Indian Journal of Natural Products and Resources
Vol. 4(1), March 2013, pp 54-60

Immunopotentiating properties of ethanolic extract of *Malvastrum tricuspidatum* A. Gray whole plant

Neelam Balekar, Santosh Ghule*, Pankaj Dixit, Dinesh Kumar Jain and Varun Tandan
College of Pharmacy, IPS Academy, Rajendranagar, A. B Road, Indore –452 012 , Madhya Pradesh, India

Received 20 October 2011; Accepted 26 April 2012

The aim of the present study was to investigate the immunomodulatory activity of ethanolic extract of *Malvastrum tricuspidatum* A. Gray (MTEE) whole plant in experimental models of immunity. Preliminary immunomodulatory activity was evaluated by studying the effect of MTEE (200, 500, and 1000 mg/kg, p.o.) on immunological organs weight and haematological parameters in Swiss albino mice. The effects were further confirmed in various models like, determination of delayed type hypersensitivity response (MTEE 200 & 500 mg/kg, p.o.), neutrophil adhesion test and humoral immunity (MTEE 500 mg/kg, p.o.). Sheep red blood cells (SRBC, 0.5×10^9cells/mL/100g) were used to immunize animals. The total leukocyte count increased significantly after acute administration of MTEE at 1000 mg/kg. Further studies confirmed the immunostimulant potential of MTEE at 500 mg/kg after 14 days of administration. The neutrophil adhesion was increased by 34.19%. Similarly, the antibody titre and DTH were also significantly increased as compared to control. It is concluded that MTEE possesses potential for augmenting immune activity by cellular and humoral mediated mechanisms at 500 mg/kg dose. As it is used for a variety of medicinal purposes, its immunomodulatory effect strengthens the rationale of its use in several Ayuvedic and Unani drugs.

Keywords: *Malvastrum tricuspidatum* A. Gray, Malvaceae, Immunomodulation, Cell mediated immunity, Humoral immunity, Neutrophil adhesion, Haemagglutination

IPC code; Int. cl. (2011.01) – A61K 36/00, A61P 37/02

Introduction

Modulator response of immune system to alleviate disease condition was major interest in Ayurveda for development of 'Rasayana' drugs. Many plants are extensively used as 'Rasayana' drugs in 'Ayurveda' for the management of neurodegenerative diseases, as well as rejuvenators, immunomodulators, aphrodiasiac and nutritional supplements[1]. Indian medicinal plants are a rich source of substances claimed to induce paraimmunity, the non-specific immunomodulation of essential granulocytes, macrophages, natural killer cells and complement functions. Ayurveda lays emphasis on promotion of health, a concept of strengthening host defenses against different diseases[2].

Immunostimulation and immunosupression need to be tackled intricately to regulate normal immunological functioning. Hence, both immunostimulating agents and immunosupressing agents have their own value and search for better agents is becoming the field of major interest all over the world[3]. A number of plant products are being investigated for immune response modifying activity. A plethora of plant-derived materials (proteins, lecithin, polysaccharides, etc.) are reported to stimulate the immune system. Some of the plants with established immunomodulatory activity are *Viscum album* L., *Panax ginseng* Mey., *Asparagus racemosus* Willd., *Azadirachta indica* A. Juss., *Tinospora cordifolia* (Willd.) Miers. ex Hook.f. & Thoms., *Ocimum sanctum* L. etc[4].

Mallow (*M. tricuspidatum* A. Gray, Family-Malvaceae) is a native plant of America, introduced into India and now found in South India, Punjab, the United Provinces, Orissa and Bengal[5]. Its whole plant is reported to contain many functional and bioactive compounds such as sigmasterols, lutein, phenylethylamine, indole alkaloids and fatty acids like palmitic acid, oleic acid, linoleic acid and sterulic acid, etc. The active constituents of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the immune system in different experimental models[6]. Therefore, the chemical profile indicates it as a good source of immunomodulatory agent. Further, it is used as hypoglycaemic, antiulcerogenic, wound healing

*Correspondent author:
E-mail: ghulesantosh1284@gmail.com
Phone/Fax no. +91-731-4041627

agent, anti-inflammatory, antibacterial, antipyretic and analgesic agent. However, till date no scientific evaluations are conducted for confirming its role as an immunomodulator. Thus, this study designed to study the immunomodulatory activity of extract of *M. tricuspidatum* in different experimental models of cellular and humoral immunity in mice.

Materials and Methods

Experimental animals

Inbred Swiss albino mice (6-8 weeks) of either sex weighing about 18-20 g, procured from Govt. Veterinary College, Mhow, Indore, M.P. were acclimatized to our animal house for at least 10 days prior to the experiments. They were fed standard rodent diet (Trimurti Feeds, Maharashtra, India) with water *ad libitum*. The present investigations employed Swiss albino mice as the same had been used by several earlier investigators for immunological studies[7]. All protocols were approved by IAEC (Approval no: CPCSEA/51/2011).

Preparation of plant extract

The whole plant of *M. tricuspidatum* was collected in August 2011 from local areas of Indore, A. B. Road and dried under shade (Plate 1). The dried material was powdered and extracted with petroleum ether (60-80%) for 48 h to remove fatty matter. The defatted marc was then subjected to Soxhlet extraction with 95% ethanol (yield: 6.78%) for 8 h. The total extract was concentrated using rotary evaporator.

Chemicals and their sources

Leishman's stain, hydrochloric acid, sodium chloride and glucose were purchased from Merck (Mumbai, India). Grower's solution (RBC diluting fluid), Turk's fluid (WBC diluting fluid) and EDTA di-sodium salt (S.D Fine-Chem limited, Mumbai, India). All other chemicals were of analytical grade and all the solutions were prepared fresh.

Plate 1 — *Malvastrum tricuspidatum* A. Gray

Antigen preparation

Fresh sheep blood was collected from local slaughter house, which was stored in sterile Alsever's solution and washed thrice with pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×10^9 cells/mL/100 g animal for immunization and challenge[8].

Preliminary phytochemical screening of extract

Preliminary phytochemical analysis was carried out to check and identify the active constituents of the ethanolic extract of *M. tricuspidatum* whole plant such as saponins, flavonoids, terpinoids, amino acids, proteins, alkaloids and carbohydrates by using foam formation test (Froth formation), Dragendroff (yellow colour formation) and Mayer test(white precipitate), lead acetate test (Reddish brown colour), Millons test (Coloured precipitate), Biuret test (violet colour) and Fehling's test (Green colour), respectively[9].

Acute toxicity studies

The acute toxicity study was carried out to select the dose by using up and down or stair case method. Two mice were given a dose of 50 mg/kg orally and examined for a period of 24 h for mortality. The subsequent doses were then increased by a factor of 1.5 to attain maximum non-lethal and minimum lethal dose. The extract was found to be safe up to the dose of 2 g/kg *p.o.* according to Organization for Economic Co-operation and Development (O.E.C.D.) guideline 425 different doses ranging from $1/10^{th}$ as lower dose and $1/50^{th}$ as the maximum safe dose were selected[10,11].

Grouping

Swiss albino mice of either sex were divided into four groups (n=5) fashioned as: Group I: control, normal saline; Group II: 200 mg/kg, MTEE *p.o.*; Group III: 500 mg/kg MTEE, *p.o.*; Group IV: 1000 mg/kg MTEE, *p.o.*

Experimental protocol

The solutions were prepared in distilled water for oral administration. Initially effect of MTEE on organ weight and haematological parameters was evaluated. Further immunomodulatory activity was checked both at cellular and humoral levels. Cellular immunity was evaluated by delayed type hypersensitivity response and neutrophil adhesion test, humoral immunity was analyzed by serum antibody titre.

Determination of effect on organ weight

Group I-IV was treated orally with saline (10 mL/kg) and extract (200, 500, and 1000 mg/kg) for 5 days, respectively. At the end of treatment schedule body weights of animals were recorded and animals were sacrificed by cervical dislocation 12 h after the last dose of drug treatment and the weights of vital organs such as liver, spleen, kidney and thymus were recorded and expressed as relative organ weight in mg/100 g mouse[12].

Determination of effect of *M. tricuspidatum* on haematological parameters

Groups I-IV were treated with saline and extract for 5 days as outlined above. At the end of treatment schedule blood was collected from retro-orbital puncture and parameters such as total RBC count, total WBC count (hemocytometer), differential count (Leishman's stain), haemoglobin content (Sahli's method) were recorded[13]. Same animals were used for determining the effect of various doses of extract on haematological parameters and organ weights.

Assessment of cellular immune functions

Determination of delayed-type hypersensitivity (DTH) response

The DTH response was determined using the method of Lagrange *et al* (1973)[14]. Mice were treated as outline above for 14 days. On the day of termination of treatment, animals in group I-III were sensitized to antigen by injecting SRBC (0.5×10^9 cells/mL/100 g suspended in saline solution, i.v.) and after 5 days they were antigenically challenged by injecting SRBC (10^8 cells/20 µL) into sub-planter region of hind paw. Increase in the thickness of paw was measured 24 h later with digital Vernier caliper and cell-mediated immunity expressed as difference in paw thickness[15,7].

Neutrophil adhesion test

Swiss albino mice in Groups I & III were administered MTEE and saline for 14 days in their respective groups. Adhesion of neutrophils to nylon fibers was determined using a modification of adherence assay[15]. Briefly, 1cc tuberculin syringe was uniformly packed with 65 mg fibers to a volume of 0.4 cc (Fig.1). This assemble was pre-warmed to 37°C in an incubator. Blood samples were collected from mice (after 14 days of drug treatment) by retro-orbital puncture and 1.0 mL added to tuberculin syringe and allowed to flow through the fibers for 2 min with the effluent collected in a test tube. Percent neutrophil adherence was calculated based on cell counts performed before and after blood sample was passed through the syringes[16]. Cell count was determined using Leishman's stain at 100× magnification using oil immersion. The neutrophil adherence was thus determined as a percentage using the formula

$$\% \text{ Neutrophil Adherence} = \frac{Nu - Nt}{Nu} \times 100$$

Where, N_u is neutrophil count in untreated blood, N_t is neutrophil count of fibre treated blood.

Assessment of humoral immune function

Serum antibody titre

After studying cell-mediated immune response, mice were lightly anesthetized with ether on 7th day and blood was withdrawn from the retro-orbital plexus. The serum was separated by centrifuging the blood at 3000 g. Serial 2 fold dilution of serum was made in 50 µL of saline in 96-well microtitre plate and mixed with 50 µL of SRBC (0.025×10^9 cells) in saline. After mixing, plate was kept at room temperature for 2h. The value of antibody titre was assigned to the highest serum dilution showing visible haemagglutination[15,7].

Statistical Analysis

Wherever necessary statistical significance was assessed using student t-test and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The values are expressed as mean ± SD and $P < 0.05$ was considered significant.

Results

Effect of plant extract on organ weight and haematological parameters

None of the doses of extract showed toxicity or mortality in the extract-treated animals. No effect was

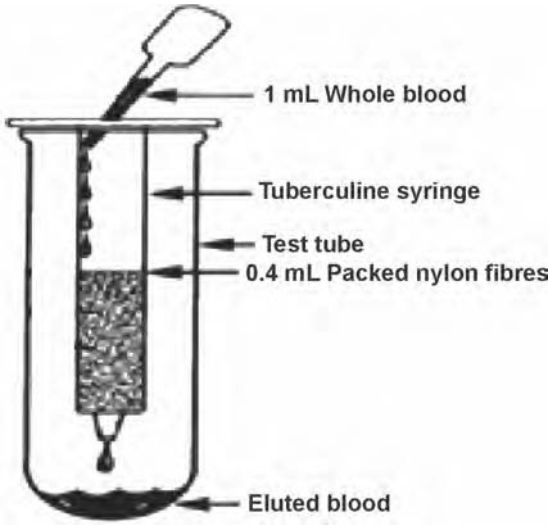

Figure 1— Schematic representation of packed nylon column

observed in the kidney weight at any dose when compared with control (normal saline-treated) animals (group I). Significantly stimulatory weight gains (P<0.05) were observed in thymus and spleen at the dose of 1000 mg/kg. But both of them were unaffected (P>0.05) at doses of 200 mg/kg and 500 mg/kg. As regard the relative organ weight of liver, a significant (P<0.05 and P<0.01) increase at doses of 500 and 1000 mg/kg was observed which did not reflect any abnormality (Fig. 2). Administration of ethanolic extract was found to increase the total WBC count (P<0.001) at a dose of 1000 mg/kg, but the same was unaffected at lower doses of 200 and 500 mg/kg (Fig. 1). There was no appreciable change in the differential count and Hb content at all three doses, whereas total RBC count showed a rise (P<0.01) at 1000 mg/kg dose.

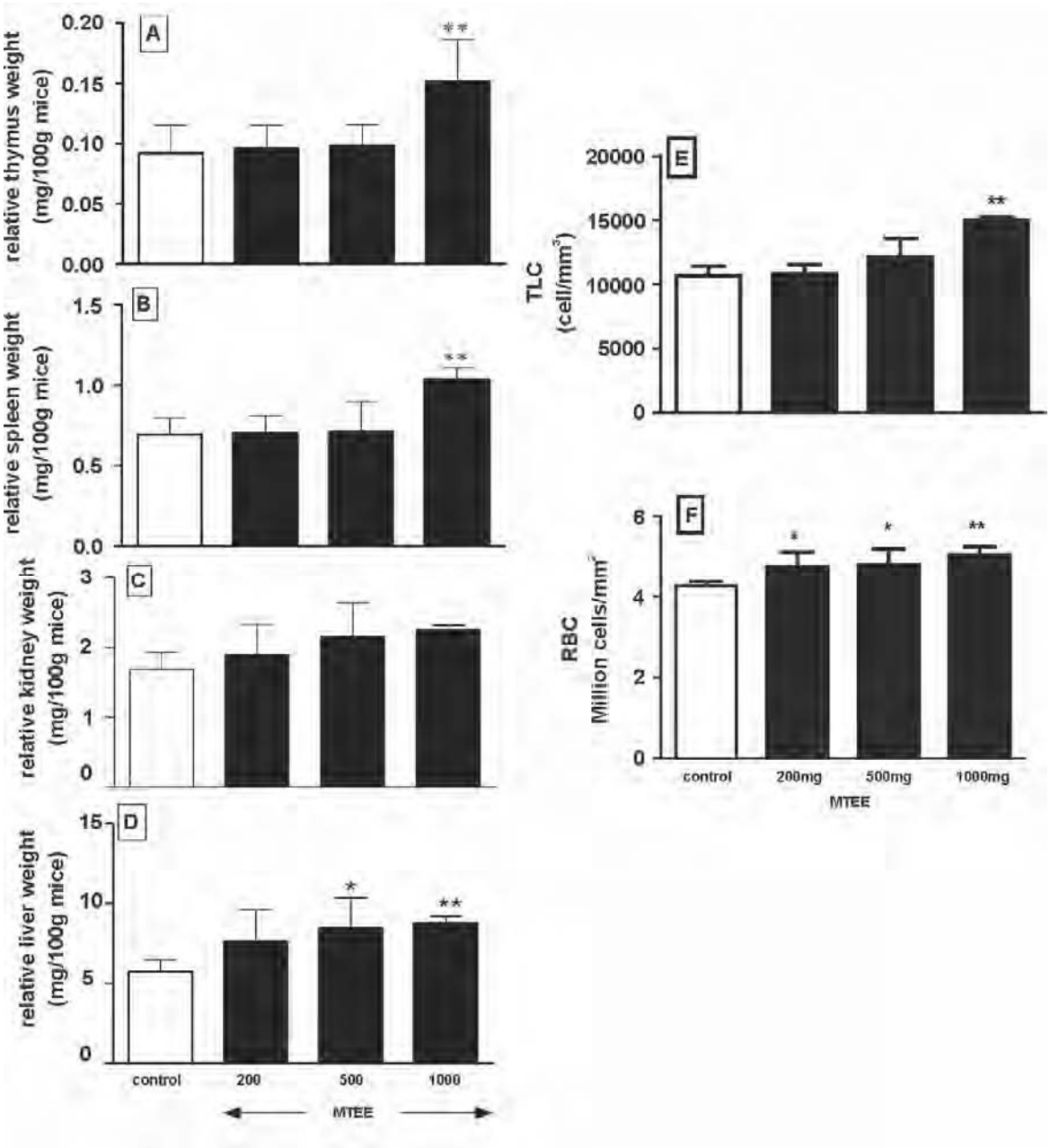

Figure 2— Effect of *Malvastrum tricuspidatum* whole plant ethanolic extract on relative organ weights & haematological parameters [(A) Relative thymus weight, (B) Relative spleen weight, (C) Relative kidney weight, (D) Relative liver weight, (E) TLC, (F) RBC]
Each bar represents mean±SD; one-way of analysis of variance, ANOVA followed by Dunnett's multiple Comparison Test (n=5), values are compared with control animals, *P<0.05; **P<0.01; MTEE = *Malvastrum tricuspidatum* whole plant ethanolic extract.

Table 1 — Effect of MTEE on DTH and neutrophil adhesion

Groups	DTH (mm)	Neutrophil Index		Per cent Neutrophil adherence
		UTB	FTB	
Control	0.54±0.25	19.20±1.30	12.40±1.14	35.22±3.55
MTEE (200mg/kg)	0.50±0.02	–	–	–
MTEE (500mg/kg)	1.25±0.36[b]	21.80±1.92	6.60±2.07	69.62±9.446[c]

Each value represents mean ±SD; DTH analysis: one-way ANOVA followed by Dunnett's Multiple Comparison Test (n=5), $^{*}P$<0.01 when compared with control. Neutrophil adhesion test: Student's t-Test (n=5), $^{\#}P$<0.01 when compared with control; MTEE = *Malvastrum tricuspidatum* whole plant ethanolic extract

Effect of plant extract on cellular immune function

Plant extract at dose 500 mg/kg elicited a significant (P<0.01) increase in DTH response as compared to control (normal saline-treated) animals (Group I) whereas a non significant (P>0.05) value was observed at 200 mg/kg dose (Table 1). Incubation of blood with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibers at a dose of 500 mg/kg. There was also rise in neutrophil count in untreated blood of all treated animals (Table 1).

Effect of plant extract on humoral immune function

In haemagglutination titre the dose of 500 mg/kg showed a mean titre value of 1:4506, while the mean titre value of control (normal saline-treated) animals (Group I) was 1:614.4, thus showing increase in the titre value.

Discussion

The species has primarily been described as an antihyperglycemic herb in laboratory animals[17]. In the present study it showed an overall stimulatory effect on the specific as well as non-specific immune functions in mice. Stimulatory effects were observed at 500 mg/kg. Though there was an increase in liver weight, it did not reflect any toxicity.

Plant extract at dose of 1000 mg/kg increased cell counts indicating its stimulatory effect on haematopoietic cells. In most of the parameters studied, 500 mg/kg dose was effective in inducing the immune functions. It appears that 500 mg/kg is the optimum dose in mice. Thus, 500 mg/kg dose of *M. tricuspidatum* seems to be pharmacologically effective dose in mouse as far as immunomodulatory effects are concerned. The response at lower dose i.e. 200 mg/kg, was either identical to control group animals or mildly stimulated as compared to control animals. The increase in thymus weight may be partially due to stimulatory effect of plant extract on the lymphocytes, which ultimately home in the thymus. However, this homing may be temporary and in due course of time normalcy may ensure. Further detailed investigations may throw light on this aspect.

Immune activation is an effective and protective approach for treating infectious diseases. Among the leukocytes, only antigen specific lymphocytes possess the diversity, specificity, memory and self-recognition indicating an adaptive immune response[18]. It was observer that MTEE caused significant increase in TLC and lymphocyte population indicating the presence of immunological effects of MTEE. Boundary of neutrophil from the blood stream requires a firm adhesion which is mediated through the interaction of the β_2 integrins present on the neutrophil. The β_2 integrins stored in the cell granules and up regulated for a firm adhesion[19,20]. In this study adherence of neutrophil to the nylon fibre was increased in treated group as compared to the control.

DTH is a part of the process of immunity to many intercellular infections, microorganisms, especially causing chronic diseases such as tuberculosis. DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilation, macrophage accumulationand activation, promoting increased phagocytic activity and increased concentration of lytic enzymes for more effective killing[2,18]. Delayed type hypersensitivity reaction is characterized by large influxes of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops when antigen activates sensitized T_{DTH} cells. These cells generally appear to be a TH1 subpopulation although sometimes TC cells are also involved. Activation of T_{DTH} cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including interleukin-2, interferon-, macrophage migration inhibition factor and tumor necrosis factor[21]. The overall effects of these cytokines are to recruits macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that DTH reaction is important in host defense against parasites and bacteria that can

live and proliferate intracellularly. Treatment of MTEE enhanced DTH reaction, which is reflected from the increased footpad thickness compared to control group suggesting heightened infiltration of macrophages to the inflammatory site. This study may be supporting a possible role of MTEE in assisting cell-mediated immune response. Increase in DTH reaction in mice in response to thymus-dependent antigen revealed the stimulatory effect of MTEE on T lymphocytes and accessory cell types for the expression of reaction[22,23].

The humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. To evaluate the effect of *M. tricuspidatum* on humoral response, its influence was tested on sheep erythrocyte specific haemagglutination antibody titre in mice. MTEE was found to significantly enhance the production of circulating antibody titre. This indicates the enhanced responsiveness of macrophages and T and B lymphocytes subsets involved in antibody synthesis[2].

The main chemical constituents of this species are: β-phenethylamine, dotriacontane, β-sitosterol, lutein, indole bases and steroids, palmitic acid, linoleic acid, sterulic acid, etc[24]. Linoleic acid, in particular is described as immunostimulating agent. Some of these constituents also possess antioxidant properties and they may induce the immunostimulant effects as several antioxidants have been reported to possesses immunomodulatory properties[25-28].

Conclusion

Findings of the present study establish that *M. tricuspidatum* has appreciable immunostimulatory activity. It is already reported for various medicinal activities such as anti-inflammatory and analgesic, wound healing agent, hypoglycemic and antibacterial agent. It is not possible at this juncture to single out the most effective immunomodulatory constituent of the plant. Administration of *Malvastrum* in humans is simple as it is readily available as wild species around. However, its reported immunomodulatory effects warrant further investigation for its use in the cases of clinical immunostimulation.

References

1 Mukherjee D, Khatua NT, Venkatesh P, Saha BP and Mukherjee PK, Immunomodulatory potential of *Nelumno nucifera* Gaertn., *J Ethnopharmacol*, 2010, **128**, 490-494.

2 Gokhle AB, Damre AS and Saraf MN, Investigations into immunomodulatory activity of *Argyreia speciosa*, *J Ethnopharmacol*, 2003, **84**, 109-114.

3 Makare N, Bodhankar S and Rangri V, Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice, *J Ethnopharmacol*, 2001, **78**, 133-137.

4 Tiwari U, Rastogi B, Singh P, Saraf D K and Vyas SP, Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals, *J Ethnopharmacol,* 2004, **92**, 113-119.

5 Kirtikar KR and Basu BD, Indian Medicinal Plants, 2nd ed., International Book Distributors, New Delhi, 1935, 305-306.

6 Shivprasad HN, Kharya MD, Rana AC and Mohan S, Preliminary immunomodulatory activities of aqueous extract of *Terminalia chebula, Pharm Biol,* 2006, **44**, 32-34.

7 Umathe SN, Dixit PV, Wanjari MM and Ullewar MP, Leuprolide –a GnRH agonist prevents restraint stress-induced immunosupression via sex steroid-independent peripheral mechanism in mice, *Int Immunopharmacol*, 2008, **8**, 71-79.

8 Ladics S G, Use of SRBC antibody responses for immunotoxicity testing methods, 2007, **41** (1), 9-19.

9 Trease GE and Evans MC, Textbook of Pharmacognosy, 12th ed., Balliere, Tindall, London, 1983, 343-383.

10 Ghosh MN, Fundamentals of experimental pharmacology, 3rd ed., Hilton and companies, Kolkata, 190-197.

11 Office of pollution prevention and toxics (OPPT) guidelines.

12 Davis L and Kuttan G, Immunomodulatory activity of *Withania somnifera*, *J Ethnopharmacol*, 2000, **71** (1-2), 193-200.

13 Sunila ES and Kuttan G, Immunomodulatory and antitumour activity of *Piper longum* Linn. and piperine, *J Ethnopharmacol*, 2004, **90** (2-3), 339-346.

14 Lagrange PH, Mackaness GB and Miller TE, Influence of dose and route of antigen injection on the immunological induction of T-cells, *J Exp Med*, 1973, **139**, 528-542.

15 Bin-hafeez B, Haque S, Parvez S, Pandey S, Sayeed I and Raisuddin S, Immunomodulatory effects of fenugreek (*Trigonella foenum-graecum* L.) extracts in mice, *Int Immunopharmacol*, 2003, **3** (2), 257-265.

16 Pfister RR, Haddox JL and Synder TL, The effect of citrate on the adherence of neutrophils to nylon fibers *in vitro*, *Investigtive Opthalmology & Visual Science*, 1988, **29** (6), 869-875.

17 Rattanajarasroj S, Bansiddhi J, Amporn KA and Chaorai B, Hypoglycemic activity of daikad (*Malvastrum coromandelianum* (L.) Garcke) in rats, *Bull Dep Med Sci*, 2005, **47**, 180-192.

18 Kindt TJ, Goldsby RA, Osborne BA, and Kuby, Immunology, W.H. Freeman and Company, New York, 35-57.

19 Smith WC, Marlin DS, Rothlein R, Toman C and Anderson CD, Cooperative interactions of LFA-1 and Mac-1 intercellular adhesion molecule-1 in facilitating adherence and transendothilial migration of human neutrophils *in vitro*, *J Clin Invest*, 1989, **83** (6), 2008-2017.

20 Springer AT, Traffic signals on endothelium for lymphocytes reticulation and leukocyte emigration, *Annu Rev Physiol*, 1995, **57**, 827-872.

21 Askenase PW and Van Loveren, M, Delayed type hypersensitivity: activation of mast cells by antigen specific T-cell factors initiates cascade of cellular interactions, *Immunology Today*, 1983, **4**, 259-264.

22 Dean HJ, Padarathsingh ML and Jerrells RT, Application of immunocompetence assays for defining immunosupression, *Annals New York Academy Sci*, 1979, **320**, 579-590.

23 Luster IL, Dean JH and Boorman GA, Cell mediated immunity and its application in toxicology, *Envoron Health Persp*, 1982, **43**, 31-36.

24 Prakash A and Verma RK, Chemical constituents from the aerial portions of *Malvastrum tricuspidatum* A. Gray, *Indian J Pharm Sci*, 1983, **45**, 102-103.

25 Fuente MDL and Victor MV, Anti-oxidants as modulators of immune function, *Immno Cell Bio*, 2000, **78**, 49-54.

26 Devasagayam TPA and Sainis KB, Immune system and antioxidants, especially those derived from Indian medicinal plants, *Indian J Exp Biol*, 2002, **40** (6), 639-655.

27 Khonsung P, Nantsupawat S, Jesadanont SN, Chantharateptawan V and Panthong A, Anti-inflammatory and analgesic activity of water extract of *Malvastrum coromandelianum* (L.) Garcke, *Thai J Pharmacol*, 2006, **28** (3), 9-15.

28 Sittiwet C, Jesadanont S, Pongpech P, Naenna, P and Pongsamart S, Antibacterial activity of *Malvastrum coromandelianum* Garcke against methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus*, *Curr Res Bacteriol,* 2008, **1** (1), 42-45.