Immunostimulatory activity of aqueous extract of
*Murraya koenigii* (Linn.) Spreng. leaves

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Leaves of *Murraya koenigii* (Linn.) Spreng. (*Curry patta*) are reported to possess antidiabetic, antimicrobial, cytotoxic and anti-inflammatory activity. In the present study immunomodulatory potential of aqueous extract of its leaves on specific (humoral and cell mediated) and non-specific (macrophage) immunity in mice was investigated. Oral administration of the aqueous extract of leaves at the doses of 250 and 500 mg/kg significantly enhanced the delayed-type hypersensitivity reaction induced by ovalbumin however, the effect was not dose related. The extract also potentiated the production of circulating antibody titre significantly in response to ovalbumin. Pre-treatment of the extract restored the myelosuppressive effect of cyclophosphamide through ameliorating total white blood cells count. Significant increase in release of nitric oxide from mouse peritoneal macrophages in culture supernatant indicates enhancement of cytotoxic activity of macrophages. The present investigation thus reveals that aqueous extract of *M. koenigii* leaves possesses immunostimulatory activity by acting on both specific and non-specific immunity.

**Keywords:** Antibody titre, Hypersensitivity, Immunomodulatory, Immunostimulatory, Curry patta, *Murraya koenigii*, Myelosuppression, Nitric oxide.

**IPC code: Int. cl.** A61K 36/00, A61K 36/75, A61K 127/00, A61P 37/00

**Introduction**

The immune system is known to be involved in the etiology as well as patho-physiological mechanisms of many diseases. Modulation of immune responses to alleviate the diseases has been of interest for many years and the concept of ‘Rasayana’ is based on related principles. Rasayana, listed as a class in the texts of traditional Indian Medicine literature, consists of a number of plants reputed to promote physical and mental health, improve defense mechanisms of the body and enhance longevity. These attributes are similar to the modern concept of adaptogenic agents, which are known to afford protection of the human physiological system against diverse stressors. Development of agents capable of moving ‘patients’ immune system from a state of immune deficiency to one of more normal function would likely to have a significant impact on disease and the patient it affects. Such agent would not be a cure, but would control the manifestation and course of disease. Phytochemicals and medicinal plants are claimed to induce para-immunity, the non-specific immunomodulation of essentially macrophages, granulocytes, NK (Natural Killer) cells, lymphocytes and complement functions.

*Murraya koenigii* (Linn.) Spreng. of Rutaceae family is an aromatic pubescent shrub or small tree commonly known as *Curry patta* in India. The plant is used in Indian system of medicine to treat various ailments. The aromatic leaves, which retain their flavour and other qualities even after drying, are slightly bitter, acrid, cooling, weakly acidic in tastes and are considered as tonic, anthelmintic, analgesic, digestive, appetizing and are widely used in Indian cookery for flavouring food stuffs.

The green leaves are used to treat piles, inflammation, itching, fresh cuts, dysentery, vomiting, bruises and dropsy. The roots are slightly purgative, stimulant and used for general body aches, whilst the bark is used to treat snakebite. The essential oil of the leaves is reported to possess antimicrobial, antifungal and pesticidal activities. *M. koenigii* leaves mixed with fat separated butter are used for the treatment of amoebiasis, diabetes and hepatitis in Ayurveda. The aqueous extract of the leaves produced hypoglycemia in normal and alloxan diabetic dogs. It is reported that the aqueous extract of these leaves has favourable effect in bringing down
the severity of diabetes by normalizing the serum level of lipids, creatinin and alkaline phosphatase\(^{19}\).

Carbazole alkaloids present in leaves possess various biological activities such as anti-tumour, antioxidative, anti-mutagenic and anti-inflammatory activities\(^{19-21}\). The aqueous extract of leaves, when administered parenterally to female guinea pigs, not only raises the phagocytic index but also mobilize a greater number of leucocytes to take part in phagocytosis\(^{22}\). The present study was therefore, undertaken to explore the acute toxicity studies and immunomodulatory activity of aqueous extract of its leaves on cellular and humoral immune responses to the antigenic challenge by ovalbumin, phagocytic activity on culture of peritoneal macrophages and on cyclophosphamide-induced myelosuppression.

Materials and Methods

Plant material and extraction

The fresh leaves were collected from the local market at Mumbai, Maharashtra. Leaves were then shade dried at room temperature. Dry material was coarsely pulverized to powdered form. The dried powdered leaves were extracted with distilled water using maceration technique. The aqueous extract was dried at 40°C using a vacuum evaporator. The yield of aqueous extract was found to be 17% w/w of dried leaves powder.

Experimental animals

Albino mice (20-22 g) were used for the study. The animals were housed under good hygienic conditions in the departmental animal house. Animals were housed under standard conditions of temperature (22±2°C), 12h/12h light and dark cycle, fed with standard pellet diet (Amrut Ind Ltd. Pune) and had access to water, \textit{ad libitum}. All the experiments were performed in accordance with the Institutional Animal Ethics Committee (IAEC) constituted as per directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of animal welfare division, Government of India, New Delhi. The registration number of the animal house in our institute is 87/1999/CPCSEA.

Chemicals

Ovalbumin, Freund’s complete adjuvant and TMB/H\(_2\)O\(_2\) were procured from Bangalore Genei, India. Streptomycin, Penicillin and HEPES buffer were procured from Himedia Pvt Ltd, India. Fetal bovine serum and PHA-M (Phytohemagglutinin) were procured from Sigma Aldrich, USA. All the other chemicals were procured from standard local source.

Acute toxicity study\(^{23}\)

Oral acute toxicity study in albino mice were carried out with aqueous extracts in accordance with OECD guideline no.425 and the extract was found to be safe at the dose 2000 mg/kg.

Humoral antibody (HA) titre and delayed-type hypersensitivity reaction

Animals were divided into 3 groups of six animals each. The control group received distilled water only as vehicle; while animals in the treatment groups were given the test extracts (250 and 500 mg/kg, p.o.) in distilled water daily for 20 days. On day 21 the animals were immunized (subcutaneously) with 3 mg of ovalbumin dissolved in 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 was carried out and the serum antibody titre was estimated by Enzyme Linked Immunosorbent Assay (ELISA)\(^{24, 25}\). Flat bottom polystyrene plates were coated with 12.5\(\mu\)g of ovalbumin dissolved in 100\(\mu\)l of sodium carbonate buffer (\(p\)H 9.6) at 4°C for 12 h. The coated plates were washed three times with phosphate buffer saline (0.15M, \(p\)H 7.2) containing 0.05% TWEEN-20 (PBS-Tween). The wells were incubated with 100\(\mu\)l of 1% BSA (bovine serum albumin) in sodium carbonate buffer at 37°C for 1h. Serial dilutions of serum in PBS-Tween were prepared and 100\(\mu\)l was incubated with coated wells for 1 h at 37°C. After washing, diluted (1:2000) Anti-mouse IgG conjugated with peroxidase (100 \(\mu\)l) was added and the plates were incubated at 37°C for 1 h. The enzyme activity was determined by addition of Trimethyl benzidiene (TMB). The enzyme reaction was stopped by addition of 50\(\mu\)l, 8N sulphuric acid and the absorbance was measured at 450nm. The antibody titre 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 (s.c.) with 50 \(\mu\)g ovalbumin in 50 \(\mu\)l saline in the left hind footpad 14 days after the immunization. The increase in footpad thickness was measured 24 h 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.

**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 cells (PEC) were collected in 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 exudates were centrifuged at 1000rpm, 25°C for 20min 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 hemocytometer and cell viability was tested by trypan-blue dye exclusion technique. The collected cells were then adjusted to required cell counts per ml, and seeded into a 96-well plate with RPMI-1640. The cells were cultured at 37°C for 2 h under a humidified atmosphere of 95% air and 5% CO₂. The growth medium was replaced by a sample dissolved in the medium and then it was maintained for 24 h under the same condition.

**Nitric oxide assay**

Nitric oxide (NO) production was determined by assaying culture supernatants for nitrite using Griess reagent by the method of Keller et al (1990). PEC at 5×10⁵ cells/well was incubated with different concentration of drug and PHA for 24 h at 37°C in 5% CO₂ atmosphere. Cell-free supernatant (75 µl) was mixed with 75 µl of Griess reagent (sulfanilamide 1%, phosphoric acid 5%, naphthylethenediamine 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.

**Cyclophosphamide induced myelosuppression**

Cyclophosphamide induced myelosuppression was studied according to the method described by Manjarekar et al. Animals were divided into 4 groups of six animals each. The control groups (vehicle and negative) received distilled water only as vehicle daily for 16 days while animals in treatment groups were given the test extract (250 and 500 mg/kg, p.o.) in distilled water daily for 16 days. On days 17, 18, 19 all the animals except in the vehicle control group were injected with cyclophosphamide (30 mg/kg, i.p.) 1 h after administration of the extract or vehicle. Blood samples were collected on day 20 and total white blood cell (WBC) count was determined.

**Statistical Analysis**

Results have been expressed as mean ± SD. Data were analyzed by one way ANOVA followed by Dunnet’s test for multiple comparisons with the level of significance chosen at \( P<0.05 \).

**Results**

**Humoral antibody (HA) titre**

The antibody titre was used to assess humoral immune response. The augmentation of the humoral immune response to ovalbumin by aqueous extract of *M. koenigii* leaves is evidenced by increase in the level of antibody titre in the serum of mice (Table 1). The humoral antibody titre value in control group was found to be 16.64. Administration of aqueous extract produced significant increase in humoral antibody titre at 500 mg/kg.

**Delayed-type hypersensitivity (DTH) reactions**

The cell-mediated immune response was assessed by DTH reaction, i.e. foot pad reaction. Aqueous extract of *M. koenigii* leaves produced a significant increase in DTH reactivity in mice irrespective of dose. The extract showed 30 and 40% increase in paw oedema at 500 and 250 mg/kg dose, respectively as compared to 20% increase in paw oedema of control. Increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of aqueous extract on T cells (Table 1).

**Phagocytic response by nitrite production**

The phagocytic activity of the reticulo-endothelium system was measured by the release of the nitric oxide (NO) from culture of peritoneal macrophage. The extract showed 40 and 88% increase in the NO production from peritoneal macrophage at 416 and 832 µg/ml, respectively (Figure 1). The extract showed dose dependent increase in nitrite production which indicates significant effect on the cytotoxic activity of the macrophages (Figure 1).
Cyclophosphamide induced myelosuppression

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the WBC count. A significant reduction in white blood cell count was observed in animals treated with cyclophosphamide alone (Negative control group) as compared to the control group. Treatment with aqueous extract of leaves resulted in a restoration of WBC count (Table 1). The extract showed dose dependent increase in WBC count with near normal level at 500 mg/kg.

Table 1 — Effect of aqueous extract of Murraya koenigii (MKA) on antibody titre, DTH reaction and WBC count in cyclophosphamide induced myelosuppression.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antibody titre</th>
<th>DTH response</th>
<th>Total WBC count per cu mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>-</td>
<td>-</td>
<td>5180±940</td>
</tr>
<tr>
<td>Control</td>
<td>16.64±0.89</td>
<td>20.35±3.68</td>
<td>9180±2120#</td>
</tr>
<tr>
<td>MKA (250 mg/kg)</td>
<td>17.47±1.17</td>
<td>40.64±3.64*</td>
<td>6800±1430</td>
</tr>
<tr>
<td>MKA (500 mg/kg)</td>
<td>21.47±0.4*</td>
<td>30.13±3.27*</td>
<td>8230±2200#</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. (n = 6)
*: Significantly different from control (P < 0.05)
#: Significantly different from negative control group (P < 0.05)

Discussion

Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy. The results obtained in the present study indicate immunostimulant property of aqueous extract of M. koenigii leaves by stimulating both the specific and non-specific immune mechanisms.

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses; IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc. The augmentation of the humoral immune response to ovalbumin by aqueous extract of M. koenigii leaves, as evidenced by increase in the antibody titre in mice indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumour immunity and delayed-type hypersensitivity reactions. Several lines of evidence suggest that DTH reaction is important in host defense against parasites and bacteria that can live and proliferate intracellularly. The extract showed the significant increase in DTH reaction but not in dose dependent manner, since immune response is not always directly related with the immunomodulator concentration. This may be partly due to different constituents present in fraction at different concentrations and saturation of cells responsible for immune response. Some of these constituents may have immunosuppressive activity, whereas other possesses immunostimulant action. Therefore, increase in DTH reaction in mice in response to ovalbumin, T cell dependent antigen revealed the stimulatory effect of aqueous extract of M. koenigii leaves on T cells.

Macrophage produces the reactive oxygen species (ROS) through partial reduction of oxygen. It is believed that the induction of nitric oxide, one of ROS, plays an important role in the protection against viral hepatitis. NO has been shown to be the principle effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation. The extract found capable to stimulate NO production in macrophage cultures, imply its stimulatory effect in the host defense against microbial infection on activated macrophage. The administration of aqueous extract of M. koenigii leaves significantly ameliorated the total WBC count which indicates the protection towards myelosuppressive effect induced by cyclophosphamide and/or stimulation of bone marrow activity.
The immunomodulatory activity of methanolic extract of *M. koenigii* leaves (MKM) was reported by Shah *et al*.55. It was mentioned that MKM stimulates humoral immunity along with phagocytic activity and provide protection towards cyclophosphamide induced myelosuppression but did not show any effect on DTH reaction, while in the present study MKA has shown significant increase in DTH reaction along with significant effect on humoral immunity and myeloprotection towards cyclophosphamide. These results indicate the role of specific water soluble phytochemical for the stimulatory action on cell mediated immune response.

Conclusion

The present investigation suggests that aqueous extract of *M. koenigii* leaves stimulates both specific and non-specific immune responses by stimulating macrophages, humoral and cell mediated response. It can therefore, be concluded that aqueous extract of *M. koenigii* is a potential immunostimulant against cytotoxic drugs and can be used as a complimentary therapeutic agent. Further, studies are in progress to find the constituents responsible for specific and non specific immunostimulatory activity.

Acknowledgements

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