge[9].

Dosages of *M. koenigii* and standard drugs used

The concentrated methanolic leaves extract of MK was dissolved in freshly prepared 1% CMC solution and administered to the animals in three different doses (250, 500 & 750 mg/kg body wt). Azathioprine was administered at a dose of 50mg/kg body wt i.p for induction of immunosuppression and the standard group was administered with Levamisole 50mg/kg body wt orally by suspending in the vehicle.

Induction of immunosuppression

The study comprises of six different groups of six animals each as follows:

- Group I: normal animals: received 2mL of 1% CMC solution for 18 days.
- Group II: disease control animals: received 50mg/kg AZP i.p daily for 18 days.
- Group III: standard animals: received Levamisole 50mg/kg orally by suspending in 1% CMC solution daily for 18 days.
- Group IV: drug treated animals: received the methanolic extract of MK at a dose of 500mg/kg orally by suspending in 1% CMC solution 1hr after the administration of AZP daily for 18 days.
- Group V: drug treated animals: received the methanolic extract of MK at a dose of 750mg/kg orally by suspending in 1% CMC solution 1hr after the administration of AZP daily for 18 days.
- Group VI: drug treated animals: received the methanolic extract of MK at a dose of 250mg/kg orally by suspending in 1% CMC solution 1hr after the administration of AZP daily for 18 days.

On the 19th day, blood was collected from the retro-orbital plexuses and then the animals were sacrificed under ether anesthesia. Hematological parameters such as RBC count, WBC count, Hb level were determined. Weight of vital organs such as thymus, spleen, liver and kidney were also recorded. Delayed Type Hypersensitivity reaction and Carbon clearance Test were also performed according to their specified methods to determine the immune functions.

Evaluation of the disease

The disease induced in experimental animals was evaluated based on the following determinations:

Determination of effect of MK on the hematological parameters

Swiss albino mice were treated with plant extracts in three different doses. Preliminary findings indicated that these doses were found to be safe as they didn't produce any toxicity. Blood parameters such as RBC count, WBC count, Hb level were recorded using auto analyser. Percentage neutrophills were determined by finding out the differential leukocyte count by fixing the blood smears and staining with Leishman's stain[10].Methanolic extract of leaves of MK was Administration of the methanolic extract of leaves of MK showed statistically significant increase ($P<0.05$) in RBC and WBC count when compared to Azathioprine (50mg/kg) treated and control mice.

Determination of effect of MK on organ weight of mice

A significant dose related increase in size and weight of the thymus and spleen after MK treatment was found. When Azathioprine was administered, it caused a significant reduction in the weight of thymus and spleen, while in the animals treated with Azathioprine along with MK, these parameters were found to be increased. There were no significant differences found in the weights of liver and kidney after administration of Azathioprine and/or MK administration.

Determination of effect of MK extract on Delayed Type Hypersensitivity Reaction

DTH was measured by the method described by Puri & Saxena with some modification[11]. The animals were divided into six groups of six mice each. The drugs and extracts were administered to animals in various groups as per their treatment protocols. Group 1 received only vehicle (CMC solution), group II serves as disease control and was given 50mg/kg body weight of Azathioprine i.p. group III was kept as standard group and was given with Levamisole 50mg/kg orally. Groups IV, V and VI received the plant extracts at doses of 250, 500 & 750 mg/kg body weight orally one hour before the administration of Azathioprine. Two hours before sensitization with $1x10^8$ cells/mm^3 SRBCs through SC route in the left hind paw, Azathioprine was administered i.p at a dose of 50mg/kg body weight to the disease control group (group II) on day zero. All the other groups were sensitized only with SRBCs on day zero. From day 1 to day 5, control and disease control groups receive only vehicle where as the other groups receive their intended treatments.

Extracts were administered by oral route using animal feeding needle and 1ml syringe. On the fifth day, the left hind paw thickness of all animals was measured. Then, the animals were again challenged with same antigen of $1x10^8$cells/mm^3 in 0.1ml in the left hind paw by SC route in all groups. At the intervals of 0, 24, 48, 72 & 96 hrs after challenge, the paw thickness was again measured with a plethysmometer[12].

Determination of the effect of MK on Phagocytic index of macrophages by Carbon clearance test [13]

Carbon clearance test was used to determine the Phagocytic index of macrophages. To serve this purpose, six groups of mice containing six animals were treated with extracts in different doses of 250, 500 & 750 mg/kg body weight orally daily for 5 consecutive days. Animals treated with 1% CMC solution were kept as control whereas those treated with AZP 50mg/kg i.p served as disease control group and animals treated with 50mg/kg b.w served as standard group. All

the animals except group I received AZP only on day 1. Colloidal ink carbon (Indian ink), which was diluted with Phosphate Buffer Saline eight times before use was injected through the tail vein at the dose of 10µl/g body weight after 48 hrs of last treatment.

At the intervals of 0 & 15 min, blood was collected from retro-orbital plexuses of the individual animals. After collection, the blood was suspended in 0.1% sodium carbonate and the absorbance was measured at 660nm. The Phagocytic index K was then calculated by the given equation:

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

Where, OD_1 and OD_2 depict the optical densities at t_1=0 and t_2=15 min respectively.

Data and statistical analysis

All the obtained data was expressed as mean ± SEM for six mice and statistical evaluation was carried out using PRISM software package (version 5.0.4). One way ANOVA followed by post turkey was used to assess the statistical significance of differences between the disease induced and treatment groups. The value of probability less than 5% ($P<0.05$) was considered statistically significant.

RESULTS

Acute toxicity testing

Acute toxicity studies of the methanolic extract of M. koenigii showed no considerable signs and symptoms such as respiratory distress, restlessness, diarrhea, convulsions and coma in the tested animals. The extracts were found to be safe upto 5000mg/kg.

Effect of MK on hematological parameters

A significant dose related increase in the total WBC count and % of neutrophills was observed in the treatment groups whereas no significant changes were observed in other hematological parameters. A significant reduction in the WBC count and % neutrophills of mice treated with AZP which were restored in the treatment groups after the combined treatment of extract and AZP. The results were as shown in the Table 1.

Effect of MK on organ weight of normal & immunocompromised mice

A significant dose related increase in size and weight of thymus and spleen after administration of MK was observed in treatment groups. A significant reduction in the weight of thymus and spleen was observed in animals administered with AZP while in the animals treated with AZP along with MK, these parameters were found to be increased (Table 2).

Effect of MK on Delayed Type Hypersensitivity reaction

Delayed Type Hypersensitivity reaction determines the immune responses i.e. the increase in foot pad thickness using vernier calipers. The findings in the given table indicates that the mice treated with the extracts (250, 500, 750 mg/kg body weight i.e. groups IV, V & VI) increases the response in foot paw edema and was found to be statistically significant ($P<0.05$) when compared to group I and group II (Table 3).

Effect of MK on Phagocytic activity

The rate of removal of carbon particles from the blood stream is used for the assessment of Phagocytic activity. Animals treated with MK extracts showed a significant dose dependent increase in the Phagocytic index and was found to be statistically significant ($P<0.05$) when compared to the Phagocytic index of the control and disease induced group i.e. groups I & II. The results are as shown in Table 4.

DISCUSSIONS

Suppression of innate immune responses as well as bone marrow activity is the major drawback of conventional chemotherapy drugs which greatly limits their use. Azathioprine is one such drug which has found many uses in the treatment of certain cancers, leukemia[12], auto immune disorders, for treating inflammatory bowel disorder etc. It has also been used as an immunosuppressant in organ transplantations to counteract graft rejection[13]. Azathioprine gets converted to Mercaptopurine which blocks the DNA producing enzymes thereby effecting cells with high dividing capacity like T and B lymphocytes[14] which paves way to many opportunistic infections in the absence of strong immune defenses. S methyl transferase is an enzyme required for the metabolism of Azathioprine[14]. Lack of this enzyme in some people genetically leads to excessive bone marrow suppression and severe anemia[16]. Therefore, an alternative source is required to stimulate the weakened immune system to fight against these impairments.

Since long time, several sources of Immunomodulatory materials are being tried to overcome these toxicities. Medicinal plants that are indigenous to India are rich source of substances which are claimed to induce para-immunity & non-specific Immunomodulation of granulocytes, macrophages, natural killer cells and complement functions.

The present study was carried out to determine the Immunomodulatory activity of MK on animal models. A significant increase in the WBC count and percentage neutrophills was observed in the mice administered with MK extract. MK also significantly reduced the luecopenia induced by Azathioprine which indicates that the extract could stimulate the haemopoetic system.

It was also shown that the administration of extracts also stimulated the increase in size and weight of the spleen as well as thymus in immunosuppressed mice but there was no effect on the kidney and liver as they donot participate in the immune activity. These results showed that MK may restore the production of immune cells, which was decreased by Azathioprine. Hence, the ameliorative effects of MK were clearly established.

Phagocytosis is an important innate defense mechanism against the ingested foreign materials. The specialized Phagocytic cells are the blood monocytes, neutrophills and tissue macrophages. The rate of clearance of the carbon from blood by Phagocytic cells is governed by the Phagocytic index (K) in carbon clearance test. In this investigation, a significant increase in the Phagocytic index in dose-dependent manner was observed which may be due to the increased production of Phagocytic cells stimulated by MK.

Murraya leaves contain significant amounts of alkaloids (carbazole and indole), sesqueterpenes, monoterpenes, coumarins etc which may be responsible for its Immunomodulatory activity. The mechanism involved in the stimulation of both cellular and the humoral immunity by MK is yet to be established. This stimulation may be due an enhanced production of growth factors. Findings of the present study showed that MK may alleviate the myelosupression and subsequent leucopenia induced by Azathioprine in mice.

Table 1: Effect of *M. koenigii* on hematological parameters of mice in Azathioprine induced immunosuppression

Group	Hb (gm/dl)	RBC (million/mm^3)	WBC (thousand/mm^3)	Neutrophils (%)
Control	12.5962±0.987	4.2183±1.2603	5.6723±1.4281	54.1218±1.0369
Disease control	9.5489±1.0281	4.3106±2.0816	1.2381±0.3473	11.6289±1.6297
Standard	10.4369±0.9654	4.4863±1.3281	4.7819±0.2815**	58.647±0.7283**
D1	11.2426±0.8793	4.5829±0.7283	2.4013±0.5302	21.7623±0.9271
D2	11.8936±1.2183	4.6231±1.2836	3.4628±0.6739*	34.2639±1.2831*
D3	12.3643±1.1928	4.8932±0.7628	4.9283±0.3783**	52.8726±0.9248**

Data are expressed as mean ± S.E.M from six rats and analyzed by One Way ANOVA *P<0.05, **P<0.01, ***P<0/001

Table 2: Effect of *M. koenigii* on organ weights (gm) in normal and immunocompromised mice

Group	Spleen	Thymus	Liver	Kidney
Control	0.143 ± 0.03	0.0213 ± 0.012	3.812 ± 0.06	0.69 ± 0.05
Disease control	0.009 ± 0.07	0.0016 ± 0.008	3.342 ± 0.05	0.621 ± 0.04
Standard	0.1736 ± 0.04*	0.9583 ± 0.015*	3.831 ± 0.12	0.711 ± 0.04
D1	0.0496 ± 0.08	0.0699 ± 0.009	3.648 ± 0.11	0.68 ± 0.03
D2	0.1677 ± 0.11	0.0821 ± 0.011	3.701 ± 0.09	0.67 ± 0.06
D3	0.2106 ± 0.08*	0.1102 ± 0.016*	3.568 ± 0.09	0.69 ± 0.04

Data are expressed as mean ± S.E.M from six rats and analyzed by One Way ANOVA *P<0.05, **P<0.01, ***P<0/001

Table 3: Effect of *Murraya koenigii* on Delayed Type Hypersensitivity Reaction

Group	Initial	24hrs	48hrs	72hrs	96hrs
Control	0.367 ± 0.028	0.682 ± 0.036	0.641 ± 0.052	0.322 ± 0.071	0.210 ± 0.081
Disease control	0.389 ± 0.016	0.812 ± 0.091	0.782 ± 0.081	0.698 ± 0.038	0.612 ± 0.019
Standard	0.354 ± 0.039	0.398 ± 0.066**	0.368 ± 0.036**	0.312 ± 0.028**	0.268 ± 0.036**
D1	0.378 ± 0.018	0.618 ± 0.012	0.581 ± 0.039	0.476 ± 0.031	0.412 ± 0.028
D2	0.394 ± 0.012	0.578 ± 0.031*	0.531 ± 0.026*	0.458 ± 0.019*	0.408 ± 0.016*
D3	0.389 ± 0.048	0.412 ± 0.026**	0.386 ± 0.011**	0.319 ± 0.029**	0.279 ± 0.036**

Data are expressed as mean ± S.E.M from six rats and analyzed by One Way ANOVA *P<0.05, **P<0.01, ***P<0/001

Table 4: Effect of *Murraya koenigii* on Phagocytic index in mice

Group	0 min	15 min
Control	0.8231 ± 0.0765	0.8498 ± 0.0976
Disease control	0.7969 ± 0.0962	0.5821 ± 0.0651
Standard	0.9032 ± 0.0851	1.9827 ± 0.0748**
D1	0.7861 ± 0.0873	1.6303 ± 0.0858
D2	0.8174 ± 0.0791	1.7812 ± 0.0672*
D3	0.8026 ± 0.0801	2.108 ± 0.0823**

Data are expressed as mean ± S.E.M from six rats and analyzed by One Way ANOVA *P<0.05, **P<0.01, ***P<0/001

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