An in vitro study of Effect of *Centella asiatica* on Phagocytosis by Human Neutrophils

*Ravindra G. Mali, and Basavraj C. Hatapakki*

Department of Pharmacognosy and Phytochemistry, K.L.E.S. College of Pharmacy, JNMC Campus, Belgaum-590 010

**ABSTRACT:** Present investigation was planned to evaluate the effect of ethanol extract of leaves of *Centella asiatica* (CA) on neutrophil phagocytic function. The various concentrations (25, 50 and 100 mg/ml) of the extract was subjected to study its effect on different in vitro methods of phagocytosis such as neutrophil locomotion and chemotaxis, immunostimulant activity by phagocytosis of killed Candida albicans and qualitative nitro blue tetrazolium test using human neutrophils. The results of this preliminary study revealed that CA extract has stimulated chemotactic, phagocytic and intracellular killing potency of human neutrophils at the concentration range of 25-100 mg/ml. These results suggest that the ethanol extract of CA stimulates cell-mediated immune system by increasing neutrophil phagocytic function.

**KEYWORDS:** *Centella asiatica*, Phagocytosis, Neutrophils, *Candida albicans*, Chemotaxis

**Introduction**

In modern medicine immunology plays an important and increasing role in understanding and diagnosis of the diseases. The immune response is evolved in the etiology as well as the pathophysio logic mechanism of many diseases. The modulation of immune response using various agents in order to alleviate the disease has been of interest since many years (Ballow and Nelson, 1997).

Some medicinal plants and their isolated constituents have been reported to induce a state of non specifically increased resistance in experimental animals and humans (Brekhman and Dardymov, 1969). The stimulation of non-specific defense mechanism system of the human organism as one concept of therapy has a long tradition in medicine. In traditional system of medicine of India, China, Korea, a number of plants are described as ‘General tonics’ (Pallabi and Dasgupta, 1998). In terms of modern medicine, this approach to therapy so much similar to prohost therapy which aim in augmenting host defense against infection by improving immunocellular function.

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 research for locating natural resources showing immunomodulatory activity (Patil et al., 2008).

**Centella asiatica** (CA) Linn. (Umbelliferae), commonly known as ‘Brahmi’ or ‘Mandukaparni’ is widely distributed in India and is of medicinal importance (Vaidyaratnam PSV, 1994). The plant is a prostrate, perennial, faintly aromatic herb found wild throughout India and Sri Lanka up to an altitude of 2,000 feet. The plant enjoys considerable reputation in Indian traditional systems of medicine as diuretic, alternative and tonic. An infusion of CA is used in India and Madagascar in treatment of leprosy and is known to ameliorate the symptoms of the disease and to improve the general health of the patient (Anonymous, 1950). Chopra et al. (1958) has mentioned that the plant is useful as alternative, tonic, and also in the treatment of skin diseases, leprosy. The leaves are taken as tonic and for improving memory and curing syphilitic skin diseases internally as well as externally (Kakkar, 1989). CA has been investigated scientifically and found to possess a number of notable pharmacological activities such as anti-psoriatic (Sampson et al., 2001), wound healing (Shakda et al., 1999; Shetty et al., 2006), hypoglycaemic (Mutayabarwa, 2003), hepatoprotective (Antony et al., 2006), anti-gastric ulcer (Cheng et al., 2004), anti-tumour (Babu et al., 1995), antimicrobial (Mantha et al., 2004), Antinociceptive and anti-inflammatory (Sommich et al., 2004), anti-oxidant (Jayashree et al., 2003; Hussin et al., 2007), nootropic and anxiolytic (Wijeweera et al., 2006).

Neutrophils play an important role in host immune mechanism system. The neutrophilic phagocyte system has many advantages. They are attracted by a limited number of stimuli, which generally signal the presence of tissue injury of unknown reason. Even the foreign bodies,
thermal or chemical burns, bacterial infections and other types of injuries can provoke an intense neutrophil response. Moreover, neutrophils are highly effective at killing certain bacteria and their ability to digest cellular debris and exogenous particulate matter provides an important step in the host defense mechanism (Berne and Levy, 1988).

In vitro immunomodulatory activity of CA on human neutrophils has not been documented in the literature. Hence, in the present study an attempt has been made to investigate cell mediated immunomodulatory potency of leaves extract of CA 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.

Material and Methods

Plant material

The leaves of CA were carefully collected from the fields near Belgaum city and were authenticated at Botanical Survey of India, Koregaon Road, Pune (Voucher specimen No. 27047). A specimen voucher of the plant has been deposited in the Department of Pharmacognosy, College of Pharmacy, Belgaum. The leaves were shade dried and then milled to coarse powder by mechanical grinder and then used for extraction.

Preparation of extract

The powder material was then subjected to exhaustive extraction using 95% ethanol in a soxhlet apparatus. The dark green liquid extract so obtained was concentrated under vacuum and the resulting dried extract was lyophilized and preserved in a desiccator until further use.

Chemicals

All organic solvents were obtained from the S.D.Fine Chemicals Private Limited (Mumbai, India) and all were of analytical grade. Nitroblue tetrazolium (NBT), Phosphate buffer saline (PBS, pH 7.2), May-Grunwald Giemsa stain, Sodium deoxycholate, Methylene blue, Casein in Hanks solution and Haematoxylin stain were all obtained from Sigma Aldrich (St. Louis, MO).

Assessment of immunomodulatory activity

A. Neutrophil locomotion and chemotaxis

Neutrophil cell suspension was prepared in phosphate buffer saline solution (PBS) at about 10^6 cells/ml. The lower compartments of the chemotaxis 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), Chamber2- Casein 1 mg/ml (standard), and chamber 3, 4 and 5 with different concentrations (25, 50 and 100 mg/ml) of test sample. The upper compartment (1ml 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 compartment was placed on to the lower compartment and Incubated at 37°C for 180 min. The upper compartment was removed and inverted to empty out 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 100 X lens and the number of neutrophil cells reached to the lower surface of the filter was counted.

B. in vitro immunostimulant activity 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 supernatant was discarded. The cell button was washed with sterile Hank’s Balanced Salt Solution (HBBS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBBS and human serum in proportion of 4:1. The cell suspension of concentration 1x 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 h. 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 repeated for different concentrations (25, 50 and 100 mg/ml) of test sample). Immunostimulation in % was calculated by using following equation:

Stimulation (%) = PI (test) - PI (control) x 100/ PI (control)

C. Qualitative nitroblue tetrazolium (NBT) test
(Willkinson, 1981)
A suspension of leucocytes (5 x 10⁶/ml) was prepared in 0.5 ml of PBS solution in 5 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 to the other 3 tubes 0.1 ml of different concentrations (25, 50 and 100 mg/ml) of test sample were added. 0.2 ml of freshly prepared 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 supernatant. 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, counterstained 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 containing blue granules/lumps.

Statistical analysis
The values are expressed in mean ± SEM (n=3). The results were analyzed by using one way analysis of variance (ANOVA) followed by Dunnett’s t-test to determine the statistical significance.

Results
The preliminary phytochemical screening of the ethanol extract of leaves of CA showed the presence of triterpenoids, saponins, glycosides, sterols and alkaloids. The results shown in Table 1. illustrates that the ethanol extract of CA has caused significant increase in movement of number of neutrophils from the upper compartment to lower surface of filter in a dose dependant manner. The mean numbers of Candida cells phagocytosed by neutrophils, in presence of extract were significantly increase (p<0.001) as compared to the control group (Table 2) and also depicted increase in percentage of NBT positive cells containing the reduced NBT dye when compared with control samples containing PBS solution (Table 3). In case of neutrophil locomotion and qualitative NBT test the results obtained with CA extract were comparable with that of standard.

Table 1. Effect of extract of Centella asiatica on neutrophil locomotion and chemotaxis.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Group</th>
<th>Concentration (mg/ml)</th>
<th>Mean number of neutrophil/Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (PBS)</td>
<td>-</td>
<td>5.26 ± 0.45</td>
</tr>
<tr>
<td>2</td>
<td>Standard (Casein)</td>
<td>01</td>
<td>72.21 ± 1.25</td>
</tr>
<tr>
<td>3</td>
<td>C.asiatica extract</td>
<td>25</td>
<td>46.38 ± 1.32*</td>
</tr>
<tr>
<td>4</td>
<td>C.asiatica extract</td>
<td>50</td>
<td>49.79 ± 1.48*</td>
</tr>
<tr>
<td>5</td>
<td>C.asiatica extract</td>
<td>100</td>
<td>54.12 ± 1.25*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n=3), * p<0.001 compared to control group
Table 2. Effect of extract of *Centella asiatica* on neutrophil phagocytosis

| Sl. No. | Group                          | Concentration (mg/ml) | % stimulation  
<table>
<thead>
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<tbody>
<tr>
<td>1</td>
<td>Control (pooled plasma serum)</td>
<td>-</td>
<td>4.26 ± 0.75</td>
</tr>
<tr>
<td>2</td>
<td><em>C. asiatica</em> extract 25</td>
<td>25</td>
<td>27.33 ± 1.12*</td>
</tr>
<tr>
<td>3</td>
<td><em>C. asiatica</em> extract 50</td>
<td>50</td>
<td>31.54 ± 1.58*</td>
</tr>
<tr>
<td>4</td>
<td><em>C. asiatica</em> extract 100</td>
<td>100</td>
<td>36.12 ± 1.45*</td>
</tr>
</tbody>
</table>

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

Table 3. Effect of extract of *Centella asiatica* on qualitative NBT test

| Sl. No. | Group                             | Concentration (mg/ml) | % NBT positive cells  
<table>
<thead>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (PBS)</td>
<td>-</td>
<td>22.21 ± 1.04</td>
</tr>
<tr>
<td>2</td>
<td>Endotoxin activated plasma</td>
<td>-</td>
<td>74.21 ± 0.82</td>
</tr>
<tr>
<td>3</td>
<td><em>C. asiatica</em> extract 25</td>
<td>25</td>
<td>61.28 ± 0.32*</td>
</tr>
<tr>
<td>4</td>
<td><em>C. asiatica</em> extract 50</td>
<td>50</td>
<td>66.49 ± 1.18*</td>
</tr>
<tr>
<td>5</td>
<td><em>C. asiatica</em> extract 100</td>
<td>100</td>
<td>79.12 ± 1.35*</td>
</tr>
</tbody>
</table>

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

Discussion

Immunomodulators are biologic response modifying (BRM’s) compounds that affect the immune response in either a positive or negative fashion. If it results in an enhancement of immune reactions, is named as immunostimulation and primarily implies stimulation of non-specific system such as stimulation of the function and efficiency of granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may be because of environmental or chemotherapeutic factors (Hennessey and Baker, 1994). Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. Both the immunostimulative and immunosuppressive agents have their own standing and search for better agents exerting these activities is becoming the field of major interest all over (Wierda and Reasor, 1986).

A number of disorders can be treated by BRM’s. These include immunodeficiency diseases and autoimmune disorders. These drugs may work on cellular or humoral immune system or both (Claman, 1992).

In earlier immunological studies CA has shown promising immunomodulatory activity. In one study, Patil et al. (1998) evaluated aqueous suspension of CA for its immunostimulant property using humoral (Haemagglutinating antibody titre) and cell mediated (Delayed type hypersensitivity) immunity responses and compared the activity with recombinant interferon alfa-2b which is used as supportive medicine in the treatment of cancer to increase immunity of patient. The results demonstrated good activity, that is comparable to about 60% that of alfa-2b interferon. In another study, the pectin and its degraded product, isolated from CA showed immunostimulating activity to different extent in vitro (Wang et al., 2005). Further, Jayathirtha and Mishra (2004) also evaluated immunomodulatory potential of methanol extract of CA using carbon clearance, antibody titer and cyclophosphamide immunosuppression parameters and claimed for significant activity of CA extract.

In our present study extract of leaves of CA significantly increased the phagocytic function of human neutrophils when compared to control indicating the possible immunostimulating effect. The extract has significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells reached the lower surface of filter. The ingestion of micro-organism after coming in contact with them studied by slide method which provides a rapid and simple means of assessing the overall phagocytic process by the neutrophils. The CA extract significantly increased ingestion of *Candida albicans* by neutrophils. A significant increase in the intracellular reduction of NBT dye to form formazan (deep blue compound) was observed confirming the intracellular killing property of phagocytosing neutrophils.
Triterpenoid saponins widely distributed in plant kingdom have been reported to exert immunomodulatory activity (Plohmenn et al., 1984; Mali et al., 2006). CA has also been found to contain triterpenoid saponins. From the results reported here, it can be concluded that the ethanol extract of CA has significant effect on phagocytosis by human neutrophils and chemotactic locomotion of neutrophils and suggest further studies to evaluate its potential as adjuvant to chemotherapeutics in the treatment of infectious diseases with an added advantage of stimulation of neutrophils for phagocytosis.

References


