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Immunomodulatory activity of methanolic extract of *Murraya koenigii* (L) Spreng. leaves

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Immunomodulatory activity of methanolic extract of *M. koenigii* leaves was evaluated on humoral and cell mediated immune response to ovalbumin, phagocytic activity by carbon clearance test, nitric oxide (NO) release from murine peritoneal macrophages and cyclophosphamide induced myelosuppression. Significant increase in the NO production by mouse peritoneal macrophages was detected in culture supernatants indicated increased phagocytic activity of macrophages. The extract showed significant increase in phagocytic index by rapid removal of carbon particles from blood stream. The extract also increased the antibody titre against the ovalbumin and protection towards the cyclophosphamide induced myelosuppression. However, the extract did not show any significant increase in delayed type hypersensitivity reaction which indicated the inability of the extract to stimulate T cells. Present study thus reveals that the extract holds promise as immunomodulatory agent, which acts by stimulating humoral immunity and phagocytic function.

Keywords: Immunomodulatory activity, *Murraya koenigii,* Myelosuppression, Nitric oxide

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and non-specific immunity[1]. Many plants used in traditional medicine are reported to have immunomodulating activities. Some of these stimulate both humoral and cell mediated immunity while others activate only the cellular components of the immune system, i.e. phagocytic function without affecting the humoral or cell mediated immunity[2].

Plants identified as '*Rasayanas*' in Indian ayurvedic system of medicine have various pharmacological properties such as immunostimulant, tonic, neurostimulant, antiageing, antibacterial, antiviral, antirheumatic, anticancer, adaptogenic, antistress etc[3]. An entire section of materia medica of Ayurveda is devoted to drugs entitled as '*Rasayana*' used for enhancement of body resistance[4]. *Murraya koenigii* is one of such plant which is reported as tonic and used in various diseases condition.

Murraya koenigii (L) Spreng. (family Rutaceae), commonly known as curry leaves is used as a spice throughout India for its aromatic value. The traditional medical literature describes its potential role as a source of many vitamins and a domestic remedy for many of the human disorders like diabetes, cancer and many others. The leaves, bark and the root are used intensively in indigenous medicine from ancient time, as a tonic for stomachache, stimulant and carminative[5]. The *M. koenigii* leaves are used in traditional medicine for the treatment of piles, headache, stomach ache, influenza, rheumatism, traumatic injury, insect, snake bites, antivomiting, curing dysentery and diarrhea[6,7]. The leaf extract significantly decreased the level of blood glucose in experimental diabetic rats[8]. A 10% curry leaf diet has shown reduction of total serum cholesterol content[9]. It has been reported that carbazole alkaloids present in the plant possess various biological activities such as anti-tumor, anti-oxidative, anti-mutagenic, and anti-inflammatory activities[10-12].

In the present study immunomodulatory potential of the methanolic extract of *Murraya koenigii* leaves on cellular and humoral immune responses to the antigenic challenge by ovalbumin, phagocytic activity and cyclophosphamide-induced myelosuppression have been reported.

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Materials and Methods

Plant material and extraction—The fresh leaves of *Murraya koenigii* were collected locally and authenticated at Ramnarayan Ruia College, Mumbai. A voucher specimen (No. 4282) was deposited at Department Pharmaceutical Science and Technology, University Institute of Chemical Technology, Mumbai. Leaves were then shade dried at room temperature. Dry material was coarsely pulverized to powdered form. The dried powdered leaves of *M. koenigii* were extracted with methanol using soxhlet extractor. The methanolic extract was dried at 40°C using a vacuum evaporator and then investigated for immunomodulatory activity. The yield of methanolic extract was found to be 18% w/w of dried leaves powder.

Animals—Albino mice (20-22 g) were housed under hygienic conditions in the departmental animal house. Animals were housed under standard conditions of temperature (25°C±5°C), 12:12 hr light and dark cycle, fed with standard pellet diet (Amrut brand, Chakan Oil Mill Ltd. Pune) and had free access to water. All the experiments were performed in accordance with the Institutional Animal Ethics Committee.

Chemicals—Ovalbumin, Freund's complete adjuvant and TMB/H_2O_2 were procured from Bangalore Genei, India. Streptomycin, penicillin and HEPES buffer were procured from Himedia Laboratories Pvt Ltd, India. Fetal bovine serum and PHA-M (Phytohemagglutinin) were procured from sigma Aldrich, USA. All the other chemicals were purchased from standard local source.

Isolation of peritoneal macrophage and culture conditions—Peritoneal macrophages were obtained from mice that had been injected intraperitoneally 3 days previously with 2 ml of 3% thioglycollate medium (Himedia, India). Three days later, the peritoneal exudates were collected in RPMI-1640. The exudates were centrifuged at 1000 rpm at 25°C for 20 min and erythrocytes were lysed by hypotonic lysis. The mixture was centrifuged and the cell pellets were washed twice and resuspended in RPMI-1640 medium. The cell numbers were determined by a hematocytometer and cell viability was tested by trypan-blue dye exclusion technique. The collected cells were then adjusting to required cell counts per ml, and seeded into a 96-well plate with RPMI-1640 containing 10% fetal bovine serum (FBS), 20 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 mM HEPES buffer. The cells were cultured at 37°C for 2 hr under a humidified atmosphere of 95% air and 5% CO_2. The growth medium was replaced to a sample dissolved in the medium, and then it was maintained for 24 hr under the same condition.

Nitric oxide assay—NO production was determined by assaying culture supernatants for nitrite using Griess reagent by the method of Keller *et al*[13]. PEC (adherent cells) at 5×10^6 cells/ml was incubated with different concentration of drug and PHA for 24 hr at 37°C in 5% CO_2 atmosphere. Cell-free supernatant (75 µl) was mixed with 75 µl of griess regent (sulfanilamide 1%, phosphoric acid 5%, napthylethylenediamine dihydrochloride 0.1%) and incubated at room temperature for 10 min cells incubated with PHA (100 µg/ml) were used as a positive control. After incubation, the absorbance of the wells was determined by using ELISA reader (Biotek, USA) equipped with a 540 nm filter. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standards.

Phagocytic activity—Phagocytic index was determined as per the method reported by Gonda *et al*[14]. Mice were divided into 3 groups, of 6 animals each. The control group received 0.2% sodium carboxy methylcellulose solution only as vehicle; while animals of the treatment groups were given test extracts (250 and 500 mg/kg, po) in 0.2% sodium carboxy methyl cellulose daily for 20 days. Carbon ink suspension was injected via tail vein to each mouse after 48 hr of 20 days treatment. Blood samples were drawn from orbital vein at 0 and 15 min. Blood (25 µl) was mixed with 0.1% sodium carbonate (2 ml) and subjected for determination optical densities at 660 nm. The phagocytic index K was calculated by using following equation: $K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$, where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively

Humoral antibody (HA) titre and delayed type hypersensitivity reaction—Animals were divided into 3 groups of 6 animals each. The control group received 0.2% sodium carboxy methylcellulose solution only as vehicle; while animals in the treatment groups were given the test extracts (250 and 500 mg/kg, po) in 0.2% sodium carboxy methyl cellulose daily for 20 days. On day 21 the animals were immunized (subcutaneously) with 3 mg of oval-

bumin dissolved in 0.1 ml of normal saline emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei).

The blood was collected by retro orbital plexus under ether anesthesia after 7 days of immunization. The serum was separated. Quantification of serum IgG were carried out and the serum antibody titer was estimated by Enzyme Linked Immunosorbent Assay (ELISA)[15,16]. Flat bottom polystyrene plates were coated with 12.5 µg of ovalbumin dissolved in 100 µl of sodium carbonate buffer (*p*H 9.6) at 4°C for 12 hr. The coated plates were washed thrice with phosphate buffer saline (0.15 M, *p*H 7.2) containing 0.05% TWEEN-20 (Tween-PBS). The wells were incubated with 100 µl of 1% BSA in sodium carbonate buffer at 37°C for 1 hr. Serial dilutions of sera in PBS-Tw were prepared and 100 µl was incubated with coated wells for 1 hr at 37°C. After washing, diluted (1:2000) anti-mouse IgG conjugated with peroxidase (100 µl) was added and the plates were incubated at 37°C for 1 hr. The enzyme activity was determined by addition of Tri methyl benzidiene (TMB). The enzyme reaction was stopped by addition of 50 µl, 8 N sulphuric acid and the absorbance was measured at 450 nm. The anti-body titer was expressed as $\log_2$ of the reciprocal of the highest dilution of the test serum showing three times more absorbance as compared with normal serum.

For determination of the delayed type hypersensitivity (DTH) reaction, the mice were challenged (sc) with 50 µg ovalbumin in 50 µl saline in the left hind footpad 14 days after the immunization. The increase in footpad thickness was measured 24 hr after the challenge with the help of a dial caliper (Mitutoyo, Japan). The right hind footpad was injected with 50 µl vehicle and this served as the control. The degree of DTH reaction was expressed as the percentage increase in footpad thickness (L-R) over the control value[17].

Cyclophosphamide induced myelosuppression—Cyclophosphamide induced myelosuppression was studied according to the method described by Manjarekar *et al*[18]. Animals were divided into 4 groups of six animals each. The control group and cyclophosphamide group received 0.2% sodium carboxy methylcellulose solution only as vehicle daily for 16 days while animals in treatment groups were given the test extracts (250 and 500 mg/kg, po) in 0.2% sodium carboxy methyl cellulose daily for 16 days. On days 17, 18, 19 all the animals except in the control group were injected with cyclophosphamide (30 mg/kg, ip) 1 hr after administration of the extracts. Blood samples were collected on day 20 and total white blood cell (WBC) count was determined.

Statistical analysis—Results are expressed as mean ± SD. Data were analyzed by analysis of variance followed by Dunnet's test for multiple comparisons with the level of significance chosen at $P < 0.05$.

Results

Increase in the nitrite production has a significant effect on the macrophages function. It increases the phagocytic activity of the macrophages. The extract showed significant increase in the NO production from peritoneal macrophage at 416 µg/ml and 834 µg/ml with 24% and 56% respectively (Fig. 1).

Methanolic extract of *M. koenigii* possess macrophage stimulatory activity as evidenced by increased phagocytic index in carbon clearance test. The phagocytic activity of reticuloendothelial is generally measured by the rate of removal of carbon particles from blood stream. The phagocytic index for control group was found to be 0.064 whereas the extract at the dose of 500 mg/kg increased it significantly (Table 1).

Humoral response to ovalbumin was checked by ELISA antibody titre. The humoral antibody titre value in control was found to be 16.64. Administration of methanolic extract produced increase in humoral antibody titre to 21.30. None of the dose showed any significant increase in paw

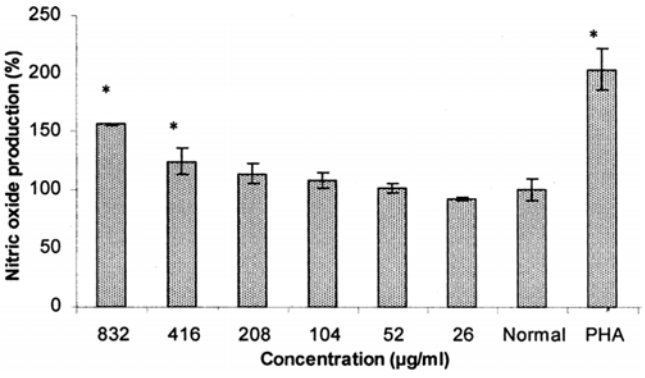

Fig. 1—Effect of methanolic extract of *Murraya koenigii* on the nitrite production of peritoneal macrophages. Values expressed as % of control, are mean ± SD. The control value was 33.21 ± 5.51 µg/ml. *: Significantly different from vehicle control group ($P < 0.05$)

Table 1—Effect of methanolic extract of *M. koenigii* (MKM) on phagocytic index, antibody titre, DTH reaction and cyclophosphamide induced myelosuppression
[Values are mean ± SD of 6 animals in each group]

Groups	Phagocytic index	Antibody titer	DTH reaction	Total WBC count per mm^3
Control/Cyclophosphamide	0.064 ± 0.004	16.64 ± 0.89	20.35 ± 3.68	5180 ± 940
MKM (250 mg/kg)	0.079 ± 0.07	16.30 ± 0.81	22.78 ± 3.87	6790 ± 1560
MKM (500 mg/kg)	0.108 ± 0.04*	21.30 ± 0.51*	24.65 ± 3.74	8140 ± 2850*

*: Significantly different from vehicle control group ($P < 0.05$)
Control/Cyclophosphamide: Data mentioned are of control group in antibody titer, DTH reaction and Phagocytic index while total WBCs count mentioned are of Cyclophosphamide group

edema as compare to control and hence did not show any effect on DTH reaction (Table 1).

Administration of cyclophosphamide has significantly lowered the levels of total WBC (5180) as compared to control group (9180) in blood. Methanolic extract was found protecting cyclophosphamide-induced myelosuppression at higher dose significantly while at marginal extent at lower dose as evidenced by increasing the levels of total WBC count (Table 1).

Discussion

Immunomodulatory activity of *M. koenigii* was explored, by evaluating its effect on phagocytic function, antibody titre, DTH reaction and cyclophosphamide induced myelosuppression in mice. Administration of methanolic extract showed immunostimulating activity *in vitro* and *in vivo*.

In this study, the methanolic extract significantly induced NO in mouse peritoneal macrophages. NO is synthesized by NO synthase (NOS)[19] and mediates diverse functions, including vasodilation, neurotransmission and inflammation[20]. NO has been shown to be the principal effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation[21]. The increase in carbon clearance index reflects the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity which probably act through the release of nitric oxide as describe before. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by opsonisation of parasite with antibodies and complement C3b leading to more rapid clearance of parasite from blood[22].

Extract showed the presence of tannins, phenolic compounds and alkaloids. Carbazole alkaloids present in *M. koenigii* are reported for its cytotoxic, antimicrobial and antibacterial activity[23-25] which indicate the probable role of these alkaloids for stimulant activity on macrophages.

Results of the present investigation showed increased antibody titre in response to ovalbumin reflecting an overall elevation of humoral immune response. DTH is antigen specific and causes erythema and induction at the site of antigen infection in immunized animals. The general characteristics of DTH are an influx of immune cells at the site of injection, macrophages and basophils in mice and induction becomes apparent within 24-72 hr. T-cells are required to initiate the reaction[26,27]. Non significant difference in the DTH response indicates that the extract has not any stimulatory effect on T-lymphocytes especially T_{DTH}-lymphocytes, and therefore no effect on cell mediated immunity.

Macrophages are acting in both nonspecific defenses by phagocytes cellular debris and pathogen and specific defense by stimulates lymphocytes and other immune cells to respond to the pathogen. Increased in humoral response and phagocytic activity indicate the stimulation of B-lymphocytes and macrophages killing activity through NO release. But non significance difference in DTH reaction indicates that the extract is unable to stimulate the macrophage function to stimulate T cell for the hypersensitivity reaction to the immunized antigen (ovalbumin). The administration of methanolic extract of *M. koenigii* significantly ameliorated the total WBCs count which indicates the restoration of the myelosuppressive effects induced by cyclophosphamide.

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