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Effect of Methanolic extract of *Ocimum Gratissimum* linn. Leaf on Phagocytosis by Human Neutrophils

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ABSTRACT

To evaluate the effect of methanolic extract of leaves of *Ocimum gratissimum* linn. on neutrophil phagocytic function. We used different concentrations (50,100,200µg/ml) of methanolic extract of *Ocimum gratissimum* 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. This preliminary study revealed that *Ocimum gratissimum* extract has stimulated chemotactic, phagocytic and intracellular killing potency of human neutrophils at the different concentration. From the results obtained it can be observed that the methanolic extract of *Ocimum gratissimum* leaves stimulates cell-mediated immune system by increasing neutrophil function phagocytic activity.

Keywords: Immunostimulant activity, *Ocimum gratissimum*, neutrophils, Phagocytosis.

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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 state¹.

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 immune stimulation or immunosuppression. The healthy state is believed to be based on a sophisticated fine-tuning of immune regulatory mechanism². Plants identified as 'Rasayanas' in Ayurvedic system of medicine have various pharmacological properties, an entire section of materia medica of Ayurveda is devoted to drugs entitled as 'Rasayana' used for enhancement of body resistance³.

Suppressive and cytotoxic activity affecting the function of immune system has reported by number of synthetic and natural therapeutic agents. Among the synthetic substances, azathioprin and cyclophosphamide is an alkylating agent resulting in the cross linking of DNA and causes inhibition of DNA synthesis. The major drawbacks of these drugs are myelosuppressive, which is undesirable. Immunomodulator of herbal origin appearing to be a better alternative to overcome the above problem⁴.

Ocimum gratissimum Linn (Lamiaceae) is a shrubs, distributed in tropical and subtropical Himalaya from Kashmir eastwards to all over in India. Commonly it's found in gardens, waste places, and road sides. In different language the plant is known as Vriddhitulsi (Sanskrit), Ramtulsi (Hindi) and Nimmatulsi (Kannada)⁵. As per the traditional claim *Ocimum gratissimum* leaves are potential source of drugs used in skin disease, inflammation, insomnia, carminative, aphrodisiac, digestive, tonic, antiemetic, antispasmodic, antineuralgic and liver diseases^{6,7}. Phytochemically the extract of leaves contain essential oil such as eugenol and cineole, ocimol, tetratriacontane, gratimissin, gratimissic acid and β -caryophyllene⁸. It has been reported that plant contains essential oil which is scientifically evaluated for wound healing⁹, antibacterial¹⁰, anthelmintic¹¹, anti-malarial¹² and hepatoprotective¹³.

MATERIALS AND METHOD

Plant Material

The leaves of *Ocimum gratissimum* were collected from the local areas of Bardoli, Gujarat in the month of November 2012 and identified from Botanical Survey of India. A voucher specimen (No. BSI/WC/Tech/2007/917) was deposited in the Herbarium of Department of Pharmacognosy, Maliba Pharmacy Collage, Tarsadi, Gujarat, India. The freshly collected leaves from the plant were shade dried at room temperature and powdered until able to pass through sieve number 40.

Preparation of extract

The powder material was subjected to maceration using methanol for 8 days. The dark brown filtrate was concentrated. That extract was lyophilized and stored at cool temperature until further used. The crude methanolic extract was subjected to phytochemical investigation¹⁴.

Preparation of test sample

Sample for *in-vitro* study were prepared by dissolving 5 gm of crude extract in 50 ml PBS (Phosphate buffer solution) to obtain a solution of 100 mg/ml. From this stock solution, different dilutions were prepared to get a concentration range of 50, 100, 200 µg/ml. Neutrophils of the blood withdrawn from normal human volunteers were used to study the activity and phosphate buffer solution were used as a vehicle.

Study of the immunomodulatory activity

*Neutrophil locomotion and chemotaxis*¹⁵

Neutrophils cell suspension was prepared in phosphate buffer solution at about 10^6 cells/ml. The lower compartment of chemo tactic chamber (5ml beaker) was filled with appropriate chemotactic reagents pre adjusted 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 (50, 100, 200 µg/ml) of test sample.

The upper compartment (1 ml 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 compartments were placed in incubator 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 5 min. The fixed filters were observed under microscope using 100X lens and the number of neutrophil cells reached to the lower surface of the filter was counted.

In Vitro Immunostimulant Studies By Slide Method

Preparation of *Candida albicans* suspension¹⁶.

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 then 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 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 granulocytes 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 (50, 100, 200 µg/ml) of test sample. Immunostimulation in % was calculated by using following equation.

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

Qualitative Nitroblue Tetrazolium Test (NBT)

A suspension of leucocytes (5×10^6 /ml) was prepared in 0.5 ml of PBS solution in 5 test tubes. 0.1 ml of PBS solution (control) and 0.1 ml of endotoxin activated plasma (standard) is added to the 1st and 2nd tube respectively and other 3 tubes of test sample contain 0.1 ml of different concentration of methanolic extract (50,100,200µg/ml). Then 0.2 ml of freshly made 0.15 % NBT solution was added to each tube and incubated at 37°C for 20 min. Centrifuged at 400 rpm 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 by tap water, dried and

observed under 100X oil emulsion objective. 200 neutrophils were counted for the % of NBT positive cells contains blue granules/lumps.

Statistical Analysis

The values are expressed in mean \pm SEM. The results were analyzed by One Way analysis of variance (ANOVA) followed by Dunnet's "t" test to determine the statistical significance.

RESULTS AND DISCUSSION:

The preliminary phytochemical investigation reveals presence of glycosides, sterols, flavonoids, volatile oils, tannins and phenolic compounds. The methanolic extract of leaves of *ocimum gratissimum* 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 (Table 3) when compared with control samples containing PBS solution. In neutrophil locomotion and chemotaxis test and qualitative NBT test, the results obtained with *ocimum gratissimum* were comparable with that of standard. 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.

Table 1: Effect of methanolic extract of leaves of *Ocimum gratissimum* on neutrophil locomotion and chemotaxis

Sl. No.	Groups	Conc. $\mu\text{g/ml}$	Mean number of neutrophil per field
1	Control (PBS)	-	7.30 \pm 0.71
2	Standard (Casein)	1000	79.29 \pm 1.05*
3	<i>O. gratissimum</i> extract	50	52.58 \pm 1.25*
4	<i>O. gratissimum</i> extract	100	59.75 \pm 1.40*
5	<i>O. gratissimum</i> extract	200	68.24 \pm 1.49*

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

Table 2: Effect of methanolic extract of leaves of *Ocimum gratissimum* on neutrophil phagocytosis

Sl. No.	Groups	Conc. $\mu\text{g/ml}$	% Stimulation
1	Control (Pooled Plasma Serum)	-	5.37 \pm 0.88
2	<i>O. gratissimum</i> extract	50	33.63 \pm 1.08*
3	<i>O. gratissimum</i> extract	100	38.48 \pm 1.22*
4	<i>O. gratissimum</i> extract	200	46.65 \pm 1.26*

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

Table 3: Effect of methanolic extract of leaves of *Ocimum gratissimum* on qualitative NBT test.

Sl. No.	Groups	Conc.µg/ml	% NBT Positive cells
1	Control (PBS)	-	23.42 ± 1.05
2	Endotoxin activated plasma	-	78.47 ± 0.93
3	<i>O. gratissimum</i> extract	50	62.76 ± 0.84*
4	<i>O. gratissimum</i> extract	100	69.22 ± 1.11*
5	<i>O. gratissimum</i> extract	200	85.66 ± 1.15*

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

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.

In the present study methanolic extract of leaves of *Ocimum gratissimum* significantly increased the phagocytic function of human neutrophils when compared to control indicating, the possible immunostimulating effect. The *Ocimum gratissimum* 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.

CONCLUSION:

The methanolic extract of *Ocimum gratissimum* has significantly increased the intercellular reduction of NBT dye to formazan (deep blue compound) by the neutrophils, confirming the intracellular killing property of phagocytosing neutrophils. From the results obtained, it can be concluded that the methanolic extract of *Ocimum gratissimum* 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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