ORIGINAL ARTICLE

Immunostimulatory effect of *Mimusops elengi* Linn stem bark in mice

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Objectives: To scrutinise the immunostimulatory activity of methanolic extract of bark of *Mimusops elengi* Linn (MEMEL) in mice. **Materials and Methods:** The MEMEL was administered orally at the dose of 10, 20, 40 mg/kg/day body weight in mice. The immunostimulatory activities on specific and non-specific immunity were studied by carbon clearance test (CCT), haemagglutination antibody (HA) and delayed type hypersensitivity, using sheep red blood cells (SRBC) as the antigen. Distilled water served as a control in all the tests and vitamin E 150 mg/kg was used as standard. **Result:** Oral administration of MEMEL showed a dose-dependent increased immunostimulatory response. Phagocytic index was found to be increased significantly ($P < 0.01$) in CCT. The production of circulatory antibody titre (humoral antibody response) was increased significantly ($P < 0.01$) and delayed type hypersensitivity reaction was found to be augmented less significantly ($P < 0.05$) by increasing the mean footpad thickness at 48 hr in response to SRBC as an antigen. **Conclusion:** This finding suggests that the MEMEL possesses potential for augmenting immune activity by cellular and humoral-mediated mechanisms.

Key words: Antibody titre, delayed type hypersensitivity, immunostimulant, *Mimusops elengi* Linn, phagocytosis

INTRODUCTION

Natural products have provided significant value to the pharmaceutical industry over the past half century[1] and played a vital role throughout the world in treating and preventing human diseases.[2] In the present day, there is widespread interest in herbal drugs. These interests mainly appear from the belief that herbal medicines are safe, inexpensive and have no adverse effects. It is no speculate that the world's one-fourth residents i.e. 1.42 billion people, are dependent on conventional medicines for the treatment of various ailments.[3] Traditional systems of medicine such as Ayurveda play a critical role in current healthcare, predominantly in the treatment of diseases where suitable treatments are not existing.[4] Modulation of immune responses to treat the diseases has been of curiosity for many years and the notion of 'Rasayana' is based on associated principles.[5] The Rasayana classes of medicinal plants in Ayurveda are reported to have a triphasic activity, i.e. the capability to develop health and longevity, enhance memory, intelligence, and youthfulness and improve complexion.[4] The use of medicinal plant commodities as immunomodulators as probable therapeutic measure is becoming a new theme of scientific investigations.[6]

Mimusops elengi linn (Sapotaceae) is a non-rasayana immunomodulatory Indian medicinal plant, usually identified as Bakul and Spanish cherry. It is considered as a blessed plant among Hindus and has obtained key place in spiritual texts as well as in prehistoric Sanskrit literature.[7] In the traditional Indian system of medicine, the Ayurveda and various folk system of medicine,[8] *Mimusops elengi* acquire several medicinal properties such as astringent, tonic and febrifuge, etc.[9] Preclinical studies have revealed that the bark possess anti-anxiety, anti-hyperlipidaemic, anti-ulcer, anti-convulsant, anti-inflammatory, analgesic, anti-pyretic, anti-oxidant, cytotoxic, antidiabetic, diuretic and hypotensive activities.[10] Chemical studies have shown that bark contains taraxerone, taraxerol, spinasterol,[11] sodium salt of betulinic acid, urosolic acid and fatty acid esters of alpha-spinasterol.[12]

A stem bark of *Mimusops elengi* Linn has been used by Ayurvedic practitioners, in rural Maharashtra, to improve the immune system and to battle a number of diseases. However, there are no scientific details available in the literature on the immunomodulatory activity of *Mimusops elengi* Linn bark extract. Also it has been reported that this plant has contained betulinic acid, a steroidal pentacyclic triterpenoid, as one of the phytomolecule which has proved to be an effective lead for immunomodulatory activity. Therefore, the present

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study was undertaken to measure the immunostimulatory activity of the methanolic extract derived from the bark of *Mimusops elengi* (MEMEL) in relation with its folklore medicinal properties.

MATERIALS AND METHODS

Plant Material

The fresh bark of *Mimusops elengi* was gathered in the month of November from Sangavi, Pune District, Maharashtra state, India. The plant was identified and authenticated by Botanical Survey of India, Pune, and a voucher specimen was deposited with a voucher specimen sample No. MIESIV 1.

Extraction

The dried bark was crushed to moderately coarse powder. The bark powder passing through a 40-mesh sieve was used for the extraction procedure. Bark powder (500 g) was fed into a Soxhlet extractor and extracted with Methanol at 35-45°C temperature for 5 days. After ensuring entire extraction, the extract was collected, filtered and dried under vacuum using a rotary vacuum evaporator (VFD-L) the yield was found to be 7% w/w. Extract was stored in an air-tight glass container till further use.

Animals

Male Swiss albino mice weighing 18-25 g were obtained from National Institute of Biosciences, Pune, and were housed in groups of 5 to 6. All mice were fed with pellet diet, and water *ad libitum*. Mice were maintained at 22 ± 1°C with 60% relative humidity, and kept under 12-h light and dark cycles. The animals were allowed to adapt to laboratory conditions prior to experimentation. All experiments were conducted during the light period of 12 hours of the day/night cycle. All the experiments were permitted and conducted as per the guidelines of Institutional Animal Ethical Committee (Approval no. CPCSEA/IAEC- 005/2012).

Acute Oral Toxicity Study

The acute toxicity study was carried out according to the limit test described in the OECD guidelines. Briefly, a test dose of 2 g/kg and 5 g/kg were given orally to the mice. All the animals survived the toxicity studies at the dose 200 mg/kg body weight. Based on the study; doses of 10, 20 and 40 mg/kg body weight were selected for animal experiments.[13]

Preparation of Antigen (Immunisation)

Sheep Red Blood Corpuscles (SRBCs)

Fresh blood was collected from sheep's sacrificed in the local slaughter house in a sterile bottle containing Alsever's solution (0.8% sodium citrate, 0.05% citric acid, 2% dextrose, and 0.42% sodium chloride) in 1:1 proportion. Blood was kept in the refrigerator and processed for the preparation of SRBCs batch, by centrifuging at 2000 rpm for 10 minutes and washing with physiological saline four to five times and then suspending into buffered saline for further use. The animals were immunised by injecting 1-ml of 20% SRBC, intraperitoneally (i.p.).[14,15]

Carbon Ink Suspension

Commercially available Camel brand black ink suspension was purchased from the local market and diluted in a ratio of 1:50 with normal saline and used for carbon clearance test (CCT) in a dose of 1 ml/200 g body weight.[16]

Evaluation of Immunomodulatory Activity

Treatment Protocol

Mice were divided into five groups consisting of six animals each randomly. In Group I, control animals received normal saline for 7 days per oral (p.o.). In Group II, animals received vitamin E (150 mg/kg i.p.) as standard. MEMEL extract was dissolved in distilled water to prepare different doses (10, 20 and 40 mg/kg body weight) and administered orally with the help of gastric canula in Groups III, IV and V, respectively. The control animals were administered with equivalent volume of distilled water as vehicle. The animals were immunised by injecting 1 ml of 20% SRBC i.p. The day of immunisation was considered as day 0.

Carbon Clearance Test

On 7th day of treatment animals of the entire group will received an intravenous injection of (0.1 ml/kg of body weight) of Indian ink dispersion (per warmed at 37°C). Blood samples will collected from retro-orbital bleeding by using glass capillaries at an interval of 0 min and 15 min after the injection of ink dispersion.[17] Blood samples were added to 4 ml of 0.1% sodium carbonate solution to lyses the erythrocytes. Absorbance of these samples will be measured at 660 nm using UV visible spectrophotometer.[16] The mean of phagocytic index will be calculated of each group of animals

$$K = \frac{\text{InoD}_1 - \text{In OD}_2}{t_2 - t_1}$$

Where, OD1 and OD2 depict the optical densities at times t_1 (0 min) and t_2 (15 min), respectively.[15,18]

Haemagglutination Titre

Blood samples were collected in micro-centrifuge tubes from individual animal by retro-orbital plexus on the 7th day and serum was separated. Antibody levels were determined by haemagglutination technique.[5] Briefly, equal volumes of individual serum samples of each group were pooled. Two-fold dilutions of pooled serum samples were made in 25-µl volumes of normal saline in micro-titration plate

and to that were added 25 µl of 1% suspension of sheep red blood cells in saline.[15] After mixing, the plates were incubated at room temperature for 1 hr and examined for haemagglutination under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.[19]

Delayed Type Hypersensitivity

On day 7th all animals from all the groups were challenged with (20 µl of 1%) SRBC in sub-plantar region of right hind paw.[20,21] Footpad oedema in mice was used for detection of cellular immune response. On 7th day, the thickness of right hind footpad was measured using digital plethysmometer and inject 20-µl volume of 1% sheep RBC in the sub-plantar region of right hind paw.[22] Footpad reaction was assessed after 24 hrs and 48 hrs i.e. on 8th and 9th day in terms of increase in the thickness of footpad due to oedema caused as a result of hypersensitivity reaction, with the help of a digital plethysmometer.[5] The footpad reaction was expressed as the difference in the thickness (mm) between the pre and post-right hind foot pad injected with SRBC.[23,15]

Statistical Analysis

Data is expressed as Mean ± SEM and was analysed for significance of variance by one-way ANOVA followed by Tukey multiple comparison tests using PRISM software.

RESULT

Acute Oral Toxicity Study

Acute oral toxicity study of MEMEL in mice did not show any changes in the behaviour, autonomic profiles and no mortality were observed in all treated and controlled groups of the mice up to the dose of 200 mg/kg.

Carbon Clearance Test

The phagocytic activity of the reticulo-endothelial system is generally calculated by the rate of removal of carbon particles from the blood stream. MEMEL improved non-specific immunity as evidenced by increased phagocytic index in the CCT. The phagocytic index for the control group was found to be 0.00388 ± 0.00076, whereas the extract increased it to 0.0218 ± 0.01028, 0.02436 ± 0.01612 and 0.03392 ± 0.01529 at doses of 10, 20 and 40 mg/kg, respectively [Table 1]. The MEMEL at a dose of 40 mg/kg showed significant ($P < 0.01$) increased in phagocytic index when compared to control group [Figure 1].

Haemagglutination Titre

The haemagglutination titre was used to assess humoral immune response. The augmentation of the humoral immune response to SRBCs by methanol extract was evidenced by increase in the antibody titre. Administration of MEMEL (10, 20 and 40 mg/kg) produced dose related increase in humoral antibody titre. The antibody titre for the control group was found to be 5.8 ± 0.83666 but at doses of 10, 20 and 40 mg/kg of MEMEL, it increased to 5.2 ± 0.83666, 7.4 ± 1.14018 and 8.2 ± 1.48324, respectively [Table 1]. The antibody titre level was found to be significantly ($P < 0.01$) high in animals treated with higher dose (40 mg/kg) of MEMEL as compared to the control group [Figure 2].

Delayed Type Hypersensitivity (DTH)

Effect of MEMEL on cell-mediated immune response by DTH-induced footpad oedema is measured by footpad thickness in the hind paw using plethysmometer. Administration of methanol extract dose not produced dose dependent increase in thickness of footpad of mice as a measure of DTH response. The mean difference (48 and 24 hrs) of paw oedema for the control group was found to be 0.178 ± 0.00837 but at doses of 10, 20 and 40 mg/kg of MEMEL, it increased to 0.179 ± 0.00837, 0.190 ± 0.01 and 0.200 ± 0.01732, respectively [Table 1]. In which 40 mg/kg dose of MEMEL elicited a less significant ($P < 0.05$) increase in footpad thickness as compared to the control group [Figure 3].

DISCUSSION

Immunomodulation involves the modulation of the immune responses through motivation or inhibition may aid in maintaining a disease-free state.[5] If the improvement of immune reactions occurs it is named as an immunostimulative which primarily implies stimulation of non-specific system, i.e. macrophages, complement, granulocytes, certain T-lymphocytes and different effectors substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may occur on account of

Table 1: Effect of *Mimusops elengi* on phagocytic activity, humoral immune response and cell-mediated immune response by DTH

Groups (*n*=6)	Treatment, dose and route	Phagocytic index	Antibody titre	Mean difference of paw oedema
Control	Distilled water, 10 ml/kg, (p.o.)	0.00388±0.00076	5.8±0.83666	0.178±0.00837
Standard	Vitamin-E, 150 mg/kg, (p.o.)	0.04358±0.00071***	8.8±0.44721***	0.396±0.0114***
ME (10 mg/kg)	1% SRBC, 0.1 ml/kg, (i.p.)+MEMEL, 10 mg/kg (p.o.)	0.0218±0.01028	5.2±0.83666	0.179±0.00837
ME (20 mg/kg)	1% SRBC, 0.1 ml/kg, (i.p.)+MEMEL, 20 mg/kg (p.o.)	0.02436±0.01612	7.4±1.14018	0.190±0.01
ME (40 mg/kg)	1% SRBC, 0.1 ml/kg, (i.p.)+MEMEL, 40 mg/kg (p.o.)	0.03392±0.01529**	8.2±1.48324**	0.200±0.01732*

Results are expressed as mean±S.E.M (*n*=6), one-way ANNOVA followed by Tukey multiple comparison test.*P<0.05, **P<0.01, ***P<0.001, When compared with control groups. DTH – Delayed type hypersensitivity; SRBC – Sheep red blood corpuscles

environmental or chemotherapeutic factors.[23] The results gained in the present study specify that *Mimusops elengi* is a potent immunostimulant drug which shows activity by stimulating both specific and non-specific immune mechanisms.

The role of phagocytosis is the elimination of microorganisms and foreign bodies, dead or injured cells.[24] Phagocytosis represents a key innate defence mechanism against ingested particulates including whole pathogenic microorganisms.[25] The particular cells that are expert of phagocytosis include neutrophils, blood monocytes and tissue macrophages. In a view of the essential role played by the macrophages in synchronising the progression and presentation of antigen to β-cells, MEMEL was estimated for its effect on macrophage phagocytic activity.[15] Increase of carbon clearance is a display of enhanced *in vivo* phagocytic activity and also potential of granulopoetic system in elimination of foreign particles. MEMEL at a dose of 40 mg/kg enhanced the phagocytic function by clearing the carbon particle produced phagocytic response.

The index of humoral immune response is the raise in antibody titre value due to boost in immune response.[15] SRBC cells decreases the antibody titre level; thus it serves as an ideal standard to evaluate whether the immune response is important. The humoral immunity involves interaction of B cells with the antigen and their consequent proliferation and differentiation into antibody-secreting plasma cells.[24] Antibody functions as the effectors of the humoral response by binding to antigen and neutralising it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells.[5] In the present study, control group showed significant inhibition in antibody titre response whereas the 40 mg/kg dose of MEMEL show a significant ($P < 0.01$) augmentation. This augmentation of the humoral response to SRBC antigen by increase in haemagglutination antibody titre indicated the enhanced responsiveness of macrophages and T- and B-lymphocyte subsets involved in antibody synthesis.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their products (lymphokines).[24] DTH reaction involves the immuno-inflammatory reaction, in which macrophages and Th1 cells play major role. These reactions require a specific antigenic substance which will release cytokines by activation with T-lymphocytes.[26] DTH is a part of the process of graft rejection, tumour immunity and most important immunity to many intracellular infectious micro-organisms, especially those causing chronic diseases viz., tuberculosis. In this study, SRBC was used as the antigenic substance which elicits the hypersensitivity reaction in mice.[15] DTH reaction is measured by footpad thickness, after 48 hr of antigenic challenge and subsequent immunisation with SRBC, the animal showed significant increase in volume of paw oedema due to production of antibodies in response to the antigen. The 40 mg/kg dose of MEMEL elicited a less significant ($P < 0.05$) increase in footpad thickness as compared to the control group, which signifies that 40 mg/kg dose of MEMEL has stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction and thus increases cell mediated immunity. Even though, the present study did not give the entire view of the mechanism whereby the methanolic extract modulates the immune system so further studies should be carried out to find out exact mechanism of action *Mimusops elengi*.

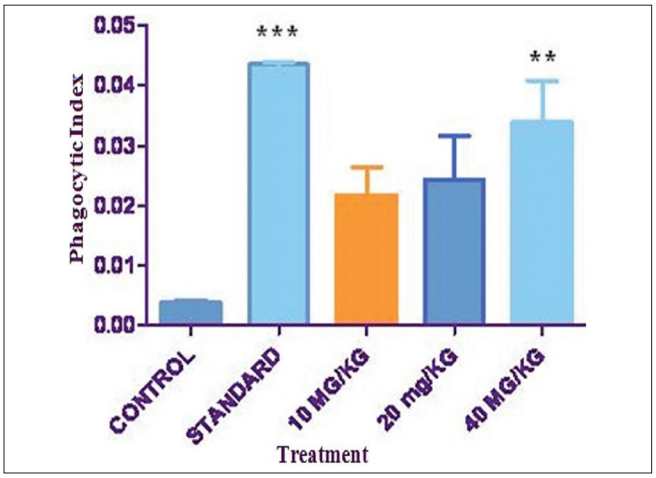

Figure 1: The effect of Mimusops elengi on phagocytic activity [Control: Group received only vehicle i.e., normal saline; Standard: Group treated with 150 mg/kg i.p. of vitamin E; 10 mg/kg: Group treated with 10 mg/kg of MEMEL; 20 mg/kg: Group treated with 20 mg/kg of MEMEL; 40 mg/kg: Group treated with 40 mg/kg of MEMEL] Results are expressed as Mean ± SEM ($n = 6$), one-way ANNOVA followed by Tukey multiple comparison test.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, when compared with control groups

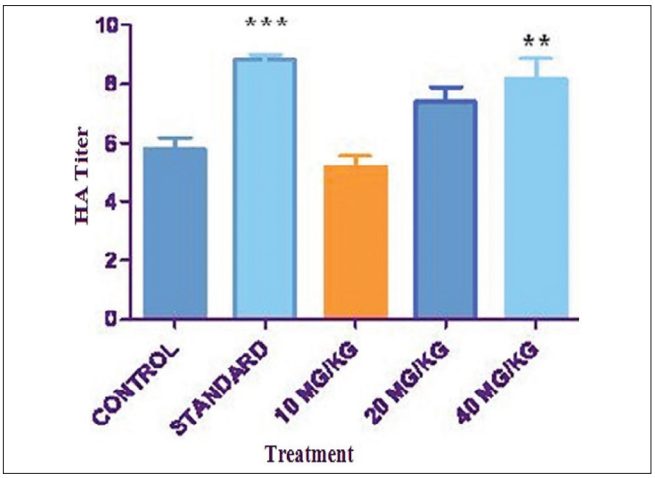

Figure 2: The effect of Mimusops elengi on humoral immune response (HA Titre)[Control: Group received only vehicle i.e., normal saline; Standard: Group treated with 150 mg/kg i.p. of vitamin E; 10 mg/kg: Group treated with 10 mg/kg of MEMEL; 20 mg/kg: Group treated with 20 mg/kg of MEMEL; 40 mg/kg: Group treated with 40 mg/kg of M EMEL] Results are expressed as Mean ± SEM ($n = 6$), one-way ANNOVA followed by Tukey multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***P < 0.001, when compared with control groups

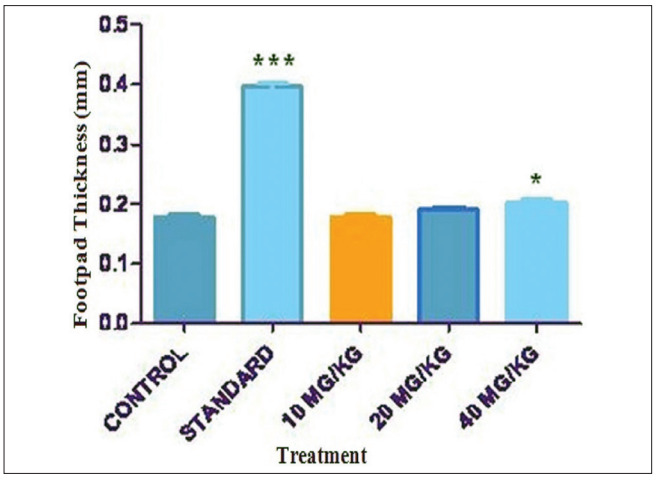

Figure 3: The effect of Mimusops elengi on cell-mediated immune response by DTH [Control: Group received only vehicle i.e., normal saline; Standard: Group treated with 150 mg/kg i.p. of vitamin E; 10 mg/kg: Group treated with 10 mg/kg of MEMEL; 20 mg/kg: Group treated with 20 mg/kg of MEMEL; 40 mg/kg: Group treated with 40 mg/kg of MEMEL] Results areexpressed as Mean ± SEM (n = 6), one-way ANNOVA followed by Tukey multiple comparison test.*P < 0.05, **P < 0.01, ***P < 0.001, when compared with control groups

Findings of the present study establish that these extracts have appreciable immunostimulatory activity. Both low dose (10 mg/kg, p.o) as well as high dose (40 mg/kg, p.o) of *Mimusops elengi* stimulates immune system by acting through cellular and humoral immunity in experimental models of immunity in animals. However, high dose was found to be most efficient than the low dose. However, further studies are required to support these conclusions. The difference in the way these extracts affected cell stimulation or inhibition perhaps indicates their various mode of actions and raises the need for further investigations in particular the possible action of the extracts in interfering with cell signalling and cytokines production.

REFERENCES

1. Baker DD, Chu M, Oza U, Rajgarhia V. The value of natural products to future pharmaceutical discovery. Nat Prod Rep 2007;24:1225-44.
2. Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. AAPS J 2006;8:E239-53.
3. Kadam PV, Deoda RS, Shivatare RS, Yadav KN, Patil MJ. Pharmacognostic, phytochemical and physiochemical studies of *Mimusops elengi* Linn stem bark (Sapotaceae). Der Pharmacia Lettre 2012;4:607-13.
4. Sharma V, Thakur M, Chauhan NS, Dixit VK. Immunomodulatory activity of petroleum ether extract of Anacyclus pyrethrum. Pharm Biol 2010;48:1247-54.
5. Satpute KL, Jadhav MM, Karodi RS, Katare YS, Patil MJ, Rub R, *et al*. Immunomodulatory activity of fruits of Randiadum etorum Lamk. J Pharmacog Phytother 2009;1:56-60.
6. Singh S, Cps Y, Noolvi MN. Immunomodulatory activity of butanol fraction of Gentiana olivieri Griseb on Balb/C mice. Asian Pac J Trop Biomed 2012;2:433-7.
7. Bakula MR. A reputed drugs of ayurveda, its history-uses in Indian medicine. Indian J Hist Sci 1981;16:169-80.
8. Nadkarni KM. Indian Materia Medica 2. 3rd ed. Mumbai: Popular Prakashan; 1996. p. 596-599.
9. Sharma PC, Yelne MB, Dennis TJ. Database on Medicinal Plants Used in Ayurveda. Vol. 1. India: Central council for Research in Ayurveda and siddha; 2000. p. 65-72.
10. Manjeshwar SB, Ramakrishna JP, Harshith PB, Princy LP, Rekha B. Chemistry and medicinal properties of the Bakul (*Mimusops elengi* Linn): A review. Food Res Int 2011;44:1823-9.
11. Mishra G, Mitra CR. Constituents of bark of Mimusops Elengi linn. Phytochemistry 1967;6:1909.
12. Jahan N, Ahmed W, Malik A. A lupene-type triterpene from Mimusops Elengi. Phytochemistry 1995;39:255-7.
13. Aher VD, Wahi VA. Pharmacological study of Tinospora Cordifolia as an immunomodulator. Int J Curr Pharm Res 2010;2:52-54.
14. Dashputre NL, Naikwade NS. Immunomodulatory activity of abutilon indicum linn on albino mice. Int J Pharm Sci Res 2010;1:178-84.
15. Patel S, Banji D, Banji OJ, Patel MM, Shah KK. Scrutinizing the role of aqueous extract of Trapa bispinosa as an immunomodulator in experimental animals. Int J Res Pharm Sci 2010;1:13-9.
16. Tilwari A, Shukla NP, Pathirissery UD. Immunomodulatory activity of the aqueous extract of seeds of Abrus precatorius Linn (Jequirity) in mice. Iran J Immunol 2011;8:96-103.
17. Ismail S, Asad M. Immunomodulatory activity of Acacia catechu. Indian J Physiol Pharmacol 2009;53:25-33.
18. Mazumder PM, Pattnayak S, Parvani H, Sasmal D, Rathinavelusamy P. Evaluation of immunomodulatory activity of Glycyrhiza glabra L roots in combination with zing. Asian Pac J Trop Biomed 2012;1:15-S20.
19. Kasote DM, Zanwar AA, Devkar ST, Hegde MV, Deshmukh KK. Immunomodulatory activity of ether insoluble phenolic components of n-butanol fraction (EPC-BF) of flaxseed in rat. Asian Pac J Trop Biomed 2012;2:623-S6.
20. Bafna AR, Mishra SH. Immunomodulatory activity of petroleum ether extract of flower-heads of Sphaeranthus indicus Linn. J Herbal Pharmacother 2007;7:25-37.
21. Patil CR, Salunkhe PS, Gaushal MH, Gadekar AR, Agrawal AM, Surana SJ. Immunomodulatory activity of Toxicodendron pubescens in experimental models. Homeopathy 2009;98:154-9.
22. Bhadoriyalb SS, Mandoriya N. Immunomodulatory effect of Tricosanthes Dioica Roxb. Asian Pac J Trop Biomed 2012;2:985-S7.
23. Makare N, Bodhankar S, Rangari V. Immunomodulatory activity of alcoholic extract of Mangifera indica L. in mice. J Ethnopharmacol 2001;78:133-7.
24. Dashputre NL, Naikwade NS. Preliminary immunomodulatory activity of aqueous and ethanolic leaves extracts of Ocimum basilicum Linn in Mice. Int J Pharm Tech Res 2010;2:1342-9.
25. Atal CK, Sharma ML, Kaul A, Khajuria A. Immunomodulating agents of plant origin. I: Preliminary screening. J Ethanopharmacol 1986;18:133-41.
26. Mukherjee D, Khatua TN, Venkatesh P, Saha BP, Mukherjee PK. Immunomodulatory potential of rhizome and seed extracts of Nelumbo nucifera Gaertn. J Ethanopharmacol 2010;128:490-4.

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