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Effect of *Enhydra fluctuans* Lour. leaf extract on phagocytosis by human neutrophils

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Abstract

Objective: To study the effect of aqueous extract of leaves of *Enhydra fluctuans* on neutrophil phagocytic function. Methods: The different concentrations of (25,50,100µg/ml) of extract of leaves of *Enhydra fluctuans* 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 Nitro Blue Tetrazolium test using human neutrophils. Result: This preliminary study revealed that *Enhydra fluctuans* extract has stimulated chemotactic, phagocytic and intracellular killing potency of human neutrophils at the different concentration. Conclusion: From the results obtained it can be observed that the aqueous extract of *Enhydra fluctuans* leaves stimulates cell-mediated immune system by increasing neutrophil function phagocytic activity.

Key Words: Immonostimulant activity, *Enhydra fluctuans*, neutrophils, Phagocytosis.

1. Introduction

The immune system is known to be involved in the etiology as well as 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 prevention and treatment of wide range of disorders, inflammatory diseases of 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 [1].

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-tunning of immunoregulatory mechanism [2].

Suppressive and cytotoxic activity affecting the function of immune system has been reported many of the synthetic and natural therapeutic agents. Among the synthetic substances, azathioprin and cyclophasphmide is an

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alkylating agent resulting in the cross linking of DNA and causes inhibition of DNA synthesis. The major drawbacks of this drug are myelosuppressive, which is undesirable. Immunomodulator of herbal origin appearing to be a better alternative to overcome the above problem [3].

Enhydra fluctuans Lour. of family Compositae is a small genus of marsh herb, distributed in tropical and subtropical regions [4]. Mainly the plant is found in Eastern Bengal, Assam and Sylhet [5]. It is the only species recorded in India which is a prostrate herb with opposite sessile, linear oblong leaves,1-3 inches long [4]. The herb is quite glabrous sometimes pubescent glandular. Stems are 0.3-0.6m, elongated simple or divaricating rooting at the nodes [6].

Survey of literature revealed that the leaves of the plant are used as laxative. Leaves are also used in the diseases of the skin and nervous system. The expressed juice of the leaves is a demulcent in gonorrhoea [7]. The plant is reported to possess analgesic activity [8] as well as antidiarrhoeal activity [9].

In our present study we have attempted to evaluate immunomodulatory potency of aqueous extract of leaves of *Enhydra fluctuans* 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.

2. Materials and Methods

2.1 Plant Material

The leaves of *Enhydra fluctuans* were collected from the local areas of Agartala, Tripura in the Month of May 2006 and authentified by Dr. Ajay Krishna Saha, Professor, Departmrnt of Botany at M.B.B College, Tripura University, Agartala, Tripura. The freshly collected leaves from the plant were shade dried at room temperature and powered until able to pass through sieve number 40.

2.2 Preparation of extract

The powder was then subjected to maceration using chloroform water IP 1996 for 6 days. The dark brown filtrate obtained was concentrated which was then lyophilized and stored at 4°C until further used. The crude aqueous extract was subjected to phytochemical investigation.

2.3 Preparation of test sample

Sample for *in-vitro* study were prepared by dissolving 2.5gm of crude extract in 25ml PBS (Phosphate buffer solution) to obtain a solution of 100mg/ml. From this stock solution, different working dilutions were prepared to get a concentration range of 25, 50,100?g/ml.neutrophils of the blood withdrawn from normal human volunteers were used to study the activity. Phosphate Buffer Solution was used as a vehicle.

2.4 Study of the immunomodulatory activity

2.4.1 Neutrophil locomotion and chemotaxis [10]

Neutrophils cell suspension was prepared in phosphate buffer solution at about 106 cells/ml. The lower compartment of chemotactic chamber (5ml beaker) was filled with appropriate chemotactic reagents preadjusted to pH of 7.2.

Eg. Chamber 1-PBS solution (Control), chamber 2-Casein 1mg/ml (standard) and chamber 3, 4, 5 with different concentration (25, 50,100µg/ml) of test sample.

The upper compartment (1 ml syringe) was filled with neutrophil cell suspension and the wet filter (millipore) of 3mm pore size was

fixed at the bottom of the upper compartment. The upper compartments were placed into the lower compartment incubate 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 heamatoxylin dye for 5min. The fixed filters were observed under microscope using 100 X lens and the number of neutrophil cells reached to the lower surface of the filter was counted.

2.4.2 In vitro immunostimulant studies by slide method

Preparation of *Candida albicans* suspension [11]

The *Candida albicans* culture was incubated in sabouraud broth overnight and then centrifuged to form a cell button at the bottom and supernant was discarded. The cell button was washed with sterile Hank's 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 proportion of 4:1.The cell suspension of concentration $1x10^8$ 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 neutrophil (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* slide and incubated at 37°C for 1 hr. The slide was drained, fixed with methanol and stained with Giemsa stain. Positive control was tested by preparing the slide in a 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 granulocyts using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was reported for different concentration (25, 50, 100μg/ml) of test

Table 1. Effect of aqueous extract of leaves of *Enhydra fluctuans* on neutrophil locomotion and chemotaxis.

Sl.No.	Groups	Concentration μg/ml	Mean number of neutrophil per field
1	Control (PBS)	-	5.60 ± 0.71
2	Standard (Casein)	1000	71.29 ± 1.05*
3	*E. fluctuans* extract	25	45.20 ± 1.25*
4	*E. fluctuans* extract	50	50.55 ± 1.40*
5	*E. fluctuans* extract	100	53.24 ± 1.49*

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

Table 2. Effect of aqueous extract of leaves of *Enhydra fluctuans* on neutrophil phagocytosis

Sl.No.	Groups	Concentration µg/ml	% Stimulation
1	Control (Pooled Plasma Serum)	-	4.89 ± 0.88
2	*E. fluctuans* extract	25	29.34 ± 1.08*
3	*E. fluctuans* extract	50	32.35 ± 1.22*
4	*E. fluctuans* extract	100	36.55 ±1.26*

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

Table 3. Effect of aqueous extract of leaves of *Enhydra fluctuans* on quanlitative NBT test.

Sl.No.	Groups	Concentration µg/ml	% NBT Positive cells
1	Control (PBS)	-	21.32 ± 1.05
2	Endotoxin activated plasma	-	75.07 ± 0.93
3	*E. fluctuans* extract	25	60.36 ± 0.84*
4	*E. fluctuans* extract	50	65.42 ± 1.11*
5	*E. fluctuans* extract	100	78.46 ± 1.15*

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

sample. Immunostimulation in % was calculated by using following equation.

$$\text{Stimulation (\%)} = \frac{\text{PI (test) - PI (control) X 10}}{\text{PI Control}}$$

2.4.3 Qualitative Nitroblue Tetrazolium Test (NBT) [10]

A suspension of leucocytes ($5X10^6$/ml) was prepared in 0.5 ml of PBS solution in 5 test tubes .0.1ml of PBS solution (control) and 0.1 ml of endotoxin activated plasma (standard) is added to the 1st and 2nd tube respectively and to the other 3 tubes of test sample 0.1 ml of different concentration (25, 50, 100µg/ml) of test sample .0.2ml of freshly made 0.15 % NBT solution was added to each tube and incubated at 37°C for 20 min. Centrifuged at 400g for 3-4 min to discard the supernant. The cells were resuspended 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-fuchsin for 15 sec. The slide was washed under tap water, dried and observed under 100X oil emulsion objective. 200 neutrophils were counted for the % of NBT positive cells containg blue granules/lumps.

2.5 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 [12].

3. Results

The preliminary phytochemical investigation reveals presence of saponins, flavonoid and Glycosides. The aqueous extract of leaves of *Enhydra fluctuans* has caused a significant increase in movement of number of neutrophils from the upper compartment to lower surface of filter in a dose dependent manner. (Table 1) Stimulation of phagocytosis of *Candida albicans* by neutrophils (table 2) and also increase in percentage of NBT positive cells containing the reduced NBT dye (table3), when compared with control samples containing PBS solution.

In neutrophil locomotion and chemotaxis test and qualitative NBT test, the results obtained with *Enhydra fluctuans* were comparable with that of standard.

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 subject such medicinal plants to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility [13, 14].

Recently there is an enthusiasm towards exploration of novel group of compounds from Natural sources that modulate the immune response of living systems and influence the disease process [15, 16]. In the present study aqueous extract of leaves of *Enhydra fluctuans* significantly increased the phagocytic function of human neutrophils when compared to control indicating, the possible immunostimulating effect. The *Enhydra fluctuans* extract has significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells reaching the microorganism by slide method which provides a rapid and simple means of assessing the overall phagocytic process by the neutrophils.

The aqueous extract of *Enhydra fluctuans* has significantly increased in ingestion of *Candida albicans* by neutrophils. The aqueous extract of *Enhydra fluctuans* has 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 aqueous extract of *Enhydra fluctuans* 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 above mentioned activities.

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