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Saussurea lappa extract modulates cell mediated and humoral immune response in mice

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ABSTRACT

In the present study immunopharmacological properties of hydroalcoholic extract of Saussurea lappa C.B. Clarke root have been investigated. After administration of extract in doses of 100 and 200 mg/kg body weight a significant increase in leukocyte count, spleen weight, phagocytic index and antibody secreting cells were noticed. Treatment with extract enhanced DTH reaction, which is reflected from the increased footpad thickness. Saussurea lappa extract treatment also reduced the total number of animals showing anaphylactic symptoms. At the low dose (100 mg/kg) Saussurea lappa extract did not affect the humoral immune response but at higher dose (200 mg/kg) produced a significant enhancement in antibody titre value. The results suggest that bio active compound of Saussurea lappa influences both humoral as well as cell mediated immune system.

Key words: Immunopharmacology, Humoral immunity, Cellular immunity, Delayed type hypersensitivity, Herbal immunomodulator

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INTRODUCTION

Traditional medicine system like Chinese, Japanese Kampo and Indian Ayurveda mentioned the many botanical medicine which claimed to be modulate the cellular and humoral function of immune system [1]. Ayurveda gives a separate class of immunomodulatory botanicals named Rasayanas. These plants, labelled as 'rasayana', have been endowed with multiple properties like delaying the onset of senescence and improving mental functions by strengthening the psycho-neuro-immune axis [2]. Many herbal medicines have been reported to modulate cytokine secretion, histamine release, immunoglobulin secretion, class switching, cellular receptor expression, lymphocyte expression, phagocytosis, and so on [1]. Botanicals produce a diverse range of phytochemicals with immunomodulating potential, including isoflavonoids, indoles, phytosterols, polysaccharides, sesquiterpenes, alkaloids, glucans and tannins. Several botanicals from these Ayurvedic texts have been studied for their immunomodulatory properties [1].

Saussurea lappa C.B. Clarke is a well known and important medicinal plant commonly known as costus, kuth, kushta, kust, muxiang, patchak, quang mu xiang is widely used in Ayurveda and Chinese systems of medicine for the treatment of various ailments, viz. asthma, inflammatory diseases, ulcer and stomach problems. Different pharmacological experiments in a number of *in vitro* and *in vivo* models have convincingly demonstrated the ability of *Saussurea costus* to exhibit anti-inflammatory, anti-ulcer, anticancer and hepatoprotective activities. Sesquiterpene lactones have been reported as the major phytoconstituents of this species. Costunolide, dehydrocostus lactone and cynaropicrin, isolated from this plant, have been identified to have potential to be developed as bioactive molecules [3, 4].

MATERIALS AND METHODS

Plant material extraction

Saussurea lappa root was purchased from the local market of the Haridwar. Quality of sample was assured by pharmacognostical characterization.

Approximately 100 g of the root powder was soaked in 1.0 L ethanol-water (70:30) mixture at room temperature and sonicated for 30 min. It was filtered through through a Whatman grade-1 filter paper in a Buchner funnel under vaccum and filtrate was evaporated to dryness on a rotary evaporator under reduced pressure. Crude hydroalcohalic extract of *Saussurea lappa* roots was obtained; which was further lyophilized to obtain dry powder.

Animals

Swiss albino mice of either sex weighing 18-28 g were used for study. Animals were procured from the Institutional animal House approved by CPCSEA. All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27°C and humidity 60-65% with 12:12 light: dark cycles). Food was provided in the form of dry pellets and water *ad libitum*. The animals were allowed to get acclimatized to the laboratory conditions for 7 days before the commencement of the experiment. All experiments involving animals complies with the ethical standards of animal handling and was approved by Institutional Animal Ethical Committee.

Experimental protocol

The drug solutions were prepared in phosphate buffer saline for oral administration. Immunomodulatory activity was checked at both cellular and humoral levels. All the experimental models had four common groups consisting of six animals each. Group I, was served as native control and received no treatment, Group II, served as vehicle control and received PBS 1 ml/100 g, p.o, group III, received the hydroalcohalic extract of *saussurea lappa* roots (100 mg/kg, p.o), whereas group IV were administered (200 mg/kg, p.o.) respectively.

Effect on leukocyte Count and spleen weight

Mice were treated according to treatment protocol for 5 consecutive days. On day 6, blood was collected from retro-orbital plexus for white blood cells (WBC) count. The animals were sacrificed by cervical dislocation and their spleens were harvested for weighing. The results of these analyses were compared with that of vehicle control [5].

Carbon clearance test

Mice were administered the doses according to treatment protocol for 5 consecutive days in their respective groups. Forty eight hours after the last dose of the tretment, animals of all the groups received intravenous injection of (0.3 ml/30 g) Indian ink (colloidal carbon) through the tail vein. Blood samples were withdrawn from each animal by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. A 50 µl blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following

$$\text{Formula: } K = (\, lg\ OD_1 - lg\ OD_2)/t_2 - t_1$$

Where, OD_1 and OD_2 are the optical densities at 0 and 15 min, respectively [6, 7].

Delayed hypersensitivity reaction

The antigen specific cellular immune response in experimental animals was measured by determining the degree of DTH response using the foot paw swelling test. Mice were given the treatment beginning on day 0 and continued till the day of challenge. The mice were primed with 0.1 ml of ship red blood cells (SRBC) suspension containing 1×10^8 cells, i.p., on day 7 and challenged on day 14 with 0.05 ml of 2×10^8 SRBC in the right hind foot pad. The left hind foot pad was injected with 50 µl of PBS as control. Foot pad swelling was measured on at 0, 24, 48 and 72 h after challenge using a calliper (Mitutoyo manufacturing Company, Japan). The difference between the mean of right and left hind footpad thickness gave a degree of foot pad swelling which was used for group comparisons as a measure of DTH reaction [8, 9].

Systemic anaphylactic

Mice were sensitized by 1 mg bovine serum albumin (BSA) subcutaneously in 0.2 ml PBS (pH 7.4) at day 0 and were shocked by intravenous injection of 1 mg BSA in PBS (pH 7.4) on day 14. During this period mice were mice were administered the doses as stated in treatment protocol, from day 0 to day 14 before shocking injection. The systemic anaphylactic reaction was observed within 30 min following the shocking injection and rated in

following fashion: **positive reaction**, mortality or animal rendered still for at least 1 min; **negative reaction** with no change or normal movement [10].

Indirect haemagglutination test

Mice of various groups were pre-treated with the drugs for 7 days and immunized with 5 × 10^9 SRBC intraperitoneally. The day of immunization was referred to as day 0. The treatment was continued for 7 more days according to protocol and blood samples were collected by retro-orbital puncture at the end of the treatment for antibody titer. Antibody titre was determined following the procedure reported by Nelson and Mildenhall. For experimentation, in two-fold serial dilutions of serum samples made in 25 µl volumes of normal saline containing 0.1% bovine serum albumin (BSA saline) in V bottom heamagglutination plates (96 well microtitre plates) were added 25 µl of 0.1% suspension of SRBC in BSA saline. After thorough mixing SRBC were allowed to settle at room temperature for 90 min until control wells showed small button of cells (negative pattern). The value of the highest serum dilution causing visible hemagglutination was considered to be the antibody titer [11, 12].

Plaque forming cell [PFC] assay

The assay was performed according to the method reported by Jerne and Nordin. After immunizing the animals with SRBC on day 0, they were treated as stated above for 5 consecutive days. Briefly, the spleen cells of SRBC immunized extract treated mice were separated in RPMI-1640 medium, washed twice and suspended in same medium. Glass petridishes were layered with 1.2% agarose in 0.15 M NaCl to form bottom layer. A mixture of 2 ml agarose (0.6%) in RPMI-1640 medium (42ºC), 0.1 ml suspension of 20% SRBC and 1×10^6 spleen cells in a volume of 0.2 ml was poured over the bottom layer of agarose followed by an incubation period of 90 min at 37ºC. A 2 ml quantity of 1:9 diluted fresh rabbit serum was added to petridish and plates were reincubated for 40 min to allow the formation of plaques. The numbers of plaques were counted immediately and values were expressed as counts per 10^6 spleen cells [13, 14].

Statistical calculation

The values were calculated as mean ± S.D. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet's t-test using Graphpad Prism 5, $P < 0.05$ or above was considered to be significant.

RESULTS

Effect on blood leukocyte count and spleen weight

Leukocyte count was increased significantly ($P < 0.05$, $P < 0.01$) on treatment with *Saussurea lappa* extract in Group III & IV compared to control. Significant ($P < 0.05$) positive effect on spleen weight was observed in Group III and IV, compared to vehicle control shown in table-1.

Table 1: Effect of *Saussurea lappa* extract on blood leukocyte count and spleen weight

Group	Treatment	Spleen weight (gm)/100 gm BW	Leukocyte count/mm3
Group 1 (Native)	(Not Treated)	0.420±0.021	3850 ± 73.21
Group 2 (Control)	PBS	0.428±0.023*	4120 ± 126.21*
Group 3	Extract 100 mg/Kg	0.527±0.037**	4590 ± 132.67**
Group 4	Extract 200 mg/Kg	0.623±0.036**	4950± 145.76***

*Values are expressed as mean±S.E.M. *P > 0.05 (insignificant compared to control); **P < 0.05 (significant compared to control); ***P < 0.01 (highly significant compared to control)*

Effect on phagocytosis

Group III shown significant ($P < 0.05$) increase in the phagocytic index as compared to control group, determined in terms of increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best when group IV at the dose of 200 mg/kg *Saussurea lappa* extract ($P < 0.01$).

Table 2: Effect of *Saussurea lappa* extract on phagocytic index and paw edema

Group	Treatment	Phagocytic index	% increase in paw volume
Group 1 (Native)	(Not Treated)	0.0398±0.002	16.78 ± 12.46
Group 2 (Control)	PBS	0.0421±0.003*	31.45 ± 14.98
Group 3	Extract 100 mg/Kg	0.0624±0.04**	59.47 ± 16.87
Group 4	Extract 200 mg/Kg	0.0885±0.02***	67.79 ± 28.38

*Values are expressed as mean±S.E.M. *P > 0.05 (insignificant compared to control); **P < 0.05 (significant compared to control); ***P < 0.01 (highly significant compared to control)*

Effect on Delayed type hepersensitivity (DTH) reaction

Treatment with *Saussurea lappa* extract supressed DTH reaction, reflected from the increased footpad thickness significantly ($P < 0.05$, $P < 0.01$ respectively) compared to control group, suggesting heightened infiltration of macrophages to the inflammatory site, shown in table-2.

Systemic anaphylaxis

In the present study, the *Saussurea lappa* extract showed a decrease in total number of animals showing anaphylactic symptoms as compared to control group, when sensitized and subsequently challenged by BSA, shown in table-3.

Table 3: Effect of *Saussurea lappa* extract on systemic anaphylaxis

Group	Sensitizing injection	Treatment	Shocking injection	Total no. mice	Anaphylactic symptoms	Deaths
Group 1 (Native)	BSA (s.c.)	(Not Treated)	BSA (i.v.)	6	6	0
Group 2 (Control)	BSA (s.c.)	PBS	BSA (i.v.)	6	6	0
Group 3	BSA (s.c.)	Extract 100 mg/Kg	BSA (i.v.)	6	4	0
Group 4	BSA (s.c.)	Extract 200 mg/Kg	BSA (i.v.)	6	3	0

Heamagglutination antibody titer

Saussurea lappa extract did not shown significant increase of heamagglutination antibody titer value in group III (100 mg/Kg) as compared to control group. However *Saussurea lappa* extract (200 mg/kg) produced significant enhancement in antibody titre value ($P < 0.05$) in group as compared to vehicle control group.

Plaque forming assay

There was no effects of *Saussurea lappa* extract (100 mg/kg) in the anti-sheep red blood cell plaque forming assay as compared to vehicle control animals. However, *Saussurea lappa* extract (200 mg/kg) produced significant ($P < 0.05$) enhancement in number of cells secreting antibodies against SRBC, which served as specific antigen, shown in table-4.

Table 4: Effect of *Saussurea lappa* extract on heamagglutination antibody titer and antibody secreting cells

Group	Treatment	heamagglutination antibody titer	Plaque forming cell/ 10^6 Spleen cells
Group 1 (Native)	(Not Treated)	67.81±11.60	288± 4.74
Group 2 (Control)	PBS	105.22±11.70*	298 ± 4.74*
Group 3	Extract 100 mg/Kg	143.61±24.50*	302 ± 2.64*
Group 4	Extract 200 mg/Kg	194.83±30.15**	400 ± 7.84**

*Values are expressed as mean±S.E.M. *P > 0.05 (insignificant compared to control); **P < 0.05 (significant compared to control); ***P < 0.01 (highly significant compared to control)*

DISCUSSION

The main objective of this study was to investigate the immunomodulatory effect of hydroalcoholic *Saussurea lappa* root extract at the dose of 100 mg/kg and 200 mg/kg.

Initially, effects on blood leukocyte count and spleen weight were determined to investigate immunomodulatory effect of *Saussurea lappa* extract. Leukocytes play a important role in both innate and adaptive immunity. Innate immunity largely depends on activity of granulocytes, while adaptive immunity depends on lymphocytes, which provide long term immunity [15]. Only the lymphocytes possess the attributes of diversity, specificity, memory and self/non self recognition. All the other cells play accessory roles, serving to activate lymphocytes, to increase the effectiveness of antigen clearance by phagocytosis or to secrete various immune effector molecules. Among different organs of immune system, spleen represents a major secondary lymphoid organ involved in elicitation of immune response. The spleen is adapted to filtering blood and trapping blood-borne antigens and thus can respond to systemic infections [10, 16]. Results from the present study revealed *Saussurea lappa* extract accelerates the proliferation of leukocytes and lymphoid tissue in dose dependent manner.

The carbon clearance test was done to evaluate the effect of drugs on the reticulo-endothelial system. The reticuloendothelial system (RES) is a diffuse system consisting of phagocytic cells like macrophages which play a vital role in the clearance of particles from the blood. Phagocytosis represents an important innate defence mechanism against ingested particulates including whole pathogenic microorganisms [7, 16]**.** Enhanced uptake of particulate matter with the treatment of *Saussurea lappa* extract is evident from carbon clearance test. The result is owing to a mechanism related to phagocytosis by macrophages. *Saussurea lappa* extract stimulates phagocytosis by macrophage activation and increases the polymorphonuclear cells, as evidenced by an increase in the rate of

carbon clearance. However, a dose proportionate response was not observed, since immune response is not always directly related with the immunomodulator concentration.

Delayed type hypersensitivity reaction develops when antigen activates sensitized T lymphocytes, which subsequently proliferates and release various cytokines including interleukin-2, interferon-γ, macrophage migration inhibition factor and tumor necrosis factor-β [15, 17]. These cytokines in turn increase vascular permeability, induce vasodilatation, recruit macrophages and activate them, promoting increased phagocytic activity [16]. Several lines of evidence suggest that DTH reaction is important in host defense against tumour immunity, and many intracellular infectious microorganisms, especially those causing chronic diseases such as tuberculosis [15]. Treatment with *Saussurea lappa* extract 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 the possible role of *Saussurea lappa* extract in assisting cell-mediated immune response.

Systemic anaphylaxis is a shock-like and often fatal state whose onset occurs within few minutes of a type I hypersensitive reaction, mediated by immunoglobulin E (IgE). IgE has high affinity to Fc receptors on the surface of mast cells and blood basophils and thus binds readily to these cells. Such IgE coated mast cells and basophils are said to be 'sensitized'. Secondry exposure to the same allergen cross-links the membrane bound IgE on sensitized mast cells causing degranulation and release the pharmacologically active mediators like histamine, leukotrienes, prostaglandins and cytokines. Human mast cells secrete IL-4, IL-5, IL-6 and TNF-α. These cytokines alter the local microenvironment, eventually leading to the recruitment of inflammatory cells such as neutrophils and eosinophils. IL-4 increases IgE production by B cells. IL-5 is especially important in the recruitment and activation of eosinophils. The high concentrations of TNF-α secreted by mast cells may contribute to shock in systemic anaphylaxis [17]. In the present study, *Saussurea lappa* extract treatment reduced the total number of animals showing anaphylactic symptoms. Thus we can conclude that through several probable mechanisms *Saussurea lappa* extract could have prevented anaphylactic symptoms, when sensitized and subsequently challenged by BSA. Probably *Saussurea lappa* extract interferes with expression of Fc receptors and various chemokines which is responsible for the humoral immune response.

Heamagglutination antibody titer was determined to establish the humoral response against SRBC. 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 [18]. *Saussurea lappa* extract at the low dose (100 mg/kg) effect on humoral immune response but at the high dose (200 mg/kg) produced a significant enhancement in antibody titre value. This indicates the enhanced responsiveness of macrophages, T and B lymphocyte subsets involved in antibody synthesis on *Saussurea lappa* extract administration.

Number of antibody secreting cells from spleen was determined using plaque forming cell assay. Since spleen contributes immensely to the humoral as well as cellular immune system, its role in generation of antibody secreting cells was studied. Antigen-activated B cells differentiate into plasma cells, which leave the follicles and secrete the IgA class of antibodies. These antibodies then are transported across the epithelial cells and released as secretory IgA into the lumen, where they can interact with antigens [17]. There were no effects of low dose of *Saussurea lappa* extract in the anti-sheep red blood cell plaque forming assay as compared to vehicle control animals. However, at higher dose (200 mg/kg) produced significant enhancement in number of cells secreting antibodies against SRBC, which served as specific antigen.

CONCLUSION

The present study has shown the immunomodulatory activity of hydroalcoholic root extract of *Saussurea lappa* in cellular arm response, phagocytic response and anaphylactic reaction at both the doses. However at the lower dose *Saussurea lappa* extract did not show significant effect on humoral immunity and number of antibody producing cells of spleen, reflecting *Saussurea lappa* has no effect on such responses on short term treatment. Higher dose of *Saussurea lappa* extract has shown potentiation of immunomodulatory activity in both humoral as well as cellular arms o fthe immune system, suggesting its therapeutics usefulness in immunocompromised patients on long term basis.

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