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Content

Effect of *Baliospermum montanum* root extract on phagocytosis by human neutrophils

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To study the effect of alcohol extract of roots of *Baliospermum montanum* on neutrophil phagocytic function. The different concentration of (25,50,100 mg/ml) extract of roots of *Baliospermum montanum* was subjected to study its effect on different *in vitro* methods of phagocytosis such as neutrophil locomotion, chemotaxis, immunostimulant activity of phagocytosis of killed *Candida albicans* and qualitative nitroblue tetrazolium test using human neutrophils. This preliminary study revealed that *Baliospermum montanum* extract has stimulated, chemotactic, phagocytic and intracellular killing potency of human neutrophils at different concentrations. From the results obtained, it can be observed that the alcohol extract of *Baliospermum montanum* stimulates cell-mediated immune system by increasing neutrophil function.

Key words: Immunostimulant, *Baliospermum montanum*, nitroblue tetrazolium test, phagocytosis

INTRODUCTION

The immune system is known to be involved in the etiology as well as the pathophysiologic mechanism of many diseases. Immunology is thus probably one of the most rapidly developing areas of biomedical research and has great promises with regard to the prevention and treatment of a wide range of disorders, inflammatory diseases of the skin, gut, respiratory tract, joints and central organs. In addition, infectious diseases are now primarily considered immunological disorders, while neoplastic diseases and organ transplantation and several autoimmune diseases may involve in an immunosuppressive state (Ziauddin, 1996).

The function and efficacy of the immune system may be influenced by many exogenous factors like food and pharmaceuticals, physical and psychological stress and hormones, etc., resulting in their immunostimulation or immunosuppression. The healthy state is believed to be based on a sophisticated fine-tuning of immunoregulatory mechanism (Mallurwar, 2006).

Suppressive and cytotoxic activity affecting the function of immune system has been reported by many of the synthetic and natural therapeutic agents. Among the synthetic substances, azathioprin and cyclophasphmide are alkylating agents resulting in the cross-linking of DNA and cause inhibition of DNA synthesis. The major drawbacks of this drug are myelosuppressive, which is undesirable. Immunomodulator of herbal origin appears to be a better alternative to overcome the above problem (Patwardhan, 1991).

Baliospermum montanum Muell Arg of family Euphorbiaceae is a stout under-shrub with herbaceous branches from the roots (Variers, 1994). It is found in tropical and subtropical Himalaya from Kashmir eastwards to Arunachal Pradesh. It is reported to contain axillarenic acid, baliospermin and montanin, which possess a wide range of activities such as anthelmintic, diuretic, purgative and bronchitis (Chattrjee, 1994). The survey of literature reveals that the whole plant and roots of *Baliospermum montanum* is found to be used in traditional system of medicine as a tonic (Variers, 1994). However, immunomodulatory activity of *Baliospermum montanum* has not been reported or scientifically investigated.

Thus, in our present study, we have attempted to evaluate the immunomodulatory potency of alcohol extract of roots of *Baliospermum montanum* using different *in vitro* methods for locomotion, phagocytic and intracellular killing potency of neutrophil which are subsequent events involved in the process of phagocytosis by neutrophils.

MATERIALS AND METHODS

Plant Material

The roots of *Baliospermum montanum* were collected from the local areas of Belgaum and authentified at the Botanical

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Survey of India, Koregaon, Pune. Voucher specimen (BSI/WC/Tech 272) of the plant material is kept at Pharmacognosy museum of K.L.E.S.'s College of Pharmacy, Belgaum. The freshly collected roots from the plant were shade-dried at room temperature and powered until able to pass through sieve number 40.

Preparation of the Extract

The standardized coarse powder of roots was subjected to successive solvent extraction using Soxhlet apparatus with different solvents in increasing polarity. The dark brown filtrate obtained was concentrated. The crude alcohol extract was subjected to phytochemical investigation.

Preparation of the Test Sample

Sample for *in vitro* study was prepared by dissolving 2.5 g of crude extract in 25 ml PBS (phosphate buffered solution) to obtain a solution of 100 mg/ml. From this stock solution, different working dilutions were prepared to get a concentration range of 25, 50 and100 µg/ml. Neutrophils of the blood withdrawn from normal human volunteers were used to study the activity. PBS was used as a vehicle.

Neutrophil Locomotion and Chemotaxis

Neutrophil cell suspension was prepared in phosphate buffered solution 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.

For example, chamber 1 - PBS solution (control), chamber 2 - casein 1 mg/ml (standard) and chamber 3, 4, 5 with different concentrations (25, 50 and 100 µg/ml) of test sample.

The upper compartment (1 ml syringe) was filled with neutrophil cell suspension and wet filter (millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartments were placed on to 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 hematoxylin dye for 5 min. The fixed filters were observed under microscope using 100× lens and the number of neutrophil cells reaching the lower surface of the filter was counted (Wilkinson, 1981).

Immunostimulant Studies by Slide Method

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 the supernatant was discarded. The cell button was washed with sterile Hanks Balanced Salt solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in a 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 the slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophil (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with a predetermined concentration of test sample and incubated at 37°C for 15 min. The PMNs were covered with *Candida albicans* slide and incubated at 37°C for 1 h. The slide was drained, fixed with methanol and stained with Giemsa stain. Positive control was tested by preparing the slide in the same way with pooled normal human serum.

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 (25, 50 and 100 mg/ml) of test sample. Immunostimulation in % was calculated by using the following equation (Wilkinson, 1981).

$$\text{Stimulation (\%)} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI Control}} \times 100$$

Qualitative Nitroblue Tetrazolium Test (NBT)

A suspension of leukocytes (5×10^6/ml) was prepared in 0.5 ml of PBS solution in five test tubes, 0.1 ml of PBS solution (control) and 0.1 ml of endotoxin-activated plasma (standard) was added to the 1st and 2nd tubes, respectively, and to the other three tubes 0.1 ml of different concentrations (25, 50, 100 µg/ml) of test sample was added. 0.2 ml of freshly made 0.15% NBT solution was added to each tube and incubated at 37°C for 20 min and centrifuged at 400 rpm for 3-4 min to discard the supernatant. The cells were re-suspended in a small volume of PBS solution.

A thin film was made with the drop on a slide, dried and fixed by heating, counter-stained by dilute Carbol-fuschin for 15 s. The slide was washed under tap water, dried and observed under 100× oil immersion objective. Two hundred neutrophils were counted for the % of NBT positive cells containing blue granules/lumps (Wilkinson, 1981).

Statistical Analysis

The values are expressed in mean ± SEM. The results were analyzed by one-way analysis of variance (ANOVA)

followed by Dunnet's '*t*' test to determine the statistical significance (Kulkarni, 1999).

RESULTS

The preliminary phytochemical investigation reveals the presence of tannins, saponins, flavonoids and glycosides. The alcohol extract of roots of *Baliospermum montanum* has caused a significant increase in the movement of a number of neutrophils from the upper compartment to the lower surface of the filter in a dose-dependent manner (Table 1), stimulation of phagocytosis of *Candida albicans* by neutrophils (Table 2) and also increase in the percentage of NBT-positive cells containing the reduced NBT dye (Table 3). When compared with control samples containing PBS solution.

Effect of alcohol extract of *Baliospermum montanum* roots on neutrophil locomotion and chemotaxis and neutrophil phagocytosis were quantified by qualitative NBT test and the results of sample groups were compared with control.

Table 1: Effect of alcohol extract of roots of *Baliospermum montanum* on neutrophil locomotion and chemotaxis

Sl. no.	Groups	Concentration mg/ml	Mean number of neutrophil/field
1	Control (PBS)	-	6.62 ± 0.70
2	Standard (Casein)	01	71.29 ± 1.28*
3	*B.montanum* extract	25	46.29 ± 1.32*
4	*B.montanum* extract	50	50.58 ± 1.42*
5	*B.montanum* extract	100	53.21 ± 1.50*

Values are mean ± SEM (n = 3). *P < 0.001 compared to control group

Table 2: Effect of alcohol extract of roots of *Baliospermum montanum* on neutrophil phagocytosis

Sl. no.	Groups	Concentration mg/ml	Mean number of neutrophil/field
1	Control (Pooled Plasma Serum)	-	4.89 ± 0.87
2	*B.montanum* extract	25	27.38 ± 1.09*
3	*B.montanum* extract	50	30.14 ± 1.25*
4	*B.montanum* extract	100	34.65 ± 1.25*

Values are mean ± SEM. *P < 0.001 compared to control group

Table 3: Effect of alcohol extract of roots of *Baliospermum montanum* on quantitative nitroblue tetrazolium test

Sl. no.	Groups	Concentration mg/ml	% NBT positive cells
1	Control (PBS)	-	21.32 ± 1.07
2	Endotoxin activated plasma	-	76.05 ± 0.95
3	*B.montanum* extract	25	61.49 ± 0.88*
4	*B.montanum* extract	50	65.38 ± 1.20*
5	*B.montanum* extract	100	85.16 ± 1.09*

Values are mean ± SEM (n = 3). *P < 0.001 compared to control group

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 subject such medicinal plants to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility (Fulzele, 2003).

Recently, there is an enthusiasm towards exploration of a novel group of compounds from natural sources that modulate the immune response of living systems and influence the disease process (Gulati, 2002).

In the present study, alcohol extract of roots of *Baliospermum montanum* significantly increased the phagocytic function of human neutrophils when compared to control indicating the possible immunostimulating effect. The *Baliospermum montanum* extract has significantly increased the neutrophil chemotactic movement indicated by the increase in the number of cell reached by the micro-organism after slide method which provides a rapid and simple means of assessing the overall phagocytic process by the neutrophils.

The alcohol extract of roots of *Baliospermum montanum* has significantly increased the ingestion of *Candida albicans* by neutrophils. The alcohol extract of roots of *Baliospermum montanum* has also significantly increased the intercellular reduction of NBT dye to formazen (deep blue compound) by the neutrophils, confirming the intracellular killing property of phagocytosing neutrophils.

From the results obtained, it can be concluded that the alcohol extract of the roots of *Baliospermum montanum* has exhibited significant effect on phagocytosis by human neutrophils and chemotactic locomotion of neutrophils. Thus, the plant can be further explored for its phytochemical profile to identify the active constituents responsible for the abovementioned activities.

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