ISSN: 0975-766X
CODEN: IJPTFI
Research Article

Available Online through
www.ijptonline.com

IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF *MADHUCA LONGIFOLIA* IN MICE

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Received on 15-01-2014 *Accepted on 08-02-2014*

Abstract

Madhucalongifolia member of the family Sapotaceae was studied for its immunomodulatory activity on albino mice in the present study. The ethanolic extract of the plant (EEML) was administered at the dose of 100 and 200mg/kg body weight in the treated groups III and IV. The antibody titre value, DTH response and effect on myelosuppression were checked against the control group I and cyclophosphomide induced myelosuppressed group II. The significant ($p<0.01$) increase in antibody titre value and DTH response in group III & IV was reported as a sign of its stimulating effect on humoral and cell mediated immunity respectively. Moreover, the restoration of Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) to normal level in EEML treated group III & IV as compared to cyclophosphomide treated group II has shown it ameliorating effect on myeloid regeneration too. Hence, the *M. longifolia* with the significant immunostimulatory activity on both the specific and non-specific immune mechanisms holds great promises for being used as an immunomodulating agent. However, more in-depth studies of various other extracts of different parts of the plant may further explore its probability to be used as immunomodulatory drug for the management of infectious diseases.

Keywords:

Madhucalongifolia, Immunomodulation, haemagglutination titre (HT), delayed type of hypersensitivity (DTH) response.

Introduction

Infectious diseases and consequent immune imbalances are major constraint in human health management throughout the world. Depth of reach of infectious is rising day by day with alarming decline in treatability of available allopathic medicines, due to emergence of mutated microbial strains and drug resistance. Immunomodulation is

looked as competent alternative treatment of the present hitch. Escalated number of publications addressing to the Immunomodulation, Immunostimulation and Immune reinforcement in national andinternational journal of higher repute is a great evidence of the same. Immunoinsufficiency and immune compromisation has been the major contributor of microbial growth in almost of all kind of infectious diseases, which can be permanently eliminated by the Immunomodulation only. Medicinal plants have been a great source of them from the ancient time. In India, earliest references are available in Rigveda[1] that a number of Indian medicinal plants and various 'rasayanas' possess immunomodulatory activity[2-6]. Plants play an essential role in the health care needed for the treatment of various diseases and to improve the immunological response against much pathology[7].

Madhucalongifolia, synonym *M. indica*, member of the family Sapotaceae, is an important economic tree growing throughout India. So far, different parts of Mahua like whole young plants, leaves, stems, barks, roots, fruits, flowers, seeds etc. have been reported to possess therapeutic potential. The areal parts have been evaluated for their anti-diabetic[8], anti-tuberculosis, and anti-arthritis activities[9]. Moreover, they are used for the treatment of cholera, paralysis, snake-bite[10], debility, tonsillitis[9], influenza, piles, low semen count, headache, Sinusitis[11], and infections. Flowers are traditionally used as cooling agent and tonic, and have been tested against helminthiasis, acute and chronic tonsillitis, pharyngitis, and bronchitis[12]. Leaves have been evaluated for therapeutic potential against the chronic bronchitis and Cushing's disease[13]. Moreover, they are applied as a poultice to relieve eczema. The bark is known as a blood purifier and a good remedy for itch, swelling, fractures and snake-bite poisoning. The methanolic extract of bark has been displayed the anti-diabetic activity in rats[8].

Madhuca being reported medicinal plants by the above studies, the immunomodulatory prospective were screened in the present study. Though Purohit*et al.*[14]have talked about the immunomodulatory activity of *Madhucalongifolia* in their study but suggested a need of comprehensive, systematic, and multi-disciplinary approaches for subsequent exploration of this activity in the stated plant.

Detail of the Plant

Madhucalongifolia, commonly known as mahwa or mahua, is an Indian tropical tree found largely in the central and north Indian plains and forests. It is a fast-growing tree that grows to approximately 20 meters in height, possesses evergreen or semi-evergreen foliage[15]. The tree is adapted to arid environments, being a prominent tree in tropical mixed deciduous forests in India in the states of Jharkhand, Uttar Pradesh, Bihar, Madhya Pradesh, Kerala, Gujarat and Orissa. It is cultivated in warm and humid regions for its oleaginous seeds flowers and wood.

Usage of plant products: The fat obtained from it is used for the skin care, to manufacture the soap or detergents, and as a vegetable butter. A product known as "illipe"is often used in sweets and chocolates. The seed cakes remained after the oil extraction is known for their very good fertilizing effect. The flowers are used to produce an alcoholic drink, country liquor in tropical India. The fruits and flowers are edible and are used as food items in Bundelkhand region. They are the rich source of sugar, protein, vitamin and minerals[16]. They appear during leanest season of agriculture (March-May). The collection of mahua flowers and fruits is one of the sources of employment for the poorest families in India. Thus, the tree is considered a boon by the tribals who are forest dwellers and keenly conserve this tree.

Materials & Methods

Antigen

Antigen Fresh blood was collected from sheep sacrificed in the local slaughter house, in a sterile bottle containing Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride). Sheep red blood cells (SRBC) was washed three times in normal saline to adjust to a concentration of 0.1ml of $0.5x10^9$ cells and $0.025x\ 10^9$ cells for immunization and challenge respectively.

Animals

Inbred colony of swiss albino mice weighing between 20-25 gm of either sex were used for the experiments. The mice were obtained from Central Drug Research Institute (CDRI), Lucknow and kept in the animal house at ambient temperature of 25^0C and 45-55% relative humidity, with 12h each of dark and light cycles. Animals were housed in the polypropylene neat cages, bottomed with husk and fed pellet diet and water ad-libitum (Principles of Laboratory Animal Care (NH publication no. 85-23, revised 1985). The mice were acclimatized to laboratory conditions for 15 days before the commencement of the experiments. The due care of animals were taken as per guidelines of CPCSEA, Department of Animal Welfare, and Government of India during the course of experimentation. Approval of all animal experiments was obtained from the Institutional Animal Ethical Committee, Bundelkhand University, Jhansi.

Drugs and Chemicals

All the drugs and chemicals were of analytical grade. The drug like Levamisole, Cyclophosphamide and colloidal carbon etc. were procured from Hi Media Lab Pvt. Ltd. FicollHypaque and bovine serum albumin (BSA) was procured from Sigma Chemical Co. All other chemicals used were of high purity.

Plant Material: The fresh fruits of *Madhucalongifolia* (known as Mahua in folk language) were collected from the local areas of Jhansi. The plant material was identified and a voucher specimen was submitted to Department of Botany, Bundelkhand University, Jhansi. The identity of the plant was also confirmed with the vouchers specimen in Regional Ayurveda Research Center, Jhansi (Central Council for Research in Ayurveda & Siddha, Govt. of India). The plant material was dried in shade and grinded to obtained coarse power and subject to solvent extraction.

Preparation of Plant Extract

As per the protocol reported by Singh *et al.*[17] the dried fruit powder was extracted in ethanol solvent using soxhlet apparatus. The solvent extract was filtered twice with whatman filter paper to remove any residual material and dried in rotary evaporator (Steroglass, Italy) under vacuum. The extract was lyophilized in (Lyophillizer-Mc-Flow, India) for complete dryness before use. The dried extract was dissolved in suitable vehicle for administration in animal model. The dissolving vehicle was invariably used in untreated control group.

Experimental design

Initial screening of the solvent extract for immunomodulatory activity was done by conducting the Haemagglutinin titre assay (HT) in normal healthy mice. In short, the animals were divided in IV groups of six animals each. Group-I was untreated control. Group II was given cyclophosphomide for myelosuppression while Group-III & IV were treated with 100 and 200mg/kg body weight of the ethanol extract respectively by gavaging. The animals were humanized 24 hr after the last dose. Body weight gain (percentage) and relative organ weight (organ weight/100g of body weight) of kidney, liver, and spleen was determined for each animal.

Immunomodulatory assays done

Cyclophosphamide-induced myelosuppression

The cyclophosphamide-induced myelosuppression was studied according to methods described by Manjarekaret al[18]. The study was carried out till 20 days followed by TLC (Total Leukocyte Count) and DLC (Differential Leukocyte Count) of the collected blood samples of experimental animals.

Assessment of humoral immune functions

Animals within the experimental groups were challenged with 0.2 mL of 10% Sheep Red Blood Cells (SRBCs), i.p. on the 10th day of initiation of experiment. The heamagglutinin titre (HT) was studied in these animals.

The haemagglutinin titre (HT) assay: This was performed as per the procedure stated by Bin Hafeez*et al*[19]. On the fifth day after immunization blood was collected from the heart of each mouse for serum preparation. Serial two fold

dilution of serum was made in 50 µl of PBS (pH 7.2) in 96 well microtitre plates and mixed with 50 µl of 1% SRBC suspension in PBS. After mixing, plates were kept at room temperature for 2 h. The value of antibody titre was assigned to the highest serum dilution showing visible haemagglutination.

Assessment of Cell mediated immune response

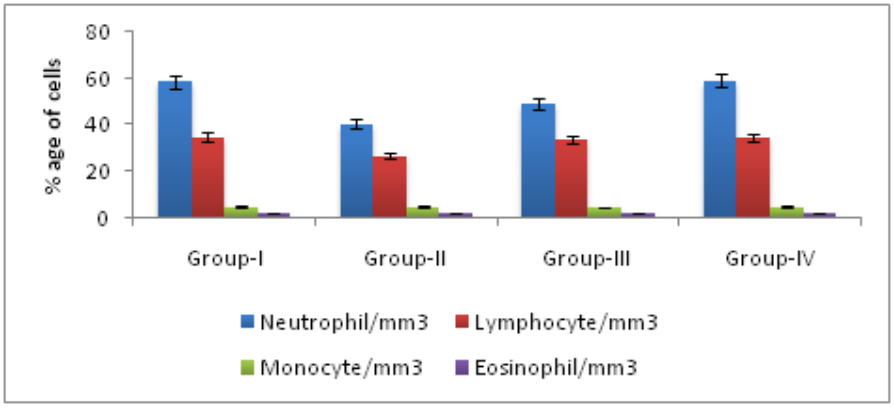
Delayed type of hypersensitivity response: The delayed type of hypersensitivity (DTH) response was determined as per the procedure given by Raisuddin*et al*[20]. On the day of termination of the treatment with plant extract; animals were immunized with $1x10^9$ SRBC, subcutaneously. On the fifth day of immunization, all the animals were again challenged with $1x10^8$ cells in the left hind footpad. The right footpad was injected with the same volume of normal saline, served as control for non-specific swelling. Increase in footpad thickness was measured 24h after the challenge, by a verniarcalipers.

Statistical Analysis: All the results are expressed as Mean±Standard Error (SEM). Data was analyzed using one-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test. P-values <0.05 was considered as statistically significant.

Results

Total Leukocyte Counts (TLC): A significant ($p<0.001$) reduction in total white blood cell count was observed in mice treated with cyclophosphamide alone (group II) as compared to control group (group I). The Ethanolic Extract of *M. longifolia* (EEML) given in the dose of 100 and 200mg/kg body weight with cyclophosphamide to the group-III and group-IV significantly ($p<0.001$) increased the levels of total WBC count as compared to cyclophosphamide treated group-II (Table-1). The total WBC count was restored back to normal in Group-IV i.e. treated with 200mg/kg body weight EEML with cyclophosphamide (Figure-1).

Figure 1: Cyclophosphomide induced myelosuppression and effect of EEML on Differential Leukocyte Count (DLC).



Differential leukocyte counts (DLC):

There was a significant ($P < 0.001$) decrease in the Neutrophils (N) and Lymphocytes (L) count in the mice treated with cyclophosphamide (group II) as compared to control group (group I). However, there was no significant reduction observed in Monocyte (M) and Eosinophil (E) count in group-II mice. EEML with cyclophosphamide at 100 mg/kg dose significantly ($P< 0.01$) increased the Neutrophils (N) count as compared to group I and group II both. Interestingly, Lymphocytes were more significantly increased as compared to group II (Figure 2). EEML with cyclophosphamide at 200 mg/kg dose in group IV was observed more effective than group II, as Neutrophil count was surprisingly very significantly increased as compared to group II ($p<0.001$) and group III ($p<0.01$). Its upraising on Monocyte count was also very significant ($p<0.001$), but failed to induce any significant difference in Monocytes (M) and Eosinphils count (Table 1).

Table 1: Effect of Ethanolic Extract of *M. Longifolia* (EEML) on Cyclophosphomide induced myelosuppression, DTH Reaction and Antibody Titre. (Given values are Mean±SEM of 6 animals in each group)

Groups	*Cells in %age*				*Cells/dL*	*mm*	
	Neutrophils Mean±SEM	**Lymphocytes Mean±SEM**	**Monocytes Mean±SEM**	**Eosinophils Mean±SEM**	**TLC Mean±SEM**	**DTH Response Paw thickness**	**Antibody Titre**
Group-I	58.66± 1.42	34.83± 0.87	4.66±0.49	2.16±0.30	9853.5±123.70	0.386±0.012	4.5±0.22
Group-II	40.33± 0.66[a]***	26.50± 1.33[a]***	4.66±0.42	1.83±0.30	4857.33± 442.08[a]***	0.418±0.015	2.0±0.36[a]***
Group-III	49.33± 2.90[ab]**	33.83± 0.94[b]***	4.33±0.21	2.16±0.16	8934.66± 286.83[b]****	0.473±0.018[a]**	5.0±0.36[b]***
Group-IV	59.00± 0.81[b]*** [&] [c]**	34.50± 0.56[b]***	4.66±0.21	1.83±0.30	9897.5± 116.57[b]***	0.461±0.017[a]*	5.5±0.22[b]***

*P<0.05; **P<0.01; ***p<0.001. a= compared with Control Group (Group-I), b= compared with Group-II, c= compared with Group-III. Statistically analysed by one-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.

Delayed Type Hypersensitivity (DTH) Responses: The administration of EEML at the dose of 100 and 200 mg/kg b.w to group III and group IV respectively have significantly ($p<0.01$) increased the Delayed Type Hypersensitivity

Responses in terms of increase in the mean difference of paw thickness of treated groups mice than control group I (Figure 3 & Table 1).

Effect of plant extract on humoral immunity parameters: a significant (P<0.001) reduction in the haemagglutinin titre value was observed in mice treated with cyclophosphomide alone (group II). Both administered doses of EEML in group III (100mg/Kg b.w.) and group IV (200mg/Kg b.w.) have significantly (P<0.001) increased the titre value in treated groups (Table 1)

Figure 2:Effect of EEML on Total Leukocyte Count (TLC).

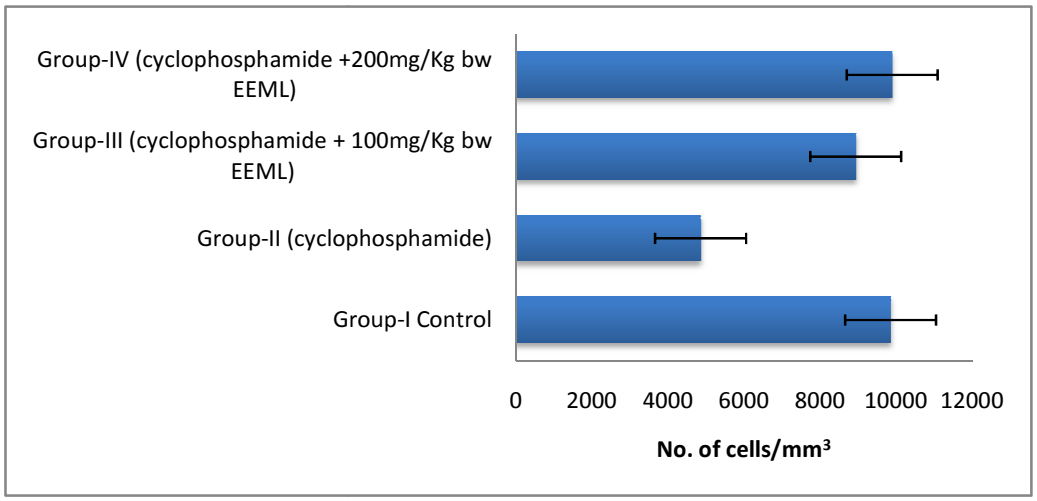
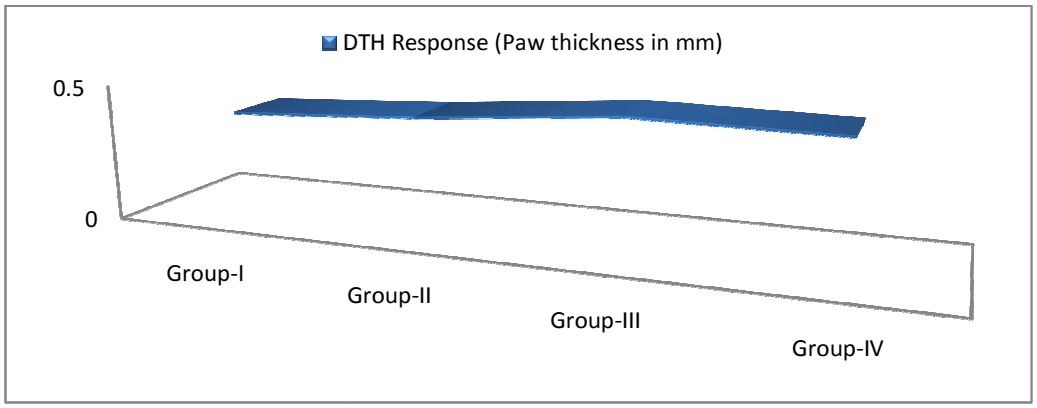


Figure 3: Effect of EEML on DTH response.



Discussion

Traditional Indian systems of medicines like Siddha and Ayurveda have suggested means to increase the body's natural resistance to diseases. A number of Indian medicinal plants and various 'rasayanas' are claimed to possess immunomodulatory activity[6]. Many of them have been recently reviewed for their immunomodulatory potential. Immunomodulation is a procedure of adjustment of the immune system by immunostimulation or immunosuppression to fight against infection. Immunostimulation and immunosuppression both needed to be tackled

in order to regulate the normal immunological functioning. The human immune system is known of two types, one which we receive by birth called innate and another which we acquire during our life time called adaptive immune system. Two subtypes of later specific immunity, Humoral and cell mediated caused by B and T lymphocytes respectively are considered the chief constituents of the immune system. In the present study, *M. longifolia* was explored for its Immunomodulatory potential on both sub types (humoral and cell mediated immunity) by evaluating its effect on antibody titre (humoral immunity parameter), DTH reaction (Cell mediated immunity parameter) and cyclophosphomide induced myelosuppression (innate immunity parameter) in mice. The administration of methanolic extract of fruit of the said plant has shown immunostimulating activity in vivo.

The enhancements in antibody titre value of the treated groups have shown its boosting effect on humoral immunity which may be a result of B-lymphocytes activation and production of antibodies from them. DTH is antigen specific and causes erythema and induction at the site of antigen infection in immunized animals. It is caused due to influx of immune cells (Macrophages and Basophils) at the site of injection which become apparent as swelling (increased paw thickness) within 24-72 hr. The significant increase in the mean difference of paw thickness of treated groups mice than control group I have reflected its immunomodulatory effect on cell mediated immunity as well. Moreover, the restoration of Neutrophils, Lymohocytes and overall WBCs to normal level in EEML treated groups III & IV as compared to cyclophosphomide induced myelosuppressed group II have revealed the ameliorating effect of *M. longifolia* on myeloid regeneration. The present immunomodulatory activity of the said plant can be attributed to the flavonoids (quercetin), alkaloids, tannins, saponin glycosides and phenolic compounds found in Maduca plants[21, 22].

Traditionally, *Madhucalongifolia* bark has been used against diabetes, rheumatism, ulcers, bleeding and tonsillitis[9]. The flowers, seeds and seed oil of madhuca have great medicinal value. Externally, the seed oil massage is very effective to alleviate pain. In skin diseases, the juice of flowers is rubbed for oleation. It is also beneficial as a nasya (nasal drops) in diseases of the head due to pitta, like sinusitis[11]. The aqueous extracts of flowers and fruits have been shown the antibacterial activities towards bacteria *Bacillus subtilis*and *Klebsiella pneumonia*[23].

Conclusions

With the significant immunostimulatory activity on both the specific and non-specific immune mechanisms, *M. longifolia* holds great promises for being used as an immunomodulating agent. However, more in-depth studies of various other extracts of different parts of the plant may further help in the exploration of immunomodulatory activity

of *Madhucalongifolia* and use that for the therapeutic purposes. Hence, the further works may provide crucial agents in form of safer, effective and economical immunomodulatory drug for the management of infectious diseases.

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