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Research Article

IMMUNOMODULATORY ACTIVITY OF ALCOHOLIC EXTRACT OF HABENARIA INTERMEDIA IN MICE

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ABSTRACT

Objective: The present study was undertaken to evaluate immunomodulatory activity of ethanolic extract of Habenaria Intermedia.

Methods: Immunomodulatory activity was evaluated by delayed-type hypersensitivity response (DTH), hematological parameters and by carbon clearance assay (phagocytic index). Swiss albino mice of either sex were divided into 4 groups (6 animal each), such as normal control, Dose I (HI lower dose as 300 mg/kg body weight), Dose II (HI lower dose as 600 mg/kg body weight), and standard group, treated with standard drug Cyclophosphamide (20 mg/kg body weight).

Result: Habenaria Intermedia at higher dose significant (P<0.0) increases the delayed-type hypersensitivity response when compared to control group. Habenaria Intermedia improves the phagocytic index in a dose dependent manner.

Conclusion: The results were comparable with that of standard drug cyclophosphamide. It was concluded that test extract is a promising drug with Immunostimulant properties.

Keywords: Habenaria Intermedia, Orchidaceae; Immunomodulatory

INTRODUCTION

The immune system is a system of biological structures and processes within an organism that protects against disease. Disorders of the immune system can result in autoimmune diseases, inflammatory diseases and cancer and immunodeficiency [1].

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 reaction, it is named as an immunostimulative drug which primarily implies stimulation of non-specific system. Immunosuppressant implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors. Immunostimulation and immunosuppression both need to be considered in order to regulate the normal immunological functioning. Hence both immunostimulating agents and immunosuppressing agents have their own standing, so search for better agents exerting these activities is becoming the field of major interest all over the world. A number of Indian medicinal plants and various 'Rasayana' have been claimed to possess Immunomodulatory activity [2].

The use of plant products as immunomodulators is still in a developing stage. These are several herbs used in indigenous system. A variety of plant-derived materials such as polysaccharides, lectins, peptides flavonoids and tannins have been reported to modulate the immune system [3]. Since ancient times, several diseases have been treated by administration of plant extracts based on traditional medicine [4].Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system [5].

The benefits of immunomodulators stem from their ability to stimulate natural and adaptive defense mechanisms, such as cytokines, which enables the body to help itself. The natural immunomodulators act to strengthen weak immune systems and to moderate immune systems that are overactive. Plant sterols and sterolins are natural immunomodulators found in some raw fruits and vegetables and in the alga, spirulina. Several plants have been used folklore medicine [6].

A traditional and folklore medicine plays an important role in the health services around the globe. About three quarters of the world population relies on the plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity. *Habenaria Intermedia* (Orchidaceae) is one of the active constituent belonging to Ashtavarga class used in Chyawanprash [7].

Habenaria Intermedia has been yet not scientifically reported for any of its immunomodulatory activity. However plants belonging to Orchidaceae extensively studied for Immunomodulatory activity [8-10]. The objective of the present work is to evaluate the immunomodulatory activity of the ethanolic extract of *Habenaria Intermedia* tubers.

MATERIAL AND METHODS

Plant Material

Plant of *Habenaria Intermedia* family Orchidaceae collected from a road side clefts between Nainital & Bhavali and also between Nainital & Haldhwani, Uttaranchal and was authenticated from Department of Botany, R. T. M. Nagpur University, Nagpur, the voucher specimen has been deposited in the office (specimen No.9567).

Preparation of Extract

Dried coarsely powdered tubers of *Habenaria Intermedia* (400 g) were defatted with petroleum ether at 50^0 - 60^0 C for 72 hrs using Soxhlet apparatus. The marc left was subsequently extracted with ethanol (95%, 60-70°C) for 72 hrs. The crude brown residue mass of extract was then concentrated, stored and preserved (2-8°C). The Percentage yield of extract (4.8w/w) was found on dry wet basis. For doing, the extract was suspended in tween-80 to prepare suitable dosage forms [11].

Experimental Animals

Albino mice (Swiss) of either sex were used. The animals were fed with standard pellet diet, water *ad libitum* and maintained under standard environment condition employed. They were housed under standard conditions (22 ± 5°C with 12 h of light/dark cycle). All experimental protocols was approved by Institutional Animal Ethical Committee Clearance (JLCCP, 2011/1/CPCSEA). J.L. Chatuvedi College of Pharmacy, Nagpur-440016(M.S), India.

Antigen

Fresh Sheep blood was collected from Veterinary College, Nagpur, in sterile Alsevar's solution (1:1 proportion). Sheep red blood cells (SRBCs) were washed three times in pyrogens free normal saline and centrifuged at 2500-3000 rpm for 10 minutes. The supernatant was removed with pasture pipette and suspended in normal saline. The concentration of 0.1 ml containing 1x 10^8/mm^3 cells was adjusted by using improved Neubaur chamber for immunization and challenge.

Chemicals and reagents

Cyclophosphamide (Khandelwal Laboratories Ltd., Mumbai) and all other solvents used for experimental work was of analytical grade.

Acute oral toxicity study

Acute oral toxicity studies of extract was carried out as per the OECD guidelines, draft guideline 423 adopted on 17th December, 2001, received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Govt. of India [12].Administration of 50 mg\kg body weight up to the dose 5000 mg\kg body weight caused no considerable signs of the toxicity in the tested animals.

Delayed Type Hypersensitivity test (DTH).

The method described by Puri and Saxena with some modification was adopted [13]. Mice were divided into four groups of six mice each. Drugs was administered in various group, group I received vehicle (1% Tween 80 solution), group II received Dose I (300 mg/kg body weight), group III received Dose II (600 mg/kg body weight), group IV received Cyclophosphamide. On day zero Cyclophosphamide (CP) 20 mg/kg was administrated IP to the animal of group IV, 2 hrs before sensitizing with 1x 10^8 SRBCs, as antigen through SC route in the left hind paw. All the group were treated as per table [Table 1] for next five days, i.e. day 1 to day 5.

All the animals were maintained on same diet and environment throughout the duration of the experiment.

Administration of extract was done by oral route using animal feeding needle and 1 ml syringe. On day 5 the left hind paw thickness was measured. The animals were then challenged with the same antigen SRBC, 1x10^8 in 0.1 ml, in the left hind paw by SC route.

The Paw thickness measurement was again done at interval of +24, +48, +72 and +96 hrs from the challenge.

Table 1: Experimental Design- Delayed Type Hypersensitivity Model in mice.

Day 0	Group IV	CP by IP , 2 hrs before Sensitization
	Group I-IV	Sensitization with 1x10^8 SRBCs
Day 1	Group I and IV	1% Tween 80
To	Group II	D_1: Dose I
Day 5	Group III	D_2: Dose II
Day 5	Group I- IV	Measurement of Paw thickness
		Challenge with 1x10^6 SRBCs
Day 6 to Day 9	Group I- IV	Measurement of Paw thickness
Day 10	Group I- IV	Collection of Blood for WBC and Total Platelet Count

On the day 10, blood was collected from the retro orbital plexus for WBC and Total Platelet Count [14].

Carbon clearance test for phagocytic activity

The method described by Hudson and Hay was followed with some modification [15].Mice were divided into four groups of six mice each. Control group I and Cyclophosphamide group IV received vehicle only while animal of treatment group II and III will be given test extract (Dose I and Dose II respectively) in 1 % Tween- 80 daily for 5 days , Cyclophosphamide will be given to group IV by IP route at day 0 only. All the groups were given 0.1 ml of carbon ink suspension through the tail vein. After 48 hours of 5 days treatment. A Blood sample was collected from the retro- orbital plexus at 0 and 15 min immediately after the injection of carbon suspension. Blood (25µl) was lysed with 2 ml of 0.1 % sodium carbonate and the absorbance was measured spectrophotometrically at 675 nm for determination of optical densities. The rate of carbon clearance, termed as phagocytic index (K), was

Calculated by using equation:

$K = (\ln OD_1 - \ln OD_2) / t_2 - t_1$

Where, OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively [16].

Statistical analysis

The mean + SEM was calculated. The variation present in the data was analysed through one way analysis of variance (ANOVA). Post-hock analysis was done by Tukey's multiple tests to estimate the significance of difference between various individual groups.

RESULTS

Effect of Habenaria Intermedia extract on mean foot paw oedema in DTH model

The results obtained in the DTH model are given in table 2.

The immune response was determined by DTH response i.e. increase in foot pad thickness using vernier calipers. The observations in Table-2 indicate that mice treated with dose i.e. 300 mg/kg (Group II) and dose 600 mg/kg (Group III) increase response in foot pad edema was found to be statistically significant ($p < 0.05$) when compared to Group I (Control) and also with Group IV (Cyclophosphamide).

Table 2: Effect of *Habenaria Intermedia* extract on mean foot paw oedema in DTH model.

Group No	Group Description	Mean Foot Pad Oedema (mm)			
		24 hrs	48 hrs	72 hrs	96 hrs
I	Control	0.625 + 0.028	0.441 + 0.025	0.233 + 0.017	0.130 + 0.018
II	D_1	0.768 + 0.031*	0.491 +0.031*	0.241 + 0.007*	0.121 + 0.009*
III	D_2	0.691 + 0.026*	0.495 + 0.029*	0.311 + 0.043*	0.115 + 0.015*
IV	CP	0.796 + 0.019	0.591 +0.025	0.285 + 0.021	0.143 + 0.012

Data was expressed as a Mean + SEM, n=6; using one way analysis variance (ANOVA) followed by Tukey's multiple comparison test. $P<0.05$ was considered as statistical significant, n=6 in each group. D_1= Dose I, D_2= Dose II, CP= Cyclophosphamide.

Effect of *Habenaria Intermedia* extract on Haematological parameters in Swiss albino mice

Table 3: Effects of Doses on WBC and Platelet Count in DTH model

Sr. No	Groups	Haematological Parameters	
		WBC count (Thousand/mm^3)	Platelet count (Thousand/mm^3)
I	Control	7.180 + 0.6198	440.8 + 75.52
II	D_1	9.338 + 0.6343*	679.7 + 67.20*
III	D_2	10.77 + 0.4190*	761.5 + 25.00*
IV	CP	6.513 + 0.3469	410.0 + 56.12

Data was expressed as a Mean + SEM, n=6; using one way analysis variance (ANOVA) followed by Tukey's multiple comparison test. P<0.05 was considered as statistical significant, n=6 in each group. D_1= Dose I, D_2= Dose II, CP= Cyclophosphamide.

Results of *Habenaria Intermedia* extract on Haematological Parameters

Administration of ethanolic extract of ***Habenaria Intermedia*** at both the levels showed statistically significant ($p < 0.05$) increase in RBC count and WBC count when compared to Cyclophosphamide (20 mg/kg) treated and control treated mice.

Effect of Habenaria Intermedia extract on Carbon clearance test for phagocytic activity in Swiss albino mice

The phagocytic activity is generally measured by the rate of removal of carbon particles from blood stream. The phagocytic index of both the levels of extract of *Habenaria Intermedia* was 1increase (1.3660 and 1.501) and was found to be statistically significant ($p < 0.05$) when compared to phagocytic index (0.9488) of vehicle treated Group I mice (Control) and also with Group IV (Cyclophosphamide).

Table 4: Effect of doses on Carbon clearance test for Phagocytic index.

Sr. No.	Groups	Phagocytic Index
I	Control	0.9488 + 0.0966
II	D_1	1.3660 + 0.0588*
III	D_2	1.501 + 0.1650**
IV	CP	0.852 + 0.0561

Data was expressed as a Mean + SEM, n=6; using one way analysis variance (ANOVA) followed by Tukey's multiple comparison test. P<0.05 was considered as statistical significant, n=6 in each group. D_1= Dose I, D_2= Dose II, CP= Cyclophosphamide.

DISCUSSION AND CONCLUSIONS

DTH is a part of the process of graft rejection, tumor immunity, and most important

Immunity to many intracellular infectious microorganisms especially those causing chronic diseases such as tuberculosis [17]. 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, induced vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentration of lytic enzyme for more effective killing [18-19]. The effect of ethanolic extract of *Habenaria Intermedia* tubers on the foot pad thickness and hematological data such as WBC and Total Platelet counts of antigenically challenged mice in [tables 2 & 3 and also Figures 1, 2 &3] showed, the (P<0.05) significant decrease in the foot pad thickness, WBC and Total Platelet counts in cyclophosphamide group as compared to control group. While in extract-treated group animals showed (P<0.05) significant increase. Control of disease by immunological means has two aspects, namely the development and improvement of protective immunity and avoidance of undesired immunological side reaction. Modulation of the immune system by the cytostatic agents is emerging as a major area in pharmacology, especially in cases, where undesired immunosuppression is the result of therapy. Cytotoxic drugs like Cyclophosphamide and azathioprin act at various levels on cells involved in defence against foreign invaders [20]. Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. They may selectively activate either cell-mediated or humoral immunity by stimulating either TH1 or TH2 type cell response, respectively. Immunomodulatory agents that are free from side effects and which can be administered for long duration to obtain a continuous immune activation are highly desirable for the prevention of diseases. There a variety of naturally and chemically derived compound discovered with the Immunomodulatory activity such as levamosole, glucan, IL-2, interferon's, etc. which are used in combination with cisplatin, adiramycin, 5-flurourcil, etc against many types of carcinomas. But most of them have side effects namely fever, myaligias fatigue, etc [21]. The role of phagocytosis is primary the removal of microorganism and foreign bodies, but also the elimination of dead or injured cells. Phagocytic defects are associated with varied pathological condition in humans [22].

In view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to β-cells. Phagocytic index [Table 4] were (P<0.05) significantly increased as compared to control and Cyclophosphamide group. Hence, increased clearance rate of carbon particles from circulation in animal reflects the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity. Phagocytosis by macrophages is important against pathogenic microorganisms and its effectiveness is markedly enhanced by opsonisation of parasite with antibodies and complement C3b leading to more rapid clearance of parasite from blood. The modulation of immune response using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations [23]. Based on results obtained, ethanolic extract of *Habenaria Intermedia* tubers was found to have a promising immunostimulant potential. It may be due to increase DTH reaction in mice in response to T-cell dependent antigen stimulatory effect on lymphocytes and necessary cell types required for the expression of reaction [24].

DTH response is direct co-related to cell-mediated immunity and was significantly increased with ethanolic extract of Habenaria *Intermedia* tubers as compared to normal control. The significant increase in Immunomodulatory potential of ethanolic of Habenaria *Intermedia* tubers could be attributed due to presence of flavonoids, tannins, steroids, alkaloids, phenolic compounds. Thus, present study validates the traditional use of *Habenaria Intermedia* tubers in Ayurvedic system of medicine.

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