INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

EVALUATION OF IMMUNOMODULATORY ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *SIDA SPINOSA* LINN.

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Article Received on: 12/03/19 Approved for publication: 22/04/19

DOI: 10.7897/2230-8407.1005186

ABSTRACT

Immunomodulatory effect of hydroalcoholic extract of *Sida spinosa* Linn was evaluated in swiss albino mice to justify the traditional claims. The assessment of immunomodulatory activity of specific immunity was carried out in immune suppressed mice by testing the humoral response to sheep RBCs in hemagglutination antibody (HA) titer and determination of total leukocyte count whereas effect on nonspecific immunity was studied by phagocytic activity of carbon particle in carbon clearance test. Immunosuppression was induced in mice by using cyclophosphamide (100 mg/kg/day, p.o.) while Levamisole (50 mg/kg/day, p.o.) was used as reference standard immune stimulating agent. The extract was administered orally at three dose levels i.e. 100, 200 and 400 mg/kg/day for the period of 21 days. The extract showed a significant ($p < 0.01$ and $p < 0.001$) increase in both primary and secondary HA titer at the dose of 200 and 400 mg/kg respectively when compared to cyclophosphamide induction control. Extract restored the cyclophosphamide induced myelosuppression in mice and showed significant ($p < 0.01$ and $p < 0.001$) increase in total leukocyte count at dose of 200 and 400 mg/kg respectively when compared to cyclophosphamide induction control. The extract also showed significant ($p < 0.01$) increase in phagocytic index at 200 and 400 mg/kg. In conclusion, present study validated traditional claims of the hydroalcoholic extract of *Sida spinosa* L. as an immunomodulatory agent.

Keywords: *Sida spinosa* L., Immunomodulation, Hemagglutination antibody titer, Phagocytic index.

INTRODUCTION

Immune system plays an important role in biological adaptation contributing to the overall maintenance of homeostasis and thereby establishment of body's integrity[1]. As per the report, there is continuous increase in stress and strain especially associated with modern life style leading to immune system related complaints[2]. The prominent complaints include rheumatoid arthritis, type-1 diabetes, psoriasis, inflammatory bowel disease, multiple sclerosis etc.[3] affecting 5 to 10 % of global population of variable age groups[4,5]. In modern medicines numerous drugs like levamisole, cyclophosphamide, azathioprine, glucocorticoid etc. are in use as immunomodulators[6,7] however, development of resistance on persistent use, associated serious side effects like nephrotoxicity, hepatotoxicity, bone marrow depression, severe hypertension, persistent myalgia etc. increases the risk of therapy by many folds as compare to benefits which adversely affects the overall outcome[8,9].

This also suggest that there is need to develop more safe, effective and patient friendly drug/s may be from alternative system of medicine that can be termed as ideal immunomodulator[10]. Discovery of clinically useful effective and patient friendly drugs like Atropine from *Atropa belladonna,* Quinine from the bark of the Cinchona tree*,* Digoxin from *Digitalis purpurea*, Vinblastine, Vincristine from *Catharanthus roseus,* Capsaicin from Capsicum species, Paclitaxel from *Taxus brevifolia* and Galantamine from *Galanthus caucasicus,* carbenoxolone from *Glycyrrhiza glabra,* gefarnate from Cabbage etc. gives indication towards exploitation of traditional claim in scientific way[11,12]. These reports created strong background to explore traditional claims which are still not well documented scientifically.

Sida spinosa Linn. (Malvaceae), traditionally claimed as rasayana plant[13] has been scientifically validated for various activities like antibacterial[14], antioxidant[15], hypoglycemic[16], anti hyperglycemic and anti hyperlipidemic[17], wound healing[18], anti ulcer[19] suggesting authenticity of its traditional claims. There is paucity of data available to label it as plant with immunomodulatory potential. On this background, present study was aimed to evaluate Immunomodulatory activity of hydroalcoholic extract of *Sida spinosa*".

MATERIAL AND METHODS

Drugs and Chemicals

All the chemicals used were analytical grade. Cyclophosphamide (Cadila Healthcare Ltd), Levamisole (Johson and Johson Pvt. Ltd.).
Carbon ink suspension: Colloidal carbon ink (Indian ink, Camel India Pvt. Ltd.), diluted eight times with normal saline and used for carbon clearance test at dose of 10 µl/g body weight of mice.

Antigenic material: Preparation of Sheep RBCs

Fresh Sheep blood was collected from local slaughter house in freshly prepared sterile Alsevere's solution in 1:1 proportion. SRBCs were centrifuged at 3000 rpm for 5 min. The sediment SRBCs were washed with physiological saline and centrifuged at

3000 rpm for 5 min. The procedure was repeated for 4-5 times. Sheep RBC batch was prepared by suspending SRBCs into buffered saline and concentration adjusted to 20% for immunization and 1 % for challenge. It was kept in refrigerator at 4°C for further use[20]

Plant Material

The leaves and stem part of the *Sida spinosa* Linn were procured from regions of Tirupati, Andhra Pradesh and authenticated by botanist, Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati, A. P., India (Voucher No.928).

Preparation of Extracts

The plant material carefully dried in shade for 15 days. After drying plant material subjected to size reduction. 500 g of powder was macerated using solvent hydroalcohlic mixture (70:30) in 1:8 ratio for 72 h at room temperature. The residue was removed by filtration; the solvent was then evaporated under reduced pressure in rotary evaporator at 42-45°C. The concentrated extract was transferred to petri dishes and dried in a vacuum oven at 40°C. The solid extract was scraped before complete drying and then dried to a constant weight. The percentage yield was 20 % and the extract was kept in airtight container until used.

Preliminary phytochemical analysis of extract

Hydroalcoholic extract (HYSS) further subjected to preliminary phytochemical analysis using standard procedure to record the presence of different classes of phytoconstituents[21].

Animals

Swiss albino mice weighing 18 to 25 g of either sex was procured from Lacsmi Biofarm Pvt. Ltd., Pune and housed in group of six. All mice were fed with standard pellet diet (Nutrivet Life Sciences, Pune) and water *ad libitum*.

Mice were maintained at 22 ± 1ºC with 60 % relative humidity and kept under 12 h light: dark cycle. The animals were allowed to acclimatize to laboratory conditions prior to experimentation. All experiments were conducted during the light period of 12 hours of the day/night cycle

Institutional animal Ethics Committee (IAEC) approved the protocol (DYPCOP/IAEC/2014/02-04) and care of animals was taken as per guidelines of CPCSEA.

Acute oral toxicity study of extracts

The acute toxicity study for hydroalcoholic extract (HYSS) was performed using OECD guideline No.423[22]. At the limit dose of 2000 mg/kg no mortality or behavioral changes recorded during the 14 days observation period. The dose of 100 mg/kg, 200 mg/kg and 400 mg/kg was used in subsequent study.

Hemagglutination Antibody titer test

36 mice were divided into six groups, each consisting six mice. All the groups were subjected to respective treatment as described below[23]

Group I: Vehicle control: Received distilled water 1 ml/100gm, p.o. from 1st to 21st day.

Group II: Cyclophosphamide (CYP)induction Control: Received Cyclophosphamide 100 mg/kg as a single oral dose on 9th and 16th day.

Group III: Levamisole + CYP: Received Levamisole50 mg/kg, p.o. from 1st to 21st day and Cyclophosphamide 100 mg/kg p.o., on 9th and 16th day

Group IV: HYSS-I + CYP: Received HYSS 100 mg/kg, p.o., from 1st to 21stday and Cyclophosphamide 100 mg/kg p.o., on 9th and 16th day

Group V: HYSS-II + CYP: Received HYSS 200 mg/kg, p.o., from 1st to 21st day and Cyclophosphamide 100 mg/kg p.o., on 9th and 16th day

Group VI: HYSS-III + CYP: Received HYSS 400 mg/kg, p.o., from 1st to 21st day and Cyclophosphamide 100 mg/kg p.o., on 9th and 16th day

Immunization was carried out on 7th and 14th day, mice from group I to VI were immunized and challenged with 0.1 ml of 20 % sheep RBC's (SRBCs) intraperitoneally.

Blood sample was collected on 14th and 21st day by retro-orbital plexus from all mice. Blood was centrifuged to obtain serum. Microtiter plates 96 wells, 'U' bottom,were used for estimation of antibody titer. Each serum sample was diluted upto two rows of wells of titer plate. Each well of a microtiter plate was filled initially with 20 µl of saline. 20 µl of serum was mixed with 20 µl of saline in the first well of microtiter 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 (24th well), so that the antibody concentration of any of the dilutions is half of the previous dilution. Normal saline was used as a diluent to prepare 1 % SRBC. 20 µl of 1 % SRBC were added to each of these dilutions and the plates were incubated at 37°C for one hour and then observed for hemagglutination. The reciprocal of highest dilution giving hemagglutination was taken as the antibody titer and mean of different groups were compared for statistical significance. Antibody titer determined on 14th and on 21st was considered as primary and secondary humoral immune response respectively.

Leucocytes from blood samples collected on day 21st were analysed using neubar's chamber (haemocytometer) to calculate the total leucocyte count

Carbon clearance test

24 mice were divided into 4 groups, each consisting six mice. The control group I received vehicle (ditilled water 1 ml/100 gm, p.o.), while mice from treatment group II to IV were administered HYSS 100, 200 and 400 mg/kg respectively for 7 days. Carbon ink suspension was injected via tail vein (10 µl/gm body weight of mouse) to each mouse after 48 h of 7 days treatment. Immediately after administraton of carbon ink suspension i.e. at 0 min and then after 15 min., 25 µl of blood samples were withdrawn from each mouse through retro orbital plexus, followed by the lysis of blood samples in 3 ml of sodium carbonate solution (0.1 %). The optical density was recorded using UV visible spectrophotometer at 660 nm (make-Labindia UV-3000).[24] The phagocytic index was calculated from the following equation

$$K = \frac{(\text{In OD1} - \text{In OD2})}{(t2 - t1)}$$

Where, K: Phagocytic index, OD_1: optical densities at time t_1, OD_2: optical densities at time t_2

Statistical analysis

All the results were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test and Dunnett's 't' test in carbon clearance test.

RESULTS

Preliminary phytochemical analysis of extract

Hydroalcoholic extract (HYSS) showed prominent presence of steroids, glycosides, flavonoids, alkaloids, tannins and phenolic compounds.

Hemagglutination Antibody titer test

Primary antibody titer response measured on day 14th and secondary antibody titer was measured on day 21st. The results are shown in Table 1.

In cyclophosphamide induction control group II, significant ($p < 0.01$) decrease in HA titer was recorded in primary and secondary antibody titer when compared to vehicle control group I. In the immunosuppressed group-III, reference standard Levamisole 50 mg/kg treatment, showed significant ($p < 0.001$) increase in primary and secondary HA titer when compared to group-II. In the immunosuppressed group V and VI, HYSS treatment showed dose dependent increase in HA titer. Significant ($p < 0.01$) increase in primary HA titer at 200 mg/kg and ($p < 0.001$) at 400 mg/kg in primary and secondary HA titer were seen when compared to induction control group II.

Total leukocyte count (Table 1) was significantly ($p < 0.001$) reduced in cyclophosphamide induction control group as compared to vehicle control group I. Cyclophosphamide induce myelosuppression was significantly ($p < 0.001$) restored with Levamisole 50 mg/kg whereas HYSS treatment showed dose dependent increase in total leukocyte count ($p < 0.01$) at 200 mg/kg and ($p < 0.001$) at 400 mg/kg.

Carbon clearance test

The phagocytic activity of reticulo-endothelial is generally measured by the rate of removal of carbon particles from blood stream. To assess the functional changes in macrophages of reticulo-endothelial system (RES), their phagocytic ability was determined. The phagocytic activity was observed as the phagocytic index of extract treated groups to the control. HYSS showed significant increase in phagocytic index ($p < 0.01$) at dose of 200 mg/kg and 400 mg/kg respectively when compared to control (Table 2).

DISCUSSION

The immune system has many interdependent components having specialized function and can be chiefly categorized into innate immune system (non specific immune response) and adaptive immune system (specific immune response) that collectively protect body from pathogens, tumors cells or infected cells etc. Alteration in immune system by interfering with its function is called as Immunomodulation. This Modulation may be specific limited to a given antigen/agent or nonspecific, with a general effect on immune response[25].

When mice were sensitized with SRBC, an antigen gets diffused in the extra vascular space and enters the lymph node via the lymphatics. Particulate antigens are taken up by macrophages lining the sinuses or disperse in the lymphoid tissues and processed. Small highly antigenic peptides are combined with MHC class II molecule. B cells with receptors for antigen binds and internalizes it into an endosomal compartment and process and presents it on MHC class II molecules to Th_2 cells.

These B lymphocytes proliferate into memory cells and antibody secreting plasma cells. Initially IgM antibodies are secreted as primary response followed by secretion of IgG antibodies. The secondary antibody response enhanced for same antigen[26,27].

In the induction control group cyclophosphamide was used as immunosuppressant, as it selectively suppresses humoral immunity by exerting depressive effect on antibody production, if given after antigenic stimulation[28]. This may be due to interference with helper T cell activity[29].

Mice treated with Levamisole significantly increase primary and secondary HA titer response when compared to induction control group indicated the stimulatory activity of positive immunomodulatory agent.

In the Immunosuppressed groups HYSS treatment showed dose dependent increase in HA titer as compared to induction control group. At dose of 200 and 400 mg/kg, HYSS showed significant increase in primary and secondary HA titer response. The enhancement of antibody responsiveness to HYSS in mice indicated the enhanced responsiveness of macrophages and B-lymphocytes subsets involving antibody synthesis. Therefore, the augmentation of antibody production response of HYSS showed effectiveness on humoral stimulation response.

Leukocytes are the cells involved in immune response continuously proliferated in bone marrow. This high degree of cell proliferation affected by cytotoxic drugs, by loss of stem cells and inability of bone marrow to regenerate new blood cells will result in thrombocytopenia and leucopenia.

In present study, in the induction control group cyclophosphamide significantly decreases total leukocyte count compared to vehicle control group. The results showed that HYSS restored the cyclophosphamide induce myelosuppression by increasing total leukocyte count significantly at the dose of 200 mg/kg and 400 mg/kg. This indicates protective effect of HYSS against myelosuppressive effect of cyclophosphamide.

The reticuloendothelial system (RES) is composed of network of reticular (supporting or structural) cells of the spleen, thymus and other lymphoid tissues, lymph nodes, capillary endothelium of the liver (Kupffers cells), free macrophages, blood monocytes, different leucocytes and the tissue macrophages (e.g. Mesangial cells in kidney, Osteoclast in bones and cartilages etc.) The proper functioning of RES by phagocyting particulate and other foreign matter constitutes first line of defense of host immune function especially against systemic bacteremia. Various dyes, carbon particles, or thorotrast (thorium dioxide), injected intravenously into animals is removed by sessile intravascular phagocytes in the liver and spleen. The Kupffer cells of liver take up approximately 90 % and the splenic macrophages 10 %.

Table 1: Effect of HYSS on Hemagglutination Antibody Titer HA Titer and Total Leukocyte Count

Group No.	Nomenclature	Primary Antibody Titer (Mean± SEM)	Secondary Antibody Titer (Mean± SEM)	Total Leukocyte Count (10^3/mm^3) (Mean± SEM)
I	Vehicle Control	58.66 ± 5.33	74.66 ± 10.66	5.389 ± 0.31
II	CYP Induction Control	4.33 ± 0.80$^{\#\#}$	7.33 ± 1.90$^{\#\#}$	3.004 ± 0.11$^{\#\#\#}$
III	Levamisole + CYP	170.66 ± 26.98***	234.66 ± 21.33***	7.905 ± 0.39***
IV	HYSS-I + CYP	37.33 ± 5.33	58.66 ± 5.33	3.540 ± 0.18
V	HYSS-II + CYP	74.66 ± 10.66**	117.33 ± 10.66***	4.571 ± 0.26**
VI	HYSS-III + CYP	117.33 ± 10.66***	149.33 ± 21.33***	5.781 ± 0.29***

Values are expressed as (Mean± SEM), n=6, CYP- Cyclophosphamide, HYSS-I, II, III: Hydroalcoholic Extract of *Sida spinosa* Linn (100, 200, 400 mg/kg respectively), $^{\#}$ = p<0.05, $^{\#\#}$ = p<0.01 and $^{\#\#\#}$ = p<0.001 as compared to vehicle control group, *= p<0.05, **=p<0.01 and ***= p<0.001 as compared to CYP Induction Control group. Statistically analyzed by one way ANOVA followed by Bonferroni multiple comparison test

Table 2: Effect of HYSS on *In-Vivo* Phagocytosis determined by Carbon Clearance Test

Group No.	Nomenclature	Phagocytic Index Mean± SEM
I	Vehicle Control	0.025 ± 0.0026
II	HYSS-I	0.030 ± 0.0032
III	HYSS-II	0.044 ± 0.0031**
IV	HYSS-III	0.050 ± 0.0027 **

Values are expressed as Mean ± S.E.M., ** = p<0.01 and ***= p<0.001, HYSS-I, II, III: Hydroalcoholic Extract of *Sida spinosa* Linn (100, 200, 400 mg/kg respectively). Statistically analyzed by One way ANOVA followed by Dunnett's test

The clearance of carbon particle at faster rate by liver and spleen phagocytes as well as from blood indicates activation of reticuloendothelial phagocytic activity[30]. In carbon clearance test, extract treated groups reported significantly high phagocytic index. Thus extract documented for its potential for stimulation of RES. This suggests possible use of extract in patients with conditions like hepatitis, liver failure etc.

CONCLUSION

In conclusion, results obtained in the present study showed that hydroalcoholic extract of *Sida spinosa* L have a significant immunomodulatory activity on both specific and nonspecific immune mechanism. It could be attributed to presence of flavonoids, steroids, saponins, phenolic compounds, glycosides etc. acting together on biological system. Therefore extract holds a promise for being used as an immunomodulatory agent and further studies have to be conducted on various fractions to determine most potent immunomodulatory fraction. Thus the study validates the traditional claim of *Sida spinosa* as a 'rasayna' in Indian Ayurvedic system of medicine.

ACKNOWLEDGMENT

The author thanks to Dr. D.Y Patil Institute of Pharmaceutical Sciences and Research, Pimpri and Dr. D. Y. Patil College of Pharmacy, Akurdi for providing laboratory facilities.

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Cite this article as:

Ashish V. Kulkarni and Niraj S. Vyawahare. Evaluation of Immunomodulatory activity of hydroalcoholic extract of *Sida spinosa* Linn. Int. Res. J. Pharm. 2019; 10(5):176-180 http://dx.doi.org/10.7897/2230-8407.1005186

Source of support: Nil, Conflict of interest: None Declared