In-vitro Immunomodulatory Effect of Hydroalcoholic Leaves Extract of Avicennia Officinalis

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The stimulation or suppression of the immune response may modulate disease free state. Herbal medicine paved the way for immunomodulation role in many immunological disorder. The present study was aimed to prove the traditional claim on the immunomodulatory activity of Avicennia officinalis against autoimmune diseases. The Avicennia officinalis leaves was authenticated and coarsely powdered then extracted successively with n-hexane, 70% hydroalcohol followed by ethylacetate. The phytoconstituents of the three extracts were analysed. Finally, all were subjected to (NBT) Nitrobluetetrazolium Assay, Candidacidal assay and Phagocytotic evaluation. The percentage yield and phytoconstituents of the extracts were determined. Among the three extract, the ethanolic extract and Ethylacetate extract showed effective immunomodulatory effect by their suppression of leucocytes and neutrophils. The results of the present study proved the immunomodulatory effect of Avicennia officinalis and further studies are essential to target this species against autoimmune diseases.

Keywords: Immunomodulation, Candidacidal assay, Nitrobluetetrazolium Assay, Phagocytosis.

The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of various diseases. This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs of natural or synthetic origin. Few drugs act as immunopotentiators and induce cytotoxicity leading to diseases and exert a variety of side effects. This has given an initiation in the search for investigating natural resources showing immunomodulatory activity. Immunology is one of the area of biomedical research has great promises with regard to prevention and treatment of wide range of disorders. The combination of phytosterols, terpenoids, minerals, fibre, phenolics and other antioxidants are probably reliable for these effects.

Avicennia officinalis belonging to Avicenniaceae is commonly available in coastal states of India which is mainly used in the treatment of rheumatism, asthma, skin disease, paralysis, ulcer and snake bites.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents were purchased from SD fine chemicals, Sisco laboratories and are analytical grade.

Preparation of extract

The plant was authenticated by Dr. Jeyaraman, Botanist, Plant Research Centre, Tambaram, and the authentication no. was PARC/2015/3171. The coarsely powdered Avicennia officinalis leaves were extracted with n-hexane, 70% hydroalcohol
and ethyl acetate successively by hot percolation process for 72 hrs. The percentage yield were calculated. The phytochemical screening of the three extracts were performed as per standard protocol. Then the extracts were subjected to in vitro immunomodulation studies.

**Nitroblue Tetrazolium Reduction Assay (NBT)**

A suspension of leucocytes was prepared in 0.5ml of PBS solution, to it 0.1ml of phosphate buffer saline solution followed by 0.1ml of endotoxin was added. The different concentration (10, 20, 50, 100, 200, 400, 800ug/ml) of test samples used was taken in 3-7 test tubes about 0.2ml of freshly prepared 0.15% NBT solution was added to each tube, incubate at 37 c for 20 mins. The resultant was centrifuged at 400mg for 3-4 mins to discard the supernatant and cells were resuspended in small volume of PBS solution and thin film was made with drop on slide and after drying, fixed by heating, counter stained with carbon fuschin for 15 seconds and the slide was washed under tap water, dried and focussed with light microscopy. 200 Neutrophils were counted for the % of NBT positive cells containing blue granules.

**Phagocytosis of candida albicans (killed)**

**Preparation of suspension of candida albicans**

*Candida albicans* culture incubated in sabouraud broth overnight, centrifuged to form cell button and supernatant discarded. The cell button was washed with Hank’s solution and centrifuged repeatedly for 3-4 times where final cell button was mixed with mixture of HBBS and human serum in proportion of 4:1.

**Preparation of slide**

Sterile glass slide containing 0.2ml of fresh human blood by finger prick and incubated at 37 degree c for 25 mins to allow clotting. The blood clot was removed gently and drained slowly with sterile normal saline without washing off adhered neutrophils. The slide containing polymorphonuclear neutrophils (PMN) was flooded with concentration of test sample and then incubated for 15 mins at 37 degree c. The slide was drained, fixed with methanol and was stained with geisma stain.

**Candidacidal assay**

The same dilution and procedure was followed as phagocytosis and the pellets of assay tubes were suspended in 100ul MEM and incubated for 30 mins at 37 degree celsius. At the end of incubation period, 0.25ml of 2.5% Na deoxycholate added to each test tube to lyse the leucocytes. 0.25ml of 0.01% methylene blue added to each test tubes and mixed well. The tubes was centrifuged, supernatant was decanted and smears prepared on slides using resultant pellets. The % of candida cells was determined.

**RESULTS AND DISCUSSION**

Immunomodulatory agents of plant and animal origin enhance the immune responsiveness of the body against pathogens by activating the non-specific immune system. However, there is a need for systemic studies on medicinal plants to potentiate the therapeutic claims made regarding their clinical utility. The primary step in this study was authentication of the plant which was done by Dr. Jeyaraman, Director PARC, West Tambaram.

The percentage yield of hydroalcoholic extract, n-hexane and ethyl acetate extract was found to be 7.30 % w/w, 1.32 % w/w and 0.86% w/w respectively. The colour of these extract were greenish brown, greenish brown and greenish yellow respectively.

**Phytochemical screening**

The preliminary phytochemical screening showed the presence alkaloids, tannins, iridoid glycosides, triterpenoids, steroids in ethanolic extract, wheras n-hexane posses steroid, triterpenoids and ethylacetate fraction showed the presence of tannins, and iridoid glycosides.

**Nitroblue Tetrazolium Assay**

In vitro NBT Assay

Nitroblue tetrazolium (NBT) assay was performed as one of the evaluation tests for the immune modulatory activity assessment. This semi-quantitative microscopic nitroblue tetrazolium (NBT) assay is used to determine the production of superoxide anion (O2(-)) in various phagocytic cells. This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium (Y-NBT) by O2(-).[^8] Nitroblue tetrazolium (NBT) is toxic to neutrophils; an effect which is greatly enhanced by endotoxin and latex particles. Cell damage, measured by the release of the cytoplasmic marker enzyme lactate dehydrogenase (LDH), was closely related to dye reduction[^9].
In NBT assay, the 70% ethanolic extract and ethyl acetate extract has suppressed the neutrophils and so reduced formazan formation. The percentage reduction of reduced neutrophils was found to be 21.32, 25.98 for ethanolic and ethyl acetate extract respectively and the results were mentioned in Table-1. Both the extracts showed effective suppressant action at higher concentration level (800µg/ml) but the n-hexane showed least action compared to other two extracts. Thus the phyto-molecule in the ethanolic extract and ethyl acetate has significant immunomodulation effect.

### Phagocytosis assay

The Polymorphonuclear neutrophils (PMN) and mononuclear phagocytes represent an important first line and effector function in the control of *Candida albicans*. This evaluation is based on the principle that the viable *Candida albicans* do not stain supravitally with methylene blue.

### Table 1. Percentage of reduced neutrophils by *Avicennia officinalis* using Nitrobluetetrazolium assay

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration of the extract (µg/ml)</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer saline</td>
<td></td>
<td>3.66±1.22</td>
<td>4.28±0.18</td>
<td>4.86±1.64</td>
<td>5.22±2.08</td>
<td>6.98±2.54</td>
</tr>
<tr>
<td>Endotoxin-activated plasma</td>
<td></td>
<td>46.33±0.88</td>
<td>52.66±1.44</td>
<td>50.02±1.22</td>
<td>53.64±2.08</td>
<td>59.42±1.42</td>
</tr>
<tr>
<td>70% ethanolic extract</td>
<td></td>
<td>16.32±2.42</td>
<td>18.06±1.44</td>
<td>18.78±2.86</td>
<td>19.42±4.20</td>
<td>21.64±1.86</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td></td>
<td>18.66±2.02</td>
<td>20.32±0.88</td>
<td>22.64±1.24</td>
<td>24.32±0.88</td>
<td>25.98±0.98</td>
</tr>
<tr>
<td>N-Hexane extract</td>
<td></td>
<td>26.44±1.28</td>
<td>30.02±1.20</td>
<td>35.28±2.64*</td>
<td>38.42±2.98</td>
<td>43.02±3.96*</td>
</tr>
</tbody>
</table>

The data are expressed as mean percentage reduced neutrophils ± standard error mean. Significant difference from Positive Control by oneway ANOVA followed by Dunnet’s ‘t’ test. (n = 4) P<0.01, P<0.5

### Table 2. Particle number phagocytosis of killed *Candida albicans* after treatment with extracts of *Avicennia officinalis*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration of the extract (µg/ml)</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled serum</td>
<td></td>
<td>2</td>
<td>3-4</td>
<td>4</td>
<td>4-5</td>
<td>7</td>
</tr>
<tr>
<td>70% ethanolic extract</td>
<td></td>
<td>4</td>
<td>2-3</td>
<td>1-2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td></td>
<td>3</td>
<td>2-3</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N-Hexane extract</td>
<td></td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

The data were expressed as mean particle number phagocytosed.

### Table 3. Percentage of Killed *Candida* after treatment with extracts of *Avicennia officinalis* leaves by Candidacidal Assay

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration of the extract (µg/ml)</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hank’s solution</td>
<td></td>
<td>15±0.58</td>
<td>16.22±1.16</td>
<td>18.68±0.24</td>
<td>19.02±1.64</td>
<td>20.64±1.28</td>
</tr>
<tr>
<td>Pooled serum</td>
<td></td>
<td>18.96±1.76</td>
<td>19.60±0.22</td>
<td>24.46±1.68</td>
<td>28.36±3.02</td>
<td>34.42±1.28*</td>
</tr>
<tr>
<td>70% ethanolic extract</td>
<td></td>
<td>32.34±1.88</td>
<td>26.86±1.52</td>
<td>21.04±1.02*</td>
<td>18.26±0.62</td>
<td>14.64±0.54</td>
</tr>
<tr>
<td>Ethyl acetate extract</td>
<td></td>
<td>14.86±1.70</td>
<td>13.42±2.08</td>
<td>12.64±1.16</td>
<td>12.43±0.33</td>
<td>1.32±1.46</td>
</tr>
<tr>
<td>N-Hexane extract</td>
<td></td>
<td>12.32±0.64</td>
<td>18.86±1.70</td>
<td>16.86±1.22*</td>
<td>15.89±1.32</td>
<td>12.32±0.64</td>
</tr>
</tbody>
</table>

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<0.5.
In this assay, the phagocytosis effect on killed candida albicans mean particle numbers were found to be 2.1 for ethanolic extract and ethyl acetate extract respectively whereas n-hexane group showed non significant effect. At lower concentration, immunosuppressant effect was least compared with higher dose. The isolated compound stimulates the phagocytosis of killed candida albicans, the mean particle was found to be 5-6.4 and 4 for isolated compound at concentration of 1000, 100, 40 µg/ml and when compared to pooled serum 6.4-5.6 at same concentration. At low concentration of 20 and 10 µg/ml the stimulation of phagocytic activity is negligible. The phagocytic assay result mentioned in Table: 2.

**Candidacidal assay**

The engulfment mechanism of neutrophils is considered as main phagocytic action of an organism and the results were mentioned in Table-3. The ethanolic extract and ethyl acetate extract of *Avicennia officinalis* has significantly increases reduced neutrophils mean particle number of killed candida allicans and also increases the percentage of candidacidal action. The percentage of candidacidal action was found to be 34.42% for pooled serum, 32.34% for ethanolic extract, 24.42% for n-hexane and 14.26% for ethyl acetate extract has least action. Thus the ethanolic extract and ethyl acetate extract of *Avicennia officinalis* has significantly increases reduced neutrophils.

**CONCLUSION**

The immunomodulatory effect of hydroalcoholic extract of *Avicennia officinalis* has proved significantly based on the reports of invitro studies namely -NBT assay, Candidacidal assay and phagocytosis assay. The immunosuppressive activity may be due to presence of active biomolecules present in it. In future, the phytomolecule has to be isolated, characterized and finally formulated for clinical trials to serve the society.

**ACKNOWLEDGEMENT**

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**REFERENCES**