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RESEARCH ARTICLE

In-vitro Immunomodulatory Activity of extracts of *Bauhinia vareigata* Linn Stem Bark on Human Neutrophils

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ABSTRACT

The in-vitro immunomodulatory activity of *Bauhinia variegata* Linn (Caesalpiniaceae) stem bark extracts on human neutrophils was carried out in the present study. The acetone-water, aqueous extracts and isolated compound (tannin) of *Bauhinia variegata* stem bark were screened for their possible immunomodulatory activity by assessing nitroblue tetrazolium test, phagocytosis of killed *Candida albicans*, candidacidal assay, neutrophil locomotion and chemotaxis. All the extracts were tested at concentrations viz. 10 μg/ml, 20 μg/ml, 50 μg/ml, 100 μg/ml and 1000 μg/ml. The acetone:water and isolated compound of *Bauhinia variegata* Linn stem bark showed predominantly significant activity on *in vitro* human neutrophils in all parameters, which are comparable to standard and control at different concentration indicating the possible immunostimulating effect.

Keywords: *Bauhinia variegata Linn stem bark, Immunomodulatory, Human neutrophils, Tannins, Acetone-water*

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases [1]. The degree to which the patient becomes abnormally susceptible to infections by this microbial environment depends on the extent of immunosuppression. The suppression of the immune system is characterized by reduction in number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This immunosuppression allows opportunistic pathogens to overwhelm the host to cause secondary infections [2]. This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs [3].

Chemotherapeutic agents available today have mainly immunosuppressive activity. Most of them are cytotoxic and exerts a variety of side effects. This has given rise to stimulation in the search for investigating natural resources showing immunomodulatory activity. Many medicinal plants are known to have immunomodulatory properties and maintain organic resistance against infection by re-establishing the body's immune system such as *Azadirachta indica* [4], *Terminalia chebula* [5] and *Lawsonia alba* [6]. The phytochemical constituents like terpenoids, steroids, proteins and tannins [7] are considered to exhibit this immunomodulatory property. Flavonoids from *Plantago species* [8] and *Syzygium samarangense* [9] have also shown immunomodulatory activity.

A number of *in vitro* and *in vivo* test systems are available for assessing immunomodulatory activity. Phagocytosis is one such widely-used method for screening the immune response [10]. Phagocytosis is the primary defence mechanism against any foreign bodies entering the body, which is offered by neutrophils and macrophages. The process of phagocytosis consists of sequential stages such as motility, adhesion to microorganisms, ingestion of microorganisms, degranulation and intracellular killing of microorganisms [11].

Bauhinia variegata Linn, commonly known as Kanchnar, Mountain Ebony, Hong Kong Orchid Tree, belonging to family Caesalpiniaceae is a medium-sized, deciduous tree, found throughout India, ascending to an altitude up to 1800 m in Himalayas. The Hong Kong Orchid Tree botanically known as genus Bauhinia. The origin of the Hong Kong Orchid Tree is China [12]. *Bauhinia variegata* Linn bark has been widely used as an Ayurvedic traditional medicine in ulcers, leprosy,

dysentery, tuberculosis, skin ailments, malaria and an antidote to snake poison [12-14]. It is also used as tonic, tissue-builder and stimulant. Beside this, the plant was proved to have antitumour activity in Dalton's ascitic lymphoma [15], N-nitrosodiethylamine-induced liver tumors and human cancer cell lines [16]. It also possess the anti-inflammatory activity due to flavonol glycoside 5,7,3',4'-tetrahydroxy-3-methoxy-7-O-alpha-l-rhamnopyranosyl(1-->3)-O-beta-galactopyranoside [17].

Phytochemical analysis of the root bark of *Bauhinia variegata* Linn yielded a flavanone (2S)-5,7-dimethoxy-3',4'-methylenedioxyflavanone and a new dihydrodibenzoxepin, 5,6-dihydro-1,7-dihydroxy-3,4-dimethoxy-2-methyldibenz [b,f] oxepin [18], 5-hydroxy 7,3',4',5'-tetra-methoxyflavone 5-O-beta-D-xylopyranosyl-(1-2)-alpha-L-rhamnopyranoside [19]. A new phenanthraquinone, named bauhinione, has been isolated from *Bauhinia variegata* Linn, and its structure has been elucidated as 2, 7-dimethoxy-3-methyl-9, 10-dihydrophenanthrene-1,4-dione on the basis of spectroscopic analysis [20]. Tannins are the chief chemical constituents present in *Bauhinia variegata* stem bark [21] and are known to possess immunomodulatory properties. The ellagic acid (tannin) present in *Terminalia arjuna* bark has shown strong antioxidant activity [22-24].

Survey of literature revealed that *Bauhinia variegata* Linn has been suggested for immunomodulatory activity [25]. In the present study, we have evaluated immunomodulatory potency of *Bauhinia variegata* stem bark extracts and isolated compound using different *in vitro* methods for locomotion, phagocytic and intracellular killing potency of neutrophils, which are subsequent events involved in the process of phagocytosis by neutrophils.

MATERIALS AND METHODS

Plant material

The stem bark of *Bauhinia variegata* Linn was collected from local area of Belgaum, Karnataka, India in the month of June 2005 and authenticated by Botanical Survey of India, Pune-1, Maharashtra, India. A voucher specimen of plant material is deposited in the herbarium of Department of Pharmacognosy, K.L.E.S. College of Pharmacy, Belgaum, Karnataka, India for future reference.

Preparation of extracts

Stem bark was shade dried at room temperature and powdered to #40 mesh particle size. The powder (250 g) of crude plant was defatted with petroleum-ether, was subjected to extraction with acetone:water (70:30), by using Soxhlet apparatus, and with chloroform-water I.P. by maceration as solvents at room temperature. The extracts were filtered and evaporated at 40°C under vacuum and the residue was freeze-dried.

Phytochemical tests [26]

Bauhinia variegata stem bark extracts were separately tested for the presence of tannins, alkaloids, carbohydrates, anthraquinones, flavonoids, saponins and coumarins.

Isolation and characterization of Tannins

About 5 g of dried acetone:water extract of stem bark of *Bauhinia variegata* was digested with boiling water for 30 min. To this saturated solution, lead acetate was added to precipitate out tannins. The solution was filtered whereby residue containing tannins and filtrate was discarded. Residue was collected in water and hydrogen sulphide (H_2S) gas was passed to remove excess of lead acetate as lead sulphide. The solution was filtered; residue, which contained excess of lead acetate, was discarded. The filtrate was concentrated to get tannins. The tannin was confirmed using benzene:glacial acetic acid (1:1) as solvent system for thin layer chromatography (TLC). The isolated tannin was also subjected to spectral characterization (UV, FT-IR and HPTLC).

Preparation of test sample

Samples solutions for *in vitro* studies were prepared by dissolving 10 mg of acetone:water and aqueous extracts of *Bauhinia variegata* stem bark in 0.5 ml water and with phosphate buffer salt solution according to concentration range from 10 μg/ml, 20 μg/ml, 40 μg/ml, 100 μg/ml and undiluted (1000 μg/ml) extract.

Study of immunomodulatory activity

Nitroblue Tetrazolium (NBT) Test [27, 28]

A suspension of leucocytes (5 x 10^6/ml) was prepared in 0.5 ml of PBS solution in 7 tubes. A volume of 0.1 ml phosphate buffer saline solution (PBS as control) and 0.1 ml of endotoxin-activated plasma (standard) was added to the 1st and 2nd tube respectively and to the other 5 tubes, 0.1ml of different concentrations (10, 20, 40, 100 and 1000 μg/ml) of test samples were added. About 0.2 ml of freshly-prepared 0.15% NBT solution was added to each tube and incubated at 37°C for 20 min and then centrifuged at 400g for 3-4 min to discard the supernatant. The cells were re-suspended in the small volume of PBS solution. A thin film was made with the drop on a slide, dried, fixed by heating, counterstained with dilute Carbol-fuchsin for 15 sec. The slide was washed under tap water, dried and focused under 100× oil immersion objective. 200 neutrophils were counted for the percentage of NBT-positive cells containing blue granules/lumps.

Phagocytosis of killed Candida albicans [27]

Preparation of Candida albicans suspension

The *Candida albicans* culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button at the bottom and supernatant was discarded. The cell button was washed with sterile Hank's Balanced Salt Solution (HBSS) and centrifuged again. This step was repeated three to four times. The final cell button was mixed with a mixture of sterile

HBSS and human serum in proportion of 4:1. The cell suspension of concentration 1×10^8 was used for the experiment.

Slide preparation

Human blood (0.2 ml) was obtained by finger prick method on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. The blood clot was removed very gently and slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with predetermined concentration of test sample and incubated at 37°C for 15 min. The PMNs were covered with *Candida albicans* suspension and incubated at 37°C for 1 hour. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis evaluation

The mean number of Candida cells phagocytosed by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (10, 20, 40, 100 and 1000 µg/ml) of test samples. A number of 200 neutrophils were examined to count the number of ingested Candida associated with each cell. The mean particle number associated with each cell was calculated.

Candidacidal Assay [27]

A suspension of leucocytes (7×10^6/ml) was prepared in 0.25 ml of Hank's solution in 7 tubes. A volume of 0.25 ml Hank's solution (control) and 0.25 ml of pooled serum (standard) was added to the 1st and 2nd tube respectively, and to the other 5 tubes added 0.25 ml of different concentrations (10, 20, 40, 100 and 1000 µg/ml) of test samples. A volume of 0.25 ml of *Candida albicans* suspension was added to each tube and incubated at 37°C in water bath for 60 min with shaking every 15 min. After 30 min, 0.1 ml solution was taken on glass slides from each tube to make thin films. Slides were stained with Giemsa stain and observed at 100× lens. These should show that the majority of the *Candida* organisms have been ingested by the leucocytes. At the end of one-hour incubation, 0.25 ml of 2.5% sodium deoxycholate was added to each tube and mixed. The deoxycholate lyses the leucocytes but does not damage the *Candida* cells. A volume of 4 ml 0.01% methylene blue was added to each tube and mixed, centrifuged at 1500 g at 4°C for 10 min. The supernatant containing methylene blue was carefully removed with a pasteur pipette leaving about 0.5 ml to resuspend the organisms. The suspension was put in an ice bath until ready for counting. About 300 *Candida* cells were counted using an improved Neubauer counting chamber. The proportions of dead cells, i.e. those which have taken up methylene blue were determined.

Neutrophil locomotion and chemotaxis test [27]

Neutrophil cell suspension was prepared in phosphate buffer saline solution (PBS) at about 10^6 cells/ml. The lower compartment of chemotactic chamber (5-ml beaker) was filled with appropriate chemotactic reagents pre-adjusted to a pH of 7.2 e.g. chamber 1-PBS solution (control); chamber 2-Casein 1 mg/ml (standard); and chamber 3, 4, 5, 6 and 7 with different concentrations (10, 20, 40, 100 and 1000 µg/ml) of test sample. The upper compartment (1ml syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37°C for 180 min. The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 2 min and then stained with Haematoxylin dye for 5 min. The fixed filters were observed under microscope using 100× lens and number of neutrophil cells reached to the lower surface of filter was counted.

Statistical analysis [29]

The values were expressed in mean ± SEM (n = 4). The results were analysed by using one-way analysis of variance (ANOVA) followed by Dunnet's 't' test determine the statistical significance.

RESULTS

The preliminary phytochemical investigation revealed the presence of tannins. Acetone:water extract, chloroform:water extract and isolated compound have shown significant activity in all the parameters only at higher concentrations.

Nitroblue tetrazolium (NBT) test

The acetone-water, aqueous extracts of stem bark and isolated compound (tannin) has stimulated the neutrophils to phagocytic activity to the extent of 67.95% and 64.95% for acetone-water extract; 60.67% and 40.83% for aqueous extract; 44.81% and 36.40% for isolated compound at concentration of 1000 µg/ml and 100 µg/ml respectively, when compared to normal control (22.53% and 22.17%) and to standard i.e. endotoxin-activated plasma (57.97% and 52.21%) at the same concentrations. However, at low concentration of 40 µg/ml, 20 µg/ml and 10 µg/ml, the stimulation of neutrophil was comparatively lower. Significant stimulation of neutrophil was observed. The results are shown in Table 1.

Table 1. Percentage of Reduced Neutrophils after treatment with extracts of *Bauhinia variegata* stem bark by Nitroblue Tetrazolium Test (NBT)

Samples (Extracts)	Concentration (µg/ml) of extracts of *Bauhinia variegata* stem bark in respective solvent				
	1000	100	40	20	10
Phosphate Buffer Saline	22.53 ± 0.32	22.17 ± 0.31	20.12 ± 0.06	19.37 ± 0.04	19.20 ± 0.03
Endotoxin-activated plasma	57.97 ± 0.95	52.21 ± 0.15	52.10 ± 0.05	51.49 ± 0.25	50.37 ± 0.11
Acetone-Water Ext.	67.95 ± 0.09*	64.95 ± 0..67*	24.06 ± 0.04	23.05 ± 0.05	18.49 ± 0.06
Aqueous Extract	60.67 ± 0.15*	40.83 ± 0.22*	38.69 ± 0.07*	31.46 ± 0.06*	25.19 ± 0.04
Isolated Compound	44.81 ± 0.18*	36.40 ± 0.11*	32.70 ± 0.05*	28.84 ± 0.07	26.81 ± 0.03

The data are expressed as mean percentage reduced neutrophils ± standard error mean. Significant difference from Positive Control (Std) by one way ANOVA followed by Dunnet's 't' test. (n = 4) * $p<0.01$

Phosphate Buffer Saline (Normal Control), Endotoxin-activated plasma (Positive Control)

Table 2. Particle number phagocytosis of killed *Candida albicans* after treatment with extracts of *Bauhinia variegata* stem bark

Samples (Extracts)	Concentration (µg/ml) of extracts of *Bauhinia variegata* stem bark in respective solvent				
	1000	100	40	20	10
Pooled Serum	6	4-5	4	3-4	3
Acetone-Water Ext.	4-5	5	4	3	3
Aqueous Extract	4	4	3	2-3	2
Isolated Compound	4-5	3	4	2	2

The data are expressed as mean particle number phagocytosed.

Pooled Serum (Positive Control)

Table 3. Percentage of Killed Candida after treatment with extracts of *Bauhinia variegata* stem bark by Candidacidal Assay

Samples (Extracts)	Concentration (µg/ml) of extracts of *Bauhinia variegata* stem bark in respective solvent				
	1000	100	40	20	10
Hank's solution	20.50 ± 0.29	18.75 ± 0.25	18.25 ± 0.25	17.50 ± 0.29	17.25 ± 0.48
Pooled Serum	33.75 ± 0.48	31.75 ± 0.25	30.50 ± 0.29	30.25 ± 0.25	29.75 ± 0.25
Acetone-Water Ext	27.75 ± 0.25*	26.00 ± 0.41*	23.50 ± 0.29*	21.75 ± 0.25	21.50 ± 0.29
Aqueous Extract	26.25 ± 0.25*	22.75 ± 0.25*	19.75 ±0.25	19.50 ± 0.28	18.25 ± 0.25
Isolated Compound	31.25 ± 0.48*	30.25 ± 0.25*	29.25 ± 0.48**	22.75 ± 0.25	19.50 ± 0.29

The data are expressed as mean percentage of killed candida. Significant difference from Positive Control (Std.) by One Way ANOVA followed by Dunnet's 't' test. (n = 4); *$p<0.01$, **$p<0.05$, Hank's solution (Normal Control), Pooled Serum (Positive Control)

Phagocytosis of killed Candida albicans assay

The acetone-water, aqueous extracts of stem bark and isolated compound (tannin) has stimulated the phagocytosis of killed *Candida albicans*. The mean particle numbers (MPN) were found to be 4-5, 5 and 4 for acetone:water extract; 4, 3 and 3 for aqueous extract; 4-5, 4 and 4 for isolated compound at concentration of 1000 µg/ml, 100 µg/ml and 40 µg/ml respectively, when compared to positive control (standard) i.e. pooled serum (6, 4-5 and 4) at the same concentrations. However, at low concentration of 20 µg/ml and 10 µg/ml, the stimulation of phagocytic activity was comparatively lower. The results are shown in Table 2.

Candidacidal assay

The acetone:water, aqueous extracts of stem bark and isolated compound (tannin) has shown significant candidacidal activity to the extent of 27.75% and 26.00% for acetone:water extract; 26.25% and 22.75% for aqueous extract; 31.25% and 30.25% for isolated compound at concentration of 1000 µg/ml and 100 µg/ml respectively, when compared to standard i.e. pooled serum (33.75% and 31.75%) and to normal control (20.50% and 18.75%) at the same concentrations. At the concentration of 40 µg/ml, isolated compound has shown 29.25% candidacidal activity. However, at low concentration of 40 µg/ml, 20 µg/ml and 10 µg/ml, the stimulation of neutrophil was comparatively lower i.e. 23.50 %, 21.75 % and 21.50% for acetone-water extract; 19.75%, 19.50% and 18.25 % for aqueous extract and 22.75%, 19.50% for isolated compound respectively. Significant stimulation of candidacidal activity was observed. The results are shown in Table 3.

Neutrophil locomotion and chemotaxis

The aqueous extract of stem bark has shown very significant chemotactic activity at all concentrations. The mean number of neutrophil per field for the acetone:water, aqueous extracts of bark and isolated compound (tannin) found to be 135.0 , 132.0 and 129.5 for acetone:water extract; 157.3, 156.5 and 150.5 for aqueous extract and 138.5, 130.8 and 130.3 for isolated compound at concentration of 1000µg/ml, 100µg/ml and 40µg/ml respectively, when compared to standard

Table 4. Number of Neutrophils per field after treatment with extracts of *Bauhinia variegata* stem bark *by Neutrophil Locomotion and Chemotactic Activity*

Samples (Extracts)	Concentration (µg/ml) of extracts of *Bauhinia variegata* stem bark in respective solvent				
	1000	100	40	20	10
Phosphate Buffer Saline	15.25 ± 0.25	14.25 ± 0.25	13.75 ± 0.49	13.25 ± 0.48	12.50 ± 0.29
Casein	151.15 ± 0.29	149.8 ± 0.25	149.0 ± 0.41	147.5 ± 0.29	146.8 ± 0.48
Acetone-Water Extract	135.0 ± 0.41*	132.0 ± 0.41*	129.5 ± 0.29*	120.3 ± 0.48	110.8 ± 0.48
Aqueous Extract	157.3 ± 0.48*	156.5 ± 0.29*	150.5 ± 0.29**	150.5 ± 0.29*	150.0 ± 0.41*
Isolated Compound	138.5 ± 0.29*	130.8 ± 0.25*	130.3 ± 0.25*	120.3 ± 0.25	108.8 ± 0.48

Mean number of Neutrophils per field ± Standard Error Mean Significant difference from Positive Control (Std.) by One way ANOVA, followed by Dunnet's 't' test. (n = 4); *p<0.01, **p<0.05, Phosphate Buffer Saline (Normal Control), Casein (Positive Control)

i.e. Casein (151.15, 149.8 and 149.0) and to normal control (15.25, 14.25 and 13.75) at the same concentrations. At concentration of 40 µg/ml, aqueous extract has shown more significant activity (150.5). However, at low concentration of 20 µg/ml and 10 µg/ml, the stimulation of chemotactic activity was comparatively lower i.e. 120.3 and 110.8 for acetone-water extract and 120.3, 108.8 for isolated compound respectively. Significant stimulation of chemotactic activity was observed. The results are shown in Table 4.

DISCUSSION

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating the non-specific immune system. However, there is a need to systemic studies on medicinal plants to substantiate the therapeutic claims made regarding their clinical utility. In the present study, the acetone:water extract of *Bauhinia variegata* Linn stem bark significantly increased the phagocytic function of human neutrophils, when compared with control indicating the possible immunostimulating effect. The engulfment of microorganisms by leukocytes called phagocytosis which is one of the main defence mechanisms of an organism. The *Bauhinia variegata* Linn stem bark extracts have significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells reached the lower surface of filter; thereby *Bauhinia variegata* Linn stem bark extracts acts as chemo-attractant. The final step of phagocytosis is the intracellular killing of micro-organisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, and activation which is also necessary for normal microbicidal activity. The *Bauhinia variegata* Linn stem bark extracts have significantly increased the intracellular reduction of NBT dye to formazan (deep blue compound) by the neutrophils confirming the intracellular killing property and overall metabolic integrity of phagocytosing neutrophils.

Tannins obtained from the stem bark of *Bauhinia variegata* Linn found to possess immunomodulatory property. Thus, our results support the immunomodulatory activity of *Bauhinia variegata*, claimed as enhancer of general immunity against various physical and mental disorders in the indigenous system of medicine. Thus, it can also be concluded that *Bauhinia variegata* Linn stem bark possess immunomodulatory activity which may be due to the presence of tannins.

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