Immunomodulatory Activity of *Abutilon Indicum* linn on Albino Mice

N. L. DASHPUTRE*, N. S. NAIKWADE

Department of Pharmacology, Appasaheb Birnale College of Pharmacy, South Shivaji Nagar, Sangli-416416, Maharashtra, India.

ABSTRACT

Purpose: To study the immunomodulatory activity of aqueous and ethanol extracts of leaves of *Abutilon indicum* Linn. (Family: Malvaceae) on albino mice.

Methods: The ethanolic and aqueous extract of leaves of *Abutilon indicum* was administered orally at the dosage levels of 200 mg/kg/day and 400 mg/kg/day body weight in mice. The assessment of immunomodulatory activity on specific and non-specific immunity were studied by heamagglutination antibody (HA) titer, delayed type hypersensitivity (DTH), neutrophil adhesion test and carbon clearance test. In order to induced immunosuppresion in mice by using cyclophosphamide (100 mg/kg/day, p.o.) and levamisole (50 mg/kg/day, p.o.) used as immunostimulating agents.

Results: Oral administration of *A. indicum* showed a significant increase in the production of circulating antibody titre in response to sheep red blood cells (SRBCs). A significant ($p<0.01$) increase in both primary and secondary HA titre was observed when compared to control group, whereas in cyclophosphamide treated group *A. indicum* showed significant ($p<0.01$) increase in HA titre. *A. indicum* showed significantly ($p<0.01$) potentiated the DTH reaction by facilitating the footpad thickness response to SRBCs in sensitized mice. Also *A. indicum* evoked a significant ($p<0.01$) increase in percentage neutrophil adhesion to nylon fibers and phagocytic activity.

Conclusion: The study demonstrates that *A. indicum* triggers both specific and non-specific responses to a greater extent. The study comprised the acute toxicity and preliminary phytochemical screening of *A. indicum*. From the results obtained and phytochemical studies the immunostimulant effect of AI could be attributed to the flavonoid content.

Keywords: *Abutilon indicum*, Immunomodulation, antibody titre, delayed type hypersensitivity, phagocytic index.

INTRODUCTION

Indian traditional systems of medicines like Siddha and Ayurveda have suggested to increase the body's natural resistance to disease.[1] Recent screening with plants has revealed many compounds (e.g. alkaloids, flavonoids, quinones, terpenoids) with pronounced antioxidant, antineoplastic, antiulcer, anti-inflammatory and immunostimulating potential.[2]

Abutilon indicum (AI) commonly known as "Atibala" in Sanskrit gives excessive tonic strength. [3] Atibala is a stronger diuretic and heart tonic.[4] AI reported in the Siddha system as a remedy for jaundice, piles, ulcer, leprosy, rakttapitta dosha and blood purifier.[5-6] Chemically it contains flavonoids (quercetin), saponins, alkaloids and phenolic compounds.[7] Hence, the present study evaluating immunomodulatory activity of AI.

MATERIALS AND METHODS

Plant material

The fresh whole plant of AI used in this study, collected at the flowering stage (month : September) from local area of Sangli, Maharashtra state, India and authenticated by Dr. U. S. Yadav of the Department of Botany, Willingdon College, Sangli, where a voucher specimen has been preserved for future identification.

Extraction

The leaves were separated from the fresh stems and dried on filter paper sheets under shade at room temperature until with changing of color of filter papers. The shade-dried, coarsely powdered leaves (500g) were successively extracted with petroleum ether (60-80^o) for 8 hr. to remove fatty matter. The defatted marc was then subjected to soxhlet extraction with 95% ethanol to obtain ethanolic extract. The dried marc was cold macerated with distilled water and chloroform (9:1) for 7 days to obtain aqueous extract. The both ethanolic and aqueous extracts were evaporated under reduced pressure at low temperature (30^0C) to dryness to yield yellowish brown color extracts of AI, stored in an airtight container in refrigerator for further experimental studies.

Preliminary phytochemical screening

Aqueous and ethanolic extracts of AI were subjected to preliminary phytochemical screening using the methods described by Kokate, Trease and Evans for the detection of various plants constituent. [8-9]

Animals

Inbred colony of swiss albino mice of weighing between 20-25 gm were housed in groups of 5 to 6. All mice were fed with pelleted diet (Pranav Agro Industries Ltd, Sangli, India) and tap water *ad libitum*. Institutional Animals Ethics Committee (IAEC) approved the experimental protocol and care of animals was taken as per guidelines of CPCSEA, Department of Animal Welfare, and Government of India. (843/ac/04/ CPCSEA).

Drugs and chemicals

All the drugs and chemicals were of analytical grade while the other drugs were procured from Levamisole (Khandelwal Pharmaceutical Ltd. Mumbai), Cyclophosphamide (Biochem pharmaceutical, Mumbai), Colloidal carbon (Indian ink, camel India Pvt. Ltd.).

Test compound formulations

The dilution of aqueous extract of leaves of AI (AEAI) was prepared in distilled water and the aqueous suspension of ethanolic extract of leaves of AI (EEAI) was prepared in 0.5 % carboxymethylcellulose (CMC) solution in distilled water prior to oral administration to animals. It was used within 7 days and stored at 8^0C while for further use, freshly prepared solution was used. The vehicle alone served as control.

Acute toxicity studies

Acute toxicity studies were performed according to organization for economic cooperation and development (OECD) guidelines, received draft guidelines 425, received from CPCSEA, Ministry of social justice and empowerment, Government of India.[10] Mice weighing between 20-25 gm in groups of five were used (n=5). The animals were fasted for 4 hr. with free access to water only. The both EEAI and AEAI extracts was administered orally in doses of 2000 and 5000 mg/kg to different groups of mice and observed over 14 days for mortality and physical/behavioral changes. The experiments were performed after the experimental protocols had been approved by the Institutional Animal Ethical committee.

Thin layer chromatography

TLC studies were carried out on the EEAI for flavonoids, using silica gel G TLC plates as the stationary phase and Chloroform : benzene : Ethanol : acetic acid : water (11 : 4 : 2 : 1 : 2) as the mobile phase. The TLC plates were spotted using the glass capillary and developed by spraying with ninhydrin reagent resulted in the formation of bright yellow spot indicating the presence of flavonoids in this extract. [11]

Experimental procedure

Antigenic material: Preparation of Sheep RBCs (SRBCs)

Sheep blood was collected in sterile Alsevere's solution in 1:1 proportion of Alsevere's solution (freshly prepared). Blood was kept in the refrigerator and processed, for the preparation of SRBCs batch, by centrifugating at 2000 rpm for 10 minutes and washing with physiological saline 4-5 times and then suspending into buffered saline for further use. [12]

Carbon ink suspension: Pelican AG, Germany, ink was diluted eight times with saline and used for carbon clearance test in a dose of 10µl/gm body weight of mice.[13]

Haemagglutination antibody (HA) titer [14]

The mice were divided into eight groups consisting of five animals each. Mice in group I received vehicle only for 21 days. Group II received cyclophosphamide (Negative Control) 100 mg/kg, p.o. on 9^{th} and 16^{th} day as a single dose. Mice in treatment group III and IV were given AEAI (400 mg/kg/day/p.o) and EEAI (200 mg/kg/day/p.o.) daily for 21 days respectively. Immunosuppresed animals in group V and VI were given AEAI (400 mg/kg/day/p.o) and EEAI (200 mg/kg/day/p.o.) plus cyclophosphamide (100 mg/kg/p.o.) on 9^{th} and 16^{th} day as a single dose respectively. Group VII received standard drug levamisole (50 mg/kg/p.o.) as an established immunostimulant agent for 21 days and group VIII received levamisole (50 mg/kg/p.o.) for 21 days plus cyclophosphamide (100 mg/kg/p.o.) on 9^{th} and 16^{th} day as a single dose.

On 7^{th} and 14^{th} day of the study, mice from all the groups (i.e. group I to VIII) were immunized and challenged respectively, with SRBCs in normal saline (0.1ml of 20% SRBCs) intraperitoneally. Blood was withdrawn on 14^{th} and 21^{st} day from retro-orbital plexus under mild ether anaesthesia from all antigenically sensitised and challenged mice respectively. Blood was centrifuged to obtain serum, normal saline was used as a diluent and SRBCs count was adjusted to (0.1% of SRBCs). Each well of a microtitre plate was filled initially with 20 µl of saline and 20 µl of serum was mixed in the first well of micro titre plate. Subsequently the 20 µl diluted serum was removed from first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were similarly carried out till the last well of the second row (24^{th} well), so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 µl SRBC (0.1% of SRBCs) were added to each of these dilutions and the plates were incubated at 37°C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titre. The antibody titres were expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance. Antibody titre obtained on 14^{th} day after immunization (on 7^{th} day) and on 21^{st} day after challenge (on 14^{th} day) with SRBCs was considered as primary and secondary humoral immune response respectively.

Delayed type hypersensitivity (DTH) response [15-16]

The drug treatment was exactly the same as described above for HA titer. On 14th day of the study, all the groups I to VIII were immunized with SRBCs (0.1ml of 20% SRBC i.p.) in normal saline. On day 21st all animals from all the groups were challenged with 0.03 ml of 20% SRBCs in subplantar region of right hind paw. Foot pad oedema in mice was used for detection of cellular immune response. On 21st day, injection of 0.1ml of 20% SRBCs in the subplantar region of right hind paw in the volume of 0.03 ml and normal saline in left hind paw in same volume. Foot pad reaction was assessed after 24 hr. on 22nd day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to oedema, the thickness of the right hind footpad was measured using vernier calliper. The footpad reaction was expressed as the difference in the thickness (m.m.) between the right foot pad injected with SRBC and the left footpad injected with normal saline.

Neutrophil adhesion test[17]

Mice were divided into four groups of five animals each. The control group I received vehicle, while animals of treatment group II and III were given AEAI (400 mg/kg/day/p.o.) and EEAI (200 mg/kg/day/p.o.) daily for 14 days respectively. Group IV received levamisole (50 mg/kg/p.o.) for 14 days. On the 14th day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anaesthesia. Blood was collected in vials pre-treated by disodium EDTA and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at 37^0C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percent neutrophil adhesion was calculated as follows,

$$\text{Neutrophil adhesion} = \frac{NI_U - NI_T \times 100}{NI_U}$$

Where,

NI_U : Neutrophil Index before incubation with nylon fiber.

NI_T : Neutrophil Index after incubation with nylon fiber.

Carbon clearance test[18]

The drug treatment was exactly the same as with the neutrophil adhesion test as described above. On 14th day, 3 hours after the last dose all the animals of each group were given colloidal carbon intravenously in a volume of 1 ml/100 g. Blood samples were then withdrawn (25 µl) from retro-orbital plexus at 0 and 15 minutes after injection of colloidal carbon ink and lysed in sodium carbonate solution (3 ml). The optical density was measured spectrophotometrically at 650 nm. The phagocytic index (K) was calculated using the formula:

$$K = \frac{(\text{In } OD_1 - \text{In } OD_2)}{(t_2 - t_1)}$$

Where, OD_1 and OD_2 are the optical densities at time t_1 and t_2 respectively.

Statistical Analysis

All the results were expressed as Mean±Standard Error (SEM). Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey-Kramer multiple comparison test. p-values <0.01 were considered as statistically significant.

RESULTS

Acute oral toxicity study

Acute oral toxicity was carried out by up-down regulation method. It is found that AEAI and EEAI were safe at limit dose 4000 mg/kg and 2000 mg/kg, with no mortality in studied subjects respectively. 1/10th of this dose *i.e.* 400 mg/kg and 200 mg/kg for AEAI and EEAI was used in the subsequent study respectively.

TLC for flavonoids

The naked eyes observed spot in bright yellow colored and in UV chamber spot observed in fluorescent yellow colored. Rf (× 100) value for the EEAI was found to be as 77 and 65 which is the near the Rf value for flavanol and flavones.

Preliminary phytochemical screening

The presence of various phytoconstituents of the extracts was detected by phytochemical screening. The AEAI found to contain carbohydrates, proteins, amino acids and flavonoids. The EEAI were found to contain carbohydrates, proteins, amino acids, saponin glycosides, flavonoids, alkaloids, tannins and phenolic compounds.

Haemagglutination antibody [HA] titre

Effect of AEAI and EEAI on primary and secondary antibody response on HA titre is shown in [Table 1]. Primary antibody response on day 14th in AEAI (400 mg/kg/p.o.) treated group with normal immune status showed significant increase (P<0.01) in HA titer titre when compared with the control group. A significant decrease (P<0.01) in the antibody titre was observed in the cyclophosphamide-treated group when compared

with the control group. In immunosuppressed groups, where the immunity was suppressed by administration of cyclophosphamide on day nine, EEAI (200 mg/kg/p.o.) administration produced a significant (P<0.01) rise in the antibody titre when compared with the cyclophosphamide-treated group. Secondary antibody titre on twenty-first day in AEAI and EEAI-treated groups with normal immune status group showed a significant rise (P<0.01) in the antibody titre when compared with the control group. In the immunosuppresed groups where the immunity was suppressed by administration of cyclophosphamide on day sixteenth AEAI and EEAI showed a significant rise (P<0.01) in HA titer when compared with the cyclophosphamide group.

Table 1. Effect of *A.indicum* on primary and secondary antibody titre.

Treatment	Primary antibody titre	Secondary antibody titre
Vehicle Control	4.00 ± 0.577	5.66 ± 0.88
Cyp-treated (Negative control)	3.00 ± 0.577** a	4.66 ± 0.33** a
AEAI	7.00 ± 0.57** a	8.00 ± 0.57** a
EEAI	6.00 ± 0.57	8.66 ± 0.33** a
LMS	8.00 ± 0.57** a	8.66 ± 0.88**a
AEAI + Cyp.	5.33 ± 0.88	7.33 ± 0.33
EEAI + Cyp.	6.33 ± 0.33** b	7.66 ± 0.33** b
LMS + Cyp.	7.33 ± 0.57** b	8.33 ± 0.33** b

The values are expressed as (Mean ± S.E.M.), n= 5, ** p< 0.01.
a: Cyp :Cyclophosphamide, only normal immune status groups *i.e*, AEAI, EEAI and LMS-treated groups were compared with vehicle control (Group I). b: Immunosuppressed test extract of AEAI, EEAI, LMS + Cyp. treated groups were compared with negative control (GroupII).

Delayed Type Hypersensitivity

Effect of AEAI and EEAI on cell mediated immune response by DTH induced footpad oedema is shown in [Table 2]. On twenty-first day cyclophosphamide-treated group showed significant (p<0.01) decrease in the mean difference of paw thickness when compared to control group. In the all groups of mice with normal immune status, of AEAI (400 mg/kg/p.o.) and EEAI (200 mg/kg/p.o.) showed significant (p<0.01) potentiated DTH response in terms of increase in the mean difference of paw thickness when compared with control group. In the all groups of mice treated with cyclophosphamide i.e. an immunosuppressed groups are, AEAI and EEAI showed significant (p<0.01) potentiated DTH response in terms of increase in the mean difference of paw thickness when compared with cyclophosphamide (negative control) group. Heightened delayed type hypersensitivity reaction suggests activation of cellular immune system.

Table 2. Effect of *A.indicum* treatment on cell mediated immune response by delayed type hypersensitivity induced footpad oedema.

Treatment	Mean diff.of paw oedema in (mm)
Vehicle Control	0.293 ± 0.088
Cyp-treated (Negative control)	0.445 ± 0.0035**a
AEAI	0.914 ± 0.0047**a
EEAI	0.992 ± 0.003** a
LMS	0.952 ± 0.003**a
AEAI + Cyp.	0.527 ± 0.035**b
EEAI + Cyp.	0.863 ± 0.007**b
LMS + Cyp.	0.956 ± 0.004**b

The values are expressed as (Mean ± S.E.M.), n= 5, ** p< 0.01.
a: Cyp :Cyclophosphamide, only normal immune status groups *i.e*, AEAI, EEAI and LMS-treated groups were compared with vehicle control (Group I). b: Immunosuppressed test extract of AEAI, EEAI, LMS + Cyp. treated groups were compared with negative control (GroupII).

Carbon Clearance Test

Effect of AEAI and EEAI on the phagocytic activity by the carbon clearance test is shown in [Table 3]. The phagocytic activity of the reticulo-endothelial system is generally measured by the rate of removal of carbon particles from the blood stream. In carbon clearance test, AI treated all groups, exhibited significantly high phagocytic index. The phagocytic index of AEAI (400 mg/kg/p.o.) and EEAI (200 mg/kg/p.o.) showed significant ($p<0.01$) increased in phagocytic index when compared to control group. This indicates stimulation of the reticuloendothelial system.

Table 3. Effect of *A.indicum* on Phagocytic activity by carbon clearance test.

Treatment	Phagocytic Index
Vehicle Control	0.029 ± 0.0004
AEAI	0.017 ± 0.0002**
EEAI	0.076 ± 0.0009**
LMS	0.083 ± 0.001**

Values are expressed as (Mean ± S.E.M.), n= 5, **$p<0.01$. All groups were compared with control group.

Neutrophil adhesion test

Effect of AIAE and AIEE on neutrophil activation by the neutrophil adhesion test is shown in [Table 4]. Cytokines are secreted by activated immune cells for margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. The percentage neutrophil adhesion was significantly ($p<0.01$) increased by AEAI (400 mg/kg/p.o.) and EEAI (200 mg/kg/p.o.) when compared with the control group, showed possible immunostimulant effect. AI significantly evoked increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels.

Table 4. Effect of *A.indicum* treatment on neutrophil activation by neutrophil adhesion test.

Treatment	% Neutrophil Adhesion
Vehicle Control	29.25± 0.57
AEAI	42.08±0.61**
EEAI	44.87± 1.42**
LMS	73.24±0.06**

Values are expressed as (Mean ± S.E.M.), n= 5, **$p<0.01$. All groups were compared with control group. Statistically analysed by one- way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

DISCUSSION

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of specific and non specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immuno-suppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factor. [19] The results obtained in the present study indicate that AI is a potent immunostimulant, stimulating specific and non-specific immune mechanisms.

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 effector 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 AI on humoral response, its influence was tested on sheep erythrocyte specific HA titre in mice. [20] Cyclophosphamide showed significant inhibition in antibody titre response, while AEAI and EEAI counteract the suppression of both primary and secondary humoral responses induced by cyclophosphamide. [21] This indicates the enhanced responsiveness of macrophages, 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). [22] 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 vasodilatation, macrophage accumulation, and activation, promoting increased phagocytic activity and increased

concentrations of lytic enzymes for more effective killing. When activated T_{H1} cells encounter certain antigens, viz. SRBCs. They secrete cytokines that induce a localised inflammatory reaction called delayed type hypersensitivity.[23] DTH comprises of two phases, an initial sensitisation phase after the primary contact with SRBC antigen. During this period T_{H1} cells are activated and clonally expanded by APC (antigen presenting cells) with class II MHC molecule (eg. langerhans cells and macrophages are APC involved in DTH response). A subsequent exposure to the SRBCs antigen induces the effector phase of the DTH response, where T_{H1} cells secrete a variety of cytokines that recruits and activates macrophages and other non specific inflammatory mediators. The delay in the onset of the response reflects the time required for the cytokines to induce the recruitment and activation of macrophages.[24] Therefore, increase in DTH reaction in mice in response to T cell dependent antigen revealed the stimulatory effect of AEAI and EEAI on T cells.

The role of phagocytosis is the removal of microorganisms and foreign bodies, dead or injured cells. The increase in the carbon clearance index reflects the enhancement of the phagocytic function of mononuclear macrophage and nonspecific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by the opsonisation of parasites with antibodies and complementing C3b, leading to a more rapid clearance of parasites from the blood. [25] AI appeared to enhance the phagocytic function by exhibiting a clearance rate of carbon by the cells of the reticulo-endothelium system.

Cytokines are secreted by activated immune cells for margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils. Significantly evoked increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels.[16] In the present study, AI evoked a significant increase in percent of neutrophils. This may help in increasing immunity of body against microbial infections.

Since AI was found to have a significant immunostimulatory activity on both the specific and non-specific immune mechanisms. The significant increase in the immunostimulatory activity of AI could be attributed to the presence of flavonoids (quercetin), alkaloids, tannins, saponin glycosides and phenolic compounds. Therefore, the plant holds promise for being used as an immunostimulating agent and an in-depth study on various fractions of the extract effective as immunomodulating entities from the plant is warranted to determine the most potent immunostimulating fraction from AI. Thus, the study validates the traditional use of AI as a 'Rasayana' in Ayurvedic system of medicine.

CONCLUSION

The results of the present study suggest that the aqueous and ethanolic extracts of *A. indicum* leaves may be beneficial in the treatment of impaired immunity. Further studies to identify the active moieties and elucidation of the mechanism of action are recommended.

ACKNOWLEDGEMENTS

The authors thank to Appasaheb Birnale College of Pharmacy, Sangli, Maharashtra for providing lab facilities.

REFERENCES

[1] H. Sagrawat, Y. Khan. Immunomodulatory Plants: A Phytopharmacological and Pharmacognosy Review, 2007; 1:2 : 248-60.
[2] H. Wagner. Search for plant derived natural products with immunostimulatory activity (recent advances). Pure and Applied Chemistry, 1990; 62:7: 1217-22.
[3] The Ayurvedic Pharmacopoeia of India Part- I Volume-I, p.12.
[4] K.R. Kirtikar, B.D. Basu. Indian Medicinal Plants. 2nd ed.vol.2, Allahabad, I: Lalit Mohan Basu, 1961. p.314-315.
[5] K.M. Nadkarni. Indian Materia Medica. In: Nadkarni KM editor. 1st ed. Mumbai: popular prakashan. 1976. p. 8-9.
[6] A.P. Deshpande, R.R. Jawlekar, S. Rande. Dravyaguna vidgnyan. Vol. I-II, Pune: Anmol prakashan; 2002. p. 367-8.
[7] K.N. Gaind , K.S. Chopra, Phytochemical investigation of *Abutilon indicum*, Planta Med., 1976; 30:2: 174-88.
[8] G.E. Trease, M.C. Evans. Textbook of Pharmacognosy, Balliere, Tindall, London, 12th edition, 1983; 343-383.
[9] C.K. Kokate, A.P. Purohit, S.B. Gokhale. Pharmacognosy. Immunomodulators, Adaptogens and Rasayana, Analytical pharmacognosy. Pune: Archana enterprises; 17th ed. 2001; 586- 8. ,114-7.
[10] OECD Guidelines for testing of chemical, revised draft guidelines 425:30, Acute Oral Toxicity-Up-and-Down Procedure: 2001.
[11] A.B. Gokhale, A.S. Damre, M.N. Saraf. Investigations into the immunomodulatory activity of *Argyreia Speciosa*. J Ethnopharmacology, 2003 ; 84 : 109-14.
[12] S. Dash, L.K. Nath, S. Bhise, P. Kar, S. Bhattacharya. Stimulation of immune function activity by the alcoholic root extract of *Heracleum nepalense* D. Don. Indian J Pharmacology, 2006; 38 :5: 336-40.
[13] A.A. Joharapurkar, M.M. Wanjari, S.P. Zambad, S.N. Umathe. Pyrogallol : A novel tool for screening immunomodulators. Indian J Pharmacology, 2004; 36: 355-9.
[14] R. Agarwal, S. Diwanay, P. Patki, B. Patwardhan. Studies on immunomodulatory activity of *Withania somnifera* (Ashwagandha) extracts in experimental immune inflammation. J Ethnopharmacology, 1999 ; 67 : 27–35.
[15] S.V. Fulzele, P.M. Satturwar, S.B. Joshi, A.K. Dorle. Study of the immunomodulatory activity of *Haridradi ghrita* in rats. Indian J Pharmacology, 2003; 35: 51-54.
[16] V. Gayathri, V.V. Asha, A. Subramoniam. Preliminary stuudies on the immuno-modulatory and antioxidant properties of *Selaginella species*. Indian J Pharmacol, 2005; 37:381-5.
[17] B.V. Ghule, G. Murugananthan, P.D. Nakhat, P.G. Yeole. Immunostimulant effects of *Capparis zeylanica* Linn. Leaves. J Ethnopharmacology, 2006; 108 : 311–15.
[18] J.B. Harborne. Phytochemical methods. A guide to modern technique of plant analysis. 3rd ed. Springer: 2005; 40-96, 170.

[19] N. Makare, S. Bodhankar, V. Rangari. Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. J Ethnopharmacology, 2001;78 : 133-7.
[20] B. Benacerraf. A hypothesis to relate the specificity of T lymphocytes and the activity of I region specific Ir genes in macrophages and B lymphocytes. J of Immunology, 1978; 120: 1809-1812.
[21] A.V. Kulkarni, B.D. Siraskar, S.S. Jadhav, S.M. Dhonde, A.S. Kulkarni, S.S. Bingi. Study of hydroalcoholic extract of *Portulaca oleracea* L. for immunomodulatory activity in mice, Indian J of green pharmacy, 2007; 4 ; 1:1: 45-9.
[22] L.E. Miller, H.R. Ludke, J.E. Peacock, R.H. Tomar, Manual of Laboratory Immunology. Lea and Febiger, London, 1991; 1-18.
[23] A.R. Bafna, S.H. Mishra, Immunomodulatory activity of methanol extract of flower-heads of *Sphaeranthus indicus* Linn. Ars Pharmaceutical, 2004; 45:3: 281-91.
[24] C.V. Rao, Immunology. Introduction, India: Rajkamal Electric press; 2006; 1-12.
[25] D.E, Pallabi, S.C. Dasgupta, A. Gomes. Immunopotentiating and immuno-prophylactic activities of Immue 21, a polyherbal product. Indian J Pharmacology, 1998; 30: 163-8.