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Original Article

Evaluation of *Cassia angustifolia* Vahl as an Immunomodulatory Agent

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ABSTRACT

Modulation of immune functions using medicinal plants and their products has currently become an accepted therapeutic approach for treatment of many diseases. The present study deals with evaluation of immunomodulatory activity of *Cassia angustifolia* Vahl on cyclophosphamide-induced immunosuppressed male Swiss albino mice. The crude methanolic leaf extract of *C. angustifolia* was administered orally to mice at dose of 2, 5 and 10 mg/kg for 14 days. For positive control, animals were treated with levamisole. On Day 15, blood samples were collected from animals by cardiac puncture and the immune response was evaluated by various immunological parameters. The administration of extract resulted in leucocytosis in animals accompanied by significant increase in neutrophil counts. The increment in neutrophil phagocytic index and delayed type hypersensitivity response was observed after extract treatment. These immune responses were found to be highest at a dose of 5 mg/kg. The values for hemagglutination antibody titer also showed dose-dependent increase in animals treated with extract. The results therefore indicate that the crude methanolic leaf extract of *C. angustifolia* stimulates both cell-mediated and humoral immune response in immunocompromised animals.

Keywords: Immunostimulation, *Cassia angustifolia*, Cell-mediated and humoral immunity, Delayed type hypersensitivity, Hemagglutination antibody titer.

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INTRODUCTION

Infectious and non-infectious diseases remain a big global risk especially in developing countries due to their demographic and socio-economic restraints [1]. Global mortality due to non-infectious diseases is predicted to raise from 59% in 2002 to 69% in 2030 and HIV/AIDS deaths from 2.8 million in 2002 to 6.5 million in 2030 [2]. According to WHO, about 61% of deaths in India are imputed to non-infectious diseases and almost 23% of population is under the risk of premature death due to such diseases [3]. In most cases, the physiology of immune system of an individual is greatly affected by such diseases and there is need to stimulate it in circumstances of immunosuppression.

The use of immunomodulatory drugs in improving host's defence is now widely acceptable alternative for the management of disease [4]. Globally, disease conditions are usually managed by either conventional medicines or medicinal herbs. Although modern medicines are available, herbal medications are often favoured for their cultural and historical reasons [5]. Herbal medicines used for immunostimulation can serve as potential substitutes to conventional therapeutics for several diseases. Modulation of immune system by variety of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported in various in vivo models [6].

India is a country with rich biodiversity and enormous treasure of herbal plants and hence called as medicinal garden of the world [7]. Traditional Indian system of medicines like Ayurveda describes various medicinal plants and emphasize on strengthening the immune system of host [8]. *Cassia angustifolia* Vahl (Caesalpiniaceae) popularly known as Senna, is one of such common herb that grows in hot arid areas of India and Pakistan. It is a reputed drug in traditional medicine and frequently used in folk medicine as a purgative [9]. Most of pharmacopoeias of the world have also recorded this plant [10]. Due to its purgative properties it is valued as a medicine and is particularly useful in habitual constipation. It is employed in the treatment of amoebic dysentery as an antihelminthic and as a mild liver stimulant [11]. It is extensively used as febrifuge in splenic enlargements, typhoid, cholera, anemia [12], laxative, genotoxicity, and toxicity in *Escherichia coli* [13]. Leaves and pods of *C. angustifolia* are traditionally used as purgatives; the most important purgative constituents are Sennosides A, B, C, D, emodin, isorhamnetin and essential oil [14]. Leaves of *C. angustifolia* are also used as a safe laxative and this plant contributes considerably to commercial drugs and has been investigated in several parts of the world for various therapeutic preparations in different ways [15].

Due to enormous usage of *C. angustifolia* in curing different diseases and the work reported in the literature concerning systemic study of chemical composition encouraged to explore its ability to modulate immune function in animal model. Thus the aim of present research is to study immunomodulatory activity of crude methanolic extract of the leaves of *C. angustifolia* on Swiss albino mice.

MATERIALS AND METHODS

Plant Material Collection and Extraction: Fresh mature leaves of *Cassia angustifolia* were purchased from Isha Agro Developers Private Limited (ISO:22000-2005), Pune. The leaves were dried in shade at room temperature. The dried, coarsely powdered leaves were soaked in absolute methanol for 3 days with occasional shaking. The mixture was filtered and the fine filtrate was dried into a semi-solid extract in an oven set at 60 °C. The dry crude extract was stored at 4 °C until the immunomodulatory experimental bioassays were carried out. The percentage yield of the extract was 12.5%.

Experimental Design: Twenty adult male Swiss albino mice were purchased from National Institute of Biosciences, Pune. Mice were randomly assigned into 5 groups with four animals per group. Animals were treated daily as shown in Table 1. Mice in all groups were treated with an immunosuppressant cyclophosphamide in normal saline at dose of 200 mg/kg bwt on day 0 of the study by subcutaneous injection. Group I was the positive control and received 50 mg/kg of Levamisole in normal saline by oral gavage for 14 days. Group II was the negative control and received 2 ml of normal saline by oral gavage for 14 days. Group III, IV and V were the extract treatment groups and received crude extract at dose of 2, 5 and 10 mg/kg bwt respectively for 14 days by oral gavage. On day 15, blood was collected by cardiac puncture into ethylenediaminetetraacetic acid (EDTA)-containing vacutainers. It was then analysed for various immunological parameters. The study was approved by Institutional Animal Ethics Committee and animals were treated following International Standard Guidelines on Laboratory Animal Handling (OECD, 1996).

Preparation of sheep red blood cells (SRBC) as antigen: Fresh blood was collected from a sheep in a sterile bottle containing Alsever's solution. The sheep red blood cells were washed in normal saline three times by centrifugation. The supernatant was discarded and the SRBCs washed again in

sterilized phosphate buffer saline (pH 7.2). Using a Neubauer counter, SRBCs were enumerated and stored at 4 °C for use within 4-6 hours.

Determination of Total and Differential Leucocyte Count: On day 15 of treatment, the freshly collected blood samples were analysed for total and differential leucocytes counts using fully automated haematology analyser (Model: XS-1000i), Sysmex India Pvt. Ltd. Whole blood (1 ml) in EDTA-containing vacutainers was aspirated by the machine which was set to automatically analyse the sample.

Determination of Neutrophil Adhesion: On day 15 of treatment, blood samples were analysed for total leucocyte counts (TLC) and differential leucocytes counts (DLC). After the initial counts, the blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at 37 °C. The incubated blood samples were again analysed for TLC and DLC. The product of TLC and % neutrophil were given as the neutrophil index (NI) of blood sample [16, 17].

Determination of the delayed type hypersensitivity (DTH) response: On day 14 of treatment, mice were challenged by sub-cutaneous administration of 20µl of 5×10^9 SRBC per ml into the hind right foot pad. The foot thickness was measured using a Vernier calliper at 0 hrs (before challenging) and 24 hrs after the challenge. The differences obtained for pre and post challenge thicknesses were then used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema [18].

Hemagglutination antibody (HA) titer determination: On day 7, all animals were immunized with 0.1 ml of SRBCs suspension containing 5×10^9 cells intra-peritoneally. All animals continued to receive their respective treatments for more 7 days. Aliquots of blood samples collected from all animals on day 15 were transferred into clean clot activated vacutainers. After clotting, the blood was centrifuged to obtain serum. Two-fold dilutions of the serum were made using normal saline. To 25 ul of serum in the micro titer plates, 25 ul of 1% (v/v) SRBCs in normal saline were added and the mixture incubated for 1 hr at 37 °C. The titer plates were then observed for hemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the hemagglutination antibody titer (HA units/µL) [19].

Statistical Analysis: All values were expressed as mean±SEM. The data were statistically analyzed using one-way ANOVA followed by Dunnett t-test for multiple comparison was applied. The values were considered significant for $p\leq0.05$ and $p\leq0.001$.

RESULTS

Determination of Total and Differential Leucocyte Count: The variation in total and differential leucocytes counts in all the groups is summarized in Table 2. Among extract treatment groups, the highest total leucocytes count was recorded in the Group III and it is comparable with Group I since it does not differ significantly. The eosinophils and basophils counts in all the groups do not showed significant ($p\leq0.001$) variation. The highest neutrophil count was observed in Group IV and it is comparable with Group I as there is no significant difference between them. The monocyte counts for Group III and Group IV were significantly ($p\leq0.001$) higher when compared to Group II. For lymphocyte counts, the values for all the groups were significantly ($p\leq0.001$) higher when compared to Group II. However, none of the values in extract treatment groups were comparable to Group I since they differ significantly ($p\leq0.001$).

Neutrophil Adhesion: The mean % neutrophil adhesion in all the groups showed significant increase ($p\leq0.001$) when compared to Group II. The highest mean % neutrophil adhesion was recorded in the Group I followed by Group IV and both values are comparable with each other since they do not differ significantly.

Delayed Type Hypersensitivity: The mean percentage increase in footpad thickness was significantly ($p\leq0.001$) higher in Group IV when compared to Group II. The values of Group I and Group IV are comparable as they differ non-significantly.

Hemagluttination antibody titer: The mean hemagglutination antibody titer to SRBC showed a dose-dependent increment for the groups treated with extract. The HAT values for Group IV and Group V were significantly ($p\leq0.001$) higher when compared to Group II. The difference in values of Group I and Group V is statistically non-significant and thus are comparable.

DISCUSSIONS

In present study, total and differential leucocytes count was carried out to determine the effect of plant extract on the hematopoietic system in animal model. From the results it is evident that a dose of 5 mg/kg of extract causes maximum elevation in total leucocytes counts. The same dose is also accountable for the maximum increase in neutrophil count in animals. These results are in line with the findings of earlier study which had

shown leucocytosis accompanied by increase in neutrophil count in Nubian goats fed with *C. angustifolia* [20]. The observed increment in leucocytes counts can be due to the presence of variety of phytochemicals in the extract. Previous studies have suggested that methanol is the most effective solvent to extract various secondary metabolites from *C. angustifolia* [21]. Most of these metabolites such as alkaloids, phenolics, flavonoids, phytosterols, tannins, etc. are potent antioxidants thereby inhibit excessive generation of reactive oxygen species and prevent damage to immune cells. The plant also contains various micronutrients such as iron, manganese, calcium, magnesium, zinc, copper, sodium, potassium, vitamin E, etc. [22,23,24]. It is possible that the methanol extracted almost all these compounds which could contribute to the observed effects on the hematological parameters. These compounds especially iron and vitamin E are essential for development and maturation of body's immune system by promoting cellular components of hemopoiesis [25]. These processes are vital in cell proliferation and survival and hence the increment of the leucocytes counts is observed in animals dosed with the extract of *C. angustifolia*. However, at a dose of 10 mg/kg, slight reduction in total leucocytes count is observed compared to a dose of 5 mg/kg. This reduction in cell count is due to the loss of certain hematopoietic nutrients caused by slight laxative effect of the extract at 10 mg/kg dose [26].

Neutrophils are part of the cell-mediated immune responses responsible for the clearance of foreign bodies by recognition and migration toward the foreign body, phagocytosis and destroying the foreign agent [27]. In present study, the neutrophil phagocytic index has elevated in extract treated animals with highest increase observed at a dose of 5 mg/kg. This increment in neutrophil phagocytic index is an indication of enhanced neutrophil migration towards foreign bodies [27]. The increased neutrophil phagocytic index can be attributed to the presence of various compounds, macronutrients and micronutrients in *C. angustifolia*. Different studies on *C. angustifolia* have shown that it contains fatty acids [23] and trace elements [24] which are important for functioning of neutrophils in cell-mediated immune response. Poly-unsaturated fatty acids have been reported to play an important role in making neutrophils responsive towards other immune mediators. Zinc also found in *C. angustifolia* is an essential element involved in the activity of cytosolic superoxide dismutase which helps in preventing oxidation reactions within the neutrophils thus prolonging their lifespan [25]. Phytocompounds in *C. angustifolia* not only facilitate the proliferation and maturation of neutrophils, but also the secretion of cytokines [25] that resulted in an increment in neutrophil migration and adhesion to the nylon fibres as observed in the study.

The DTH reaction is a type IV cell-mediated immune response according to the Coombs and Gell classification of hypersensitivity reactions, 1975 [28]. The test provides a functional in vivo assessment of the cell-mediated immunity. The increment in footpad thickness of the animals treated with extract in this study is found to be highest at a dose of 5 mg/kg. This increment can be attributed to the ability of extract to activate lymphocytes and their accessory cell types leading to enhanced cell-mediated immunity. The extract has been reported to contain vitamins E and zinc [23,24]. These compounds stimulate the immune system by enhancing T-cell proliferation and increasing cytokine production [25] both of which are important in the inflammatory response that can be seen as an increment in the foot pad thickness.

The hemagglutination test was performed to determine the effect of methanolic leaf extract of *C. angustifolia* on the humoral immune response. The results demonstrate that methanolic leaf extract of *C. angustifolia* has stimulatory effect on the humoral immune response of animals in a dose-dependent manner. In previous study, the alcoholic extract of *C. angustifolia* has been reported to stimulate synthesis of serum proteins [29] that are important in defence mechanism of the body [30]. The copper, which is also present in *C. angustifolia*, is essential for the functioning of the enzyme cerruloplasmin which plays role in the humoral immune response. Other compounds such as fatty acids, zinc and vitamin E are also essential for the maturation of B-lymphocytes in the bone marrow which are one of the important components of humoral immune response [27].

CONCLUSION

Immunomostimulatory agents enhance the immune responsiveness of an organism against a pathogen by activating the immune system. While some of the immunostimulatory agents stimulate both humoral and cell-mediated immunity, others activate only the cellular components of the immune system i.e. phagocytic function without affecting the humoral immunity. The present investigation demonstrates that crude methanolic leaf extract of *C. angustifolia* stimulates both cell-mediated and humoral immune response in immunosupressed animals. The extract therefore can be used in the treatment of diseases associated with compromised immune system. The dose of 5 mg/kg shows maximum stimulation of immune response in animal model. Further studies utilizing pure extracts in order to pin-point the exact

mechanism responsible for immunomodulation are required for more conclusive results.

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TABLE 1: TREATMENT ADMINISTERED TO DIFFERENT GROUPS

Group	Day 0	Day 1 - 14	Day 7	Day 14
Group I (Positive Control)	Cyclophosphamide 200 mg/kg bwt s.c.	Levamisole 50 mg/kg bwt p.o.	0.1 ml of 5×10^9 SRBC per ml i.p.	20µl of 5×10^9 SRBC per ml into the hind right foot pad and 20µl of normal saline into the hind left foot pad s.c.
Group II (Negative Control)	Cyclophosphamide 200 mg/kg bwt s.c.	Normal saline 2 ml p.o.		
Group III (2 mg/kg Extract)	Cyclophosphamide 200 mg/kg bwt s.c.	Crude plant extract 2 mg/kg bwt p.o.		
Group IV (5 mg/kg Extract)	Cyclophosphamide 200 mg/kg bwt s.c.	Crude plant extract 5 mg/kg bwt p.o.		
Group V (10 mg/kg Extract)	Cyclophosphamide 200 mg/kg bwt s.c.	Crude plant extract 10 mg/kg bwt p.o.		

s.c.- subcutaneous, p.o.- per os (oral), i.p.- intraperitoneal

TABLE 2: EFFECTS ON TOTAL AND DIFFERENTIAL LEUCOCYTES COUNTS

Group	**Total Leucocyte Count ($\times10^3$cells/ul)**	**Differential Leucocyte Count ($\times10^3$ cells/ul)**				
		Eosinophils	**Basophils**	**Neutrophils**	**Monocytes**	**Lymphocytes**
I	6.48 ± 0.10^{b}	0.26 ± 0.06	0.04 ± 0.03	1.47 ± 0.07	0.23 ± 0.04^{b}	4.49 ± 0.05^{b}
II	3.16 ± 0.14	0.20 ± 0.01	0.01 ± 0.01	0.45 ± 0.00^{d}	0.02 ± 0.01	2.48 ± 0.12
III	6.11 ± 0.10^{bc}	0.29 ± 0.03^{a}	0.02 ± 0.03	1.26 ± 0.09^{bc}	0.34 ± 0.03^{bc}	4.20 ± 0.05^{bc}
IV	6.63 ± 0.15^{b}	0.35 ± 0.05^{a}	0.11 ± 0.04^{a}	1.50 ± 0.06^{b}	0.35 ± 0.08^{a}	4.31 ± 0.09^{bc}
V	5.12 ± 0.10^{bd}	0.22 ± 0.02	0.05 ± 0.00^{a}	0.97 ± 0.09^{bc}	0.20 ± 0.05^{a}	3.67 ± 0.07^{bd}

Values expressed as Mean±SEM, $^{a}p\leq0.05$ significantly different from Group II, $^{b}p\leq0.001$ significantly different from Group II, $^{c}p\leq0.05$ significantly different from Group I, $^{d}p\leq0.001$ significantly different from Group I.

TABLE 3: EFFECTS ON NEUTROPHIL PHAGOCYTIC INDEX, DELAYED TYPE HYPERSENSITIVITY RESPONSE AND HEMAGGLUTINATION ANTIBODY TITER

Group	Neutrophil Index (%)	Increment in footpad thickness (%)	Hemagglutination Antibody Titer (HA units/ul)
I	69.25±0.93[b]	46.63±2.96[b]	23.33±0.57[b]
II	29.57±0.47	27.38±2.06	4.66±0.57
III	66.27±0.89[bc]	43.33±5.77[a]	12±1.73[ad]
IV	68.65±0.63[b]	51.51±2.62[b]	20±1.00[bc]
V	58.64±2.20[bc]	46.29±3.21[a]	23±1.00[b]

Values expressed as Mean±SEM, [a]$p \leq 0.05$ significantly different from Group II, [b]$p \leq 0.001$ significantly different from Group II, [c]$p \leq 0.05$ significantly different from Group I, [d]$p \leq 0.001$ significantly different from Group I.

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