ORIGINAL ARTICLE

Evaluation of the immunomodulatory activity of ethanolic extract of the stem bark of *Bauhinia variegata* Linn

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To evaluate the immunomodulatory activity of the ethanolic extract of the stem bark of *Bauhinia variegata* Linn, to justify the traditional claim endowed upon this herbal drug as a *rasayana* in Ayurveda. The effect of the ethanolic extract of the stem bark of *Bauhinia variegata* (EBV) on the primary and secondary antibody responses was evaluated by the humoral antibody response for a specific immune response. The effect of EBV on the phagocytic activity was evaluated by the carbon clearance test and neutrophil activation was evaluated by the neutrophil adhesion test for a nonspecific immune response. The data was analysed by one-way ANOVA followed by Tukey-Kramar multiple comparison tests. On oral administration, EBV showed a significant increase in the primary and secondary humoral antibody responses, by increasing the hemagglutinating antibody titre at doses of 250 and 500 mg/kg/p.o. There was a significant increase in the phagocytic index and percentage neutrophil adhesion at doses of 250 and 500 mg/kg/p.o. The present study reveals that the ethanolic extract of the stem bark of *Bauhinia variegata* Linn holds a promise as an immunomodulatory agent, which acts probably by stimulating both the specific and nonspecific arms of immunity.

Key words: Antibody titre, *Bauhinia variegata*, immunomodulator, neutrophil adhesion, phagocytic activity, rasayana

INTRODUCTION

Herbal drugs are known to possess immunomodulatory properties and generally act by stimulating both specific and nonspecific immunity.[1] Many plants used in traditional medicine have immunomodulating activities. Some of these stimulate both humoral and cell-mediated immunity, while others activate only the cellular components of the immune system, i.e., the phagocytic function, without affecting the humoral or cell-mediated immunity. Some of these plants also suppress both humoral and cell-mediated immunity.[2] About 34 plants are identified as rasayanas in the Indian ayurvedic system of medicine having various pharmcological properties such as immunostimulant, tonic, neurostimulant, antiageing, antibacterial, antirheumatic, anticancer, adaptogenic and antistress.[3] An entire section of Materia Medica of Ayurveda is devoted to drugs entitled as 'Rasayana' used for enhancement of body resistance.[4] With a *rasayana*, "one obtains longevity, regains youth, gets a sharp memory and intellect, freedom from diseases, gets a lustrous complexion and the strength of a horse". More specifically, a rasayana can be described as one which "is antiageing, increases the life-span, promotes intelligence and memory and increases resistance to disease" (presumably infections, therefore indicating potential immunostimulant effects). Rasayana herbs act as adaptogens, immunomodulators, pro-host probiotics and antimutagenics.

Many plants with potential immunomodulatory activity are reported, some of these have already been undertaken for evaluation of their activities in animals, and also to some extent in humans. A lot more are still to be explored and offer scope for further investigation. *Bauhinia variegata* is an indigenous medicinal plant with pharmacological properties similar to rasayanas.[3]

Bauhinia variegata Linn. (Caesalpiniaceae) is a commonly found plant in moist waste ground and open plantations. It is cultivated throughout India and in the forest lands in central India. According to Ayurveda, *Bauhinia variegata* Linn is used as a tonic for the liver, in treatment of leprosy, menorrhagia, impurities of blood, tuberculous glands, wounds, ulcers, asthma, etc.[5,6] The bark powder of the plant is a major ingredient of the herbal tonic *Kanchanar guggul*, an ayurvedic remedy prescribed to increase the white blood cells. Phytochemical characterization shows the presence of tannins, steroids, alkaloids, flavonoids and saponin in the stem bark of *Bauhinia variegata* Linn.[7] The ethanolic extract of stem bark of *Bauhinia variegata* Linn contains β sitosterol, lupeol, vitamin C, kaempferol, flavonone and quercetin. Some studies have reported its antitumour, anti-ulcer, antibacterial and antifungal

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activities.[8-11] The present investigation is aimed at studying the immunomodulatory activity of the ethanolic extract of the stem bark of *Bauhinia variegata* Linn, in order to justify the traditional claims endowed upon this herbal drug as a rasayana.

MATERIALS AND METHODS

Plant Material

The stem bark of *Bauhinia variegata* Linn was collected from Pune, India. The plant specimen was authenticated by the "Botanical Survey of India" Pune, India, (voucher specimen No. Sasp 2)

Preparation of Ethanolic Extract of Stem Bark of *Bauhinia variegata* Linn

The stem bark of *Bauhinia variegata* Linn was dried in the shade and pulverized. The powder was treated with petroleum ether for dewaxing as well as to remove chlorophyll. The powder was cold macerated using ethanol (95%) as a solvent. The extract was dried at 60°C (yield - 9% w/w) and then suspended in 5% gum acacia for pharmacological studies.

Phytochemical studies showed the presence of flavonoids, triterpenes and saponin in the stem bark of *Bauhinia variegata* Linn, which are the class of compounds known to possess immunostimulant properties.

Experimental Animal

All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee.

Swiss albino mice weighing between 18 to 25 gm of either sex were used. The above-mentioned animals of either sex were purchased from the National Toxicology Center, Pune. Animals had free access to standard pellet diet and water *ad libitum*. Fresh animals were used for each experiment.

Drugs and Chemicals

Cyclophosphamide 100 mg/kg was used as a standard immunosuppressant. Carbon ink suspension: Pelican AG ink, Germany, was diluted eight times with saline and used for the carbon clearance test in a dose of 10 µl/gm body weight of mice.

Sheep red blood cells (SRBCs) were washed thrice with normal saline and adjusted to the required concentration for immunization and for challenge as an antigen.

Humoral Antibody Response[12]

The animals were divided into eight groups of six animals each. Animals in group I received orally 1l of 5% gum acacia, for 21 days. Group II received cyclophosphamide 100 mg/kg, p.o, on the nineth and sixteenth days. Animals in groups III, IV, and V with normal immune status were administered EBV at doses of 125, 250, 500 mg/kg/day, p.o, respectively, for 21 days. Animals in groups VI, VII and VIII received EBV at doses of 125, 250, 500 mg/kg/day, p.o, respectively, for 21 days plus cyclophosphamide 100 mg/kg, p.o, on the nineth and sixteenth days. On the seventh and fourteenth days of the study, mice from all the groups were immunized and challenged respectively, with 0.1 ml of 20% sheep RBC's (SRBC) in normal saline, intraperitoneally. Blood was withdrawn from all animals on the fourteenth and twenty-first days, from the retro-orbital plexus, under mild ether anesthesia, and centrifuged to obtain the serum. The antibody titre was determined using microtitre plates. Each well of a microtitre plate was filled initially with 25 µl of normal saline and 25 µl of serum was mixed with 25 µl of normal saline in the first well of the microtitre plate. Subsequently the 25 µl diluted serum was removed from the 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 carried out till the last well of the second row (twenty-first well), so that the antibody concentration of any of the dilutions is half of the previous dilution. Twenty-five microlitres of 1% SRBC was added to each well and the microtitre plates were incubated at 37°C for one hour and then observed for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titre. The antibody titre was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1, and the mean ranks of different groups were compared for statistical significance. The antibody titre obtained on the fourteenth day after immunization and on the twenty-first day after challenge with SRBCs were considered as the primary and secondary humoral immune responses, respectively.

Carbon Clearance Test[13]

The mice were divided into four groups of six animals each. The control group I orally received 1 ml of 5% gum acacia, while animals of treatment group II, III and IV were administered EBV at doses of 125, 250, 500 mg/kg/day, p.o., respectively for five days. Carbon ink suspension was injected via the tail vein to each mouse 48 hours after the five-day treatment. Blood samples (25 µl) were then withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 minutes after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (3 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula

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

where OD1 and OD2 are the optical densities at time t_1 and t_2, respectively.

Neutrophil Adhesion Test[14]

The mice were divided into four groups of six animals each. The control group I received 1 ml of 5% gum acacia orally, while animals of treatment groups II, III and IV were administered EBV at doses of 125, 250, 500 mg/kg/day, p.o., respectively daily for seven days. On the seventh day of treatment, blood samples from all the groups were collected by puncturing the retro-orbital plexus under mild ether anaesthesia. Blood was collected in vials, pre-treated with disodium EDTA, and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC), by fixing blood smears and staining with Field stain I and Leishman's stain. After the initial counts, blood samples were incubated with nylon fibre (80 mg/ml of blood sample) for 15 min at 37°C. The incubated blood samples were again analysed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Percent neutrophil adhesion was calculated as follows

$$\text{Neutrophil adhesion} = \frac{(\text{NIu} - \text{NIt})}{\text{NIu}} \times 100$$

Where,

NIu: Neutrophil Index before incubation with nylon fibres
NIt: Neutrophil Index after incubation with nylon fibres

Statistical Analysis

The results are expressed as mean ± S.E.M. Data was analysed by one-way ANOVA followed by the Tukey-Kramar multiple comparison test. Value of *P* less than 5% (i.e., $P < 0.05$) was considered statistically significant.

RESULTS

Humoral Antibody Response

Effect of ethanolic extract of stem bark of *Bauhinia variegata* on primary and secondary antibody response on H.A. Titre is shown in Table 1.

On day fourteenth day the EBV (500 mg/kg)-treated group with normal immune status showed significant rise ($P < 0.05$) in the H.A. titre when compared with the control group. A significant decrease ($P < 0.01$) in the H.A. 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, EBV administration produced a significant ($P < 0.05$) rise in the H.A. titre at doses of 250 and 500 mg/kg/p.o, respectively, when compared with the cyclophosphamide-treated group. On the twenty-first day the EBV-treated groups with normal immune status group showed a significant rise ($P < 0.05$) in the H.A. titre at a dose of 500 mg/kg/p.o when compared with the control group. A significant decrease ($P < 0.001$) in the H.A. titre was seen in the cyclophosphamide group when compared with the control group. In the immunosuppresed groups where the immunity was suppressed by administration of cyclophosphamide on day 16, EBV showed a significant rise ($P < 0.01$) in the H.A. titer at doses of 250 and 500 mg/kg/p.o, respectively, when compared with the cyclophosphamide group.

Carbon Clearance Test

Effect of the ethanolic extract of the stem bark of *Bauhinia variegata* on the phagocytic activity by the carbon clearance test is shown in Table 2.

The phagocytic activity of the reticuloendothelial system is generally measured by the rate of removal of carbon particles from the blood stream. There was a significant increase in phagocytic activity ($P < 0.01$) in the EBV 250 mg/kg- and 500 mg/kg-treated groups when compared with the control, indicating that EBV possesses a macrophage stimulatory activity.

Neutrophil Adhesion Test

Effect of the ethanolic extract of the stem bark of *Bauhinia variegata* on neutrophil activation by the Neutrophil Adhesion Test is shown in Table 3.

Bauhinia variegata increased the adhesion of neutrophils to nylon fibres, which correlated to the process of margination of neutrophils in blood vessels. The neutrophil adhesion was significantly increased ($P < 0.01$) in the EBV 250 mg/kg- and 500 mg/kg-treated groups when compared with the control.

Table 1: Humoral antibody response
Effect of ethanolic extract of stem bark of *Bauhinia variegata* on the primary and secondary antibody response on H.A.titre

Group no.	Group	Primary	Secondary
I	Control	4.66 ± 0.33	7.5 ± 0.22
II	Cyp	3.00 ± 0.25**a	5.5 ± 0.22***a
III	EBV	5.55 ± 0.34	8.0 ± 0.44
IV	EBV	5.83 ± 0.30	8.3 ± 0.33
V	EBV	6.16 ± 0.30*a	9.0 ± 0.36*a
VI	EBV + Cyp	3.83 ± 0.40	6.16 ± 0.40
VII	EBV + Cyp	4.66 ± 0.42*b	7.83 ± 0.55**b
VIII	EBV + Cyp	4.83 ± 0.40**b	8.33 ± 0.21***b

EBV: Ethanolic extract of stem bark of *Bauhinia variegate* as (Mean ± S.E.M), n = 6, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$; Cyp: Cyclophosphamide; Values are expressed, a: when compared with control (Group I); b: when compared with Cyclophosphamide Control (Group II); (Statistically analysed by one way ANOVA followed by Tukey-Kramar multiple comparison tests)

Table 2: Carbon clearance test
Effect of the ethanolic extract of stem bark of *Bauhinia variegata* on phagocytic activity by carbon clearance test

Group no.	Group	Phagocytic index
I	Control	0.011 ± 0.00096
II	EBV	0.016 ± 0.0016
III	EBV	0.019 ± 0.0029**
IV	EBV	0.023 ± 0.0017***

EBV: ethanolic extract of stem bark of *Bauhinia variegata*; Values are expressed as (Mean ± S.E.M), n = 6, ** $P < 0.01$ and *** $P < 0.001$; EBV-treated groups were compared with control; (Statistically analysed by one way ANOVA followed by Tukey-Kramar multiple comparison tests)

Table 3: Neutrophil adhesion test
Effect of ethanolic extract of stem bark of *Bauhinia variegata* on neutrophil activation by neutrophil adhesion test

Group no.	Group	% Neutrophil adhesion
I	Control	30.77 ± 1.26
II	EBV	31.25 ± 1.46
III	EBV	39.48 ± 1.66**
IV	EBV	41.95 ± 1.58***

EBV: ethanolic extract of stem bark of *Bauhinia variegata*; Values are expressed as (Mean ± S.E.M), n = 6, ** $P < 0.01$ and *** $P < 0.001$; EBV-treated groups were compared with control; (Statistically analysed by one way ANOVA followed by Tukey-Kramar multiple comparison tests)

DISCUSSION

Immunomodulatory agents of the plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system.[15] Different types of immune responses fall in two categories, specific or adaptive immune response and nonadaptive or nonspecific immune response. Specific immune response responds to the challenge with a high degree of specificity as well as the remarkable property of "memory". Typically, there is an adaptive immune response against an antigen within five or six days after the initial exposure to a particular antigen. Exposure to the same antigen some time in the future results in a memory response. The immune response to the second challenge occurs more quickly than the first, is stronger, and is often more effective in neutralizing and clearing the pathogen. Memory response generates a life-long immunity following an infection. The two key features of the adaptive immune response are thus specificity and memory. The major agents of adaptive immunity are lymphocytes, antibodies and the other molecules they produce. Because adaptive immune responses require some time to marshal, innate immunity provides the first line of defence during the critical period, just after the host's exposure to a pathogen. In general, most of the micro-organisms encountered by a healthy individual are readily cleared within a few days by the defence mechanism of the innate immune system before they activate the adaptive immune system. The nonspecific immune response provides the first line defence against infection. Most components of innate immunity are present before the onset of infection and constitute a set of disease resistance mechanisms that are not specific to particular pathogens, but which include cellular and molecular components that recognize frequently encountered pathogens. Phagocytic cells, such as macrophages and neutrophils, barriers such as skin and a variety of antimicrobial compounds synthesized by the host, all play important roles in innate immunity.

When mice are 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 are processed. Small highly antigenic peptides are combined with MHC class II molecules. B cells with receptors for antigens bind and internalize it into an endosomal compartment and process and present it on MHC class II molecules to the TH_2 cells. These B cells are triggered to proliferate, giving rise to clones of a large number of daughter cells. Some of the cells of these expanding clones serve as memory cells, others differentiate and become plasma cells that make and secrete large quantities of specific antibodies. During a primary response, IgM is secreted initially, often followed by a switch to an increasing proportion of IgG.[16,17] The magnitude of the secondary antibody response to the same antigen is amplified in terms of antibody production. In the present study, assessment of humoral immunity was carried out using the H.A. titre. The anti-SRBC antibody titre was raised in EBV-treated groups in doses of 125, 250 and 500 mg/kg with normal immune status, but was found statistically significant only at the dose of 500 mg/kg, when compared to the control group in both primary and secondary antibody titre responses.

In the immunosuppressed groups Cyclophosphamide was used as an immunosuppressant, as it selectively suppressed humoral immunity, by exerting a depressive effect on antibody production, if given after antigenic stimulation.[18] This may be due to the interference with helper T-cell activity.[19] In the immunosuppressed group EBV 250, 500 mg/kg/p.o significantly inhibited cyclophosphamide-induced suppression of humoral immunity, indicating that EBV counteracts the suppression of both primary and secondary humoral responses induced by cyclophosphamide.

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.[20] EBV significantly increases the phagocytic activity when compared to the control.

Cytokines are secreted by activated immune cells, for margination and extravasation of the phagocytes, mainly polymorphonuclear neutrophils, often just called neutrophils. These constitute the majority of the blood leucocytes. Similar to monocytes they too migrate into tissues, in response to certain stimuli. Experimentally, activation of neutrophils can be studied by the neutrophil adhesion test. EBV significantly evokes an increase in the adhesion of neutrophils to nylon fibres, which co-relates to the process of margination of cells in blood vessels. Neutrophil adhesion is significantly increased by EBV when compared to the control.

The present investigation therefore reveals that EBV certainly possesses immunomodulatory properties. However, further studies are warranted to elucidate the exact mechanism of action.

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