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Study of the immunosuppressive activity of methanolic extract of *Madhuca longifolia* (Koenig)

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SUMMARY

The immunosuppressive activity of the Methanol extract of bark of *Madhuca longifolia* (Koenig) consisting of a mixture of saponins, flavonoids, tannins, steroids, phenol and glycosides was studied on the immune responses in mice. Methanol extract of *Madhuca longifolia* (MLL) was administered orally at doses of 50, 100 and 150 mg/kg/day to healthy mice divided into four groups consisting of six animals each. The assessment of immunomodulatory activity was carried out by testing the humoral (antibody titre) and cellular (foot pad swelling) immune responses to the antigenic challenge by sheep RBCs. Furthermore, the effect on hematological parameters as well as relative organ weight was determined. On oral administration MML showed a significant decrease delayed type hypersensitivity (DTH) response whereas the humoral response to sheep RBCs was unaffected. Thus MLL significantly suppressed the cellular immunity by decreasing the footpad thickness response to sheep RBCs in sensitized mice. With a dose of 100 and 150 mg/kg/day the DTH response was 7.66 ± 2.75 and 6.41 ± 1.21 respectively in comparison to corresponding value of 14.50 ± 2.38 for untreated control group. These differences in DTH response were statistically significant ($P < 0.05$). The study demonstrates that MLL shows preferential suppression of the components of cell-mediated immunity and shows no effect on the humoral immunity.

Key words: Immunosuppressive; *Madhuca longifolia;* Delayed type hypersensitivity; Haemagglutinating antibody titre; Methanolic extract

INTRODUCTION

Clinical transplant immunosuppression aims not only to prevent host immune responses against antigens on the transplanted organ, thereby avoiding rejection, but to prevent undesirable complications of immunodeficiency (eg, infection and malignancy) and to minimize nonimmune toxicities (eg, nephrotoxicity, hyperlipidemia, bone marrow suppression, and cushingoid effects). While physical methods such as irradiation can be used, in practice, immunosuppression for solid organ transplantation is usually achieved by immunosuppressive drugs (ISDs).

It is an interesting paradox that many of the currently used ISDs, while responsible for drastic improvement in short-term outcomes, potentially compromise long-term graft and patient survival through complex toxic mechanisms. Therefore, modification of immune and nonimmune responses to ISDs through individualization of immunosuppressive agents and regimens for specific patients and

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groups of patients is a major priority. Clinicians caring for transplant recipients must consider the evidence for the best outcomes with the lowest toxicity (www.medscape.com).

Some of the plants employed in traditional medicine were shown to possess immunosuppressive activity (Zhu, 1990). *Cordyceps sinesis* (Zhu, 1990) and *Allium cepa* (Vyas *et al.*, 1983) are two examples of such plants.

Madhuca longifolia (Koeng) Gmelin syn. *Maduca indica*, *Madhuka latifolia*, *Bassia latifolia* belongs to the family sapotiaceae, commanly knowns as Mahua. Mahua is a large shady, deciduous tree doting much of central Indian landscape, both wild and cultivated. the tree is a valued for its oil bearing seeds and flowers, which are utilized for alcoholic beverages production. Mahua seeds are economic importance as they are good source of edible fats (Ramadhan, 2005). The expectrorant flowers are used to treat cheast problem such as bronchitis and also taken to increase lactation. The distilled juice of flower is considered a tonic, both nutritional and cooling and also in treatment of helminthes, acute and chronic tonsillitis, pharyngitis (Nadkarni, 1954) as well as bronchitis (Varier, 1995). The leaves are applied as a poultice to relieve the eczema. in Indian folk medicine, the leaf ash is mixed with ghee to make a dressing for wounds and burn. Mahua preparations are used for removing intestinal worm in respiratory infection and in case of debility and emaciation. The astringent bark extract is used for dental related problems, rheumatism and diabetes (www.herbnet.com).

The medicinal properties attributed to this plant are stimulant, demulcent, emollient, heating and astringent. The bark is good remedy for itch, swelling, fracture and snake bite poisoning, internally employed in diabetes mellitus. Some of the recent findings reported that seeds of *Madhuca indica* possess anti-inflammatory, antiulcer and hypoglycemic activity (Seshagiri, 2007).

The present hypothesis tested the immunosuppressant activity of methanolic extract of bark of *Madhuca longifolia* to prove its traditional medicinal importance.

MATERIALS AND METHODS

Preparation of methanolic extract of *Madhuca longifolia* (Koenig)

Plant bark of *Madhuca longifolia* were obtained from the foothills of Yercaud (Tamil Nadu, India) in the month of December 2008 and were authenticated at Plant Anatomy Research Centre, Chennai, India and voucher specimen (MDIndica - 99) has been deposited in University herbarium for future reference. The dried and coarsely powdered bark (400 g) extracted successively with 1.5 l each of petroleum ether (60 – 80 °C), methanol and in a Soxhlet extractor for 72 h. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40 – 50 °C). The petroleum ether extract yielded a pale brown sticky semisolid, weighing 3 g (3%) The methanol extracts yielded reddish brown and semi-solid residues, weighing 7.0 g (7.0%). Methanolic extract of *Madhuca longifolia* (MML) has been selected for further studies.

Phytochemical screening

The presence of phytochemicals alkaloids (Draggendorff's), flavonoids (Shibata's reaction), saponins (Frothing test), tannins (5% ferric chloride), terpenoids (2,4 dinitrophenylhydrazine), glycosides (fehling's solution), steroids (Liebermann's Burchard test) were evaluated.

Animals

Healthy male albino mice (25 – 30 g) were selected for the study. Animals were housed in standard isolation cages (45 × 35 × 25 cm) under environmentally controlled conditions with 12 - h light/12 - h dark cycle. Mice were allowed free access to water, standard laboratory rat chow (Hindustan Liver Pvt. Ltd, Mumbai) throughout the experiment. Fresh sheep red blood cells (SRBC) in Alsever's solution were prepared in host department after collecting

fresh sheep blood from local slaughter house.

Antigen

SRBC collected in Alsever's solution, were washed three times in large volumes of pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×10^9 cells/ml for immunization and challenge.

Treatment

The animals were divided into four groups consisting of six animals each. A group of six untreated rats were taken as control (Group I). The methanolic extract of *Madhuca longifolia* (MML) was fed orally for 14 days at a dose of 50 mg/kg/ day (Group II), 100 mg/kg/day (Group III) and 150 mg/ kg/day (Group IV) for assessment of immunomodulation effect. The animal experimental protocols were approved by the Institute Animal Ethics Committee.

Haemagglutinating antibody (HA) titre

Haemagglutinating antibody titre was determined according to the method of Puri *et al* (1993). Mice of group II, III and IV were pretreated with MML for 14 days and each mouse was immunized with 0.5×10^9 SRBC/mouse by *i.p.* route, including control mice. The day of immunization was referred to as day 0. The animals were treated with MML for 14 more days and blood samples were collected from each mouse on day 15 for HA titre. The titre was determined by titrating serum dilutions with SRBC (0.025×10^9 cells). The microtitre plates were incubated at room temperature for two hours and examined visually for agglutination. The highest number dilution of serum showing haemagglutination has been expressed as HA titre.

On 15th day of treatment, all the mice were sacrificed and blood was collected in heparinized vials. Blood samples for animals of each group were subjected for hematological studies such as total WBC count and spleen leukocyte count. Spleen and thymus were dissected out and embedded in 10% formalin solution to record their weight.

Delayed type hypersensitivity (DTH) response

Six animals per group (control and treated) were immunized on day 0 by *i.p.* administration of 0.5×10^9 SRBC/mouse and challenged by a intraplanter administration of 0.025×10^9 SRBC/ml into right hind foot pad on 7th day. The MML was administered orally from day 1until day 7. DTH response was measured at 24 h after SRBC challenge on day 8 and expressed as mean percent decrease in paw volume (plethysmometrically) (Puri, 1993).

Statistical analysis

The data were analysed using One-way analysis of variance (ANOVA) followed by Dunnett test. *P* values < 0.05 were considered significant.

RESULTS

The phytochemical screening of the MML indicated the presence of saponins, flavonoids, tannins, steroids, phenol and glycosides. The results of HA titre and DTH response are shown in Table 1. Even with the administration of increasing doses of MLL, the HA titre did not show any significant increase as compared to untreated control group indicating that the *Madhuca longifolia* has no effect on the humoral immunity. The DTH response to SRBC

Table 1. Effect of *Madhuca longifolia* on HA titre and DTH response to antigenic challenge by sheep RBCs in mice

Groups	HA titre	DTH response (% decrease in paw volume)
I (Untreated)	5.20 ± 0.22	14.50 ± 2.38
II (50 mg/kg, *p.o.*)	5.09 ± 0.81	10.52 ± 3.12*
III (100 mg/kg, *p.o.*)	5.16 ± 0.75	7.66 ± 2.75**
IV (150 mg/kg, *p.o.*)	4.90 ± 0.63	6.41 ± 1.21**

The values are mean ± SD of 6 mice in each group. One-way ANOVA followed by Dunnet multiple comparisons test; *$P < 0.05$, **$P < 0.01$ *Vs* group I.

Table 2. Effect of *Madhuca longifolia* on WBC, spleen leukocytes count and relative organ weight in mice

Groups	WBC (thousand/cmm)	Spleen leukocyte (thousand/cmm)	Thymus weight (g/100 g B.W)	Spleen weight (g/100 g B.W)
I (Untreated)	11.3 ± 0.47	48.5 ± 9.9	0.08 ± 0.02	0.35 ± 0.041
II (50 mg/kg, *p.o.*)	10.9 ± 0.38^{ns}	42.9 ± 8.4^{ns}	0.07 ± 0.02^{ns}	0.31 ± 0.027^{ns}
III (100 mg/kg, *p.o.*)	10.6 ± 0.45*	35.1 ± 7.2*	0.05 ± 0.02*	0.29 ± 0.031*
IV (150 mg/kg, *p.o.*)	9.54 ± 0.46**	28.4 ± 6.5**	0.03 ± 0.01**	0.23 ± 0.027*

The values are mean ± SD of 6 mice in each group. One-way ANOVA followed by Dunnet multiple comparisons test; $^{ns}P > 0.05$,*$P < 0.05$, **$P < 0.01$ *Vs* group I.

which corresponds to cell mediated immunity showed a significant dose dependent decrease due to treatment with MLL with dose of 100 mg/kg/ day and 150 mg/kg/day. The DTH response was 7.66 ± 2.75 and 6.41 ± 1.21 respectively in comparison to corresponding value of 14.50 ± 2.38 for untreated control group. The dose dependant differences in DTH response were statistically significant ($P < 0.05$). Thus MML treatment induced marked inhibition of DTH response to SRBC in the animals. Finally, the effects of MML on WBC, spleen leukocytes count and relative organ weight in mice are shown in Table 2. MML at the dose of 100 mg/kg and 150 mg/kg., p.o caused a significant reduction in the WBC, Spleen leukocyte counts as well as relative spleen weight and thymus weight. But the effect was more pronounced at dose of 150 mg/kg ($P < 0.01$) as compared to 100 mg/kg p.o dose of MLL ($P < 0.05$).

DISCUSSION

A wide range of immunosuppressive drugs have now been adopted to control unwanted immune responses, particularly those giving autoimmune disease and transplant rejection.

The clinical application of immunosuppressants has significantly improved patient survival with first-year survival up to 90% for renal transplant. (Waldmann., 2003). But unfortunately immunosuppressants are suffers from a number of serious adverse effects among which nephrotoxicity, hepatotoxicity, induction of diabetes, induction of hypertension and neurotoxicity are most notorious for cyclosporine and tacrolimus (Serkova *et al.*, 1996). As a consequence, there continues to be a high demand for new immunosuppressants. The immunosuppressants without any side effects are still a challenge to the medical system. Suppression of immune response by medicinal plant products as a possible therapeutic measure has become a subject of scientific investigation recently (Agarwal and Singh, 1999). In an effort to search for new immunosuppressants, we identified clinically useful and safe product from medicinal plants that could suppress immune response and may have future in clinic. This study reported the effect of MML on the humoral and cellular immune responses to mice subcutaneously immunized with SRBCs.

In the experiments undertaken to study the effect of MML on haemagglutination antibody titre against SRBC, it was observed that even with the administration of increasing doses of MML, the titre did not show any significant increase as compared to untreated control group indicating that the MLL has no effect on the humoral immunity.

The DTH response, which is a direct correlate of cell mediated immunity (CMI), was found to be significantly decreased at a dose of 100 and 150 mg/kg/day of the MML. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive (inflammatory) reaction. In our studies, foot volume was decreased

after MML treatment suggesting cell mediated immune suppression (Sen *et al*., 1992). In the DTH response indicates that MML has inhibitory effect on lymphocytes and accessory cell types required for the expression of the reaction (Mitra., 1999). This supports the reported anti-inflammatory activity of MML (Seshagiri., 2007). Here it is interesting to note that the treatment, while augmenting the CMI, did not affect the antibody titres.

In the present study, the immunosuppressant activity of *Madhuca longifolia*, an important plant in indigenous medicinal practice was explored. Administration of *Madhuca longifolia* was found to decrease total WBC count and spleen leukocyte count significantly indicating that the extract could suppress the non-specific immune system. Moreover there was decreased in the relative spleen weight and thymus weight supports these findings.

Madhuca longifolia has been shown to contain Madhucosides A and B (Pawar and Bhutani, 2004) and steroidal lactones. At present we do not know whether these compounds are responsible for the immunosuppressant activity produced by extract. Further studies using isolated compounds are in progress.

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