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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 way for immunmodulation role in many mmunological disorder. The present study was aimed to prove the traditional claim on the theimmunomodulatory activity of*Avicennia officinalis* against autoimmune diseases. The *Avicenniaofficinalis* leaves was authentified and coarsely powdered then extracted successively with n-hexane,70% hydroalcohol followed by, ethylacetate. The phytoconstituents ofthe 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 thethree extract, the ethanolic extract and Ethylacetateextract 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 effectsm[1]. 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[2]. The combination of phytosterols, terpenoids, minerals, fibre, phenolics and other antioxidants are probably reliable for these effects[3].

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.[4]

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 authentified by Dr.Jeyaraman, Botanist, Plant Research Centre, Tambaram, and the authentification no. was **PARC/2015/3171.**The coarsely powdered *Avicenniaofficinalis* leaves were extracted with n-hexane, 70% hydroalcohol

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and ethyl acetate successively by hot percolation process for 72hrs. The percentage yield were calculated. The phytochemical screening of the three extracts were performed as per standard protocol.Then the extracts were subjected to*invitro*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. Thr resultant was centrifuged at 400mg for 3-4mins 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 strained with carbon fuschin for 15 seconds and the slide was washed under tap water, dried and focussed under 100x magnification oil immersion.200 Neutrophils were counted for the % of NBT positive cells containing blue granules[5].

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[6].

Preparation of slide

Sterile glass slide containing 0.2ml of fresh human blood by finger prick and incubated at 37 degree c for 25mins to allowclotting.The blood clot was removed gently and drained slowly with sterile normal saline without washing off adhered neutrophil.The slide containing polymorphonuclear neutrophils (PMN) was flooded with concentation of test sample and then incubated for 15mins at 37degree 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 100ulMEM and incubated for 30mins at 37degree 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[7].

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 wasauthentification 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/wand 0.86% w/w respectively. The colour of these extract were greenishbrown, 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 respectivelyand 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 phytomolecule 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 *Candidaalbicans*[10]. This evaluation is based on the principle that the viable *Candida albicans*do not stain supravitally with methylene blue[11].

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

Extract	Concentration of the extract (µg/ml)				
	50	100	200	400	800
Phosphatebuffer saline	3.66±1.22	4.28±0.18	4.86±1.64	5.22±2.08	6.98±2.54
Endotoxin-activated plasma	46.33±0.88	52.66±0.14*	50.02±1.22	53.64±2.08	59.42±1.42*
70%ethanolic extract	16.32±2.42	18.06±1.44	18.78±2.86	19.42±4.20	21.64±1.86*
Ethyl acetate extract	18.66±2.02	20.32±0.88	22.64±1.24	24.32±0.88	25.98±0.98
N-Hexane extract	26.44±1.28	30.02±1.20	35.28±2.64*	38.42± 2.98	43.02±3.96*

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*<0.5Phosphate Buffer Saline (Normal Control), Endotoxin-activated plasma (Positive Control)

Table 2. Particle number phagocytosis of killed Candida albicans after treatment with extracts of Avicenniaofficinalis

	Extract Concentration of the extract (µg/ml)				
	50	100	200	400	800
Pooled serum	2	3-4	4	4-5	7
70% ethanolicexract	4	2-3	1-2	1	0
Ethyl acetate extract	3	2-3	2	1	1
N-Hexane extract	2	2	3	3	4

The dataswere expressed as mean particle number phagocytosed.

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

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

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 wheras 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 1000,100,40ug/ml and when compared to pooled serum 6,4-5,6 at same concentration. At low concentration of 20 and 10ug/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[12] 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 alicans and also increases the percentage of candidacidal action The percentage of candidacidal activity 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 extact 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.

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REFERENCES

1. Krzystyniak, K., Tryphonas, H., Fournier, M. *Environmental Health Perspectives,* 1995; **103**(9): 17-22.
2. Wagner H. (Ed). In. Immunomodulatory agents from plants, Basel: Birkhauser, Germany, 1999; 1-35.
3. Ruxton, C., Gardner, E.,Walker, D. *International Journal of Food Science and Nutrition,* 2006; **57**: 249-272.
4. Choi HS1, Kim JW, Cha YN, Kim C. A quantitative nitroblue tetrazolium assay fordetermining intracellular superoxide anion production in phagocytic cells. *J Immunoassay. Immunochem,* 2006; **27**(1):31-44.
5. Segal AW, Levi AJCell damage and dye reduction in the quantitative nitroblue tetrazolium (NBT) test. *ClinExpImmunol.*, 1975; **19**(2):309-18.
6. Vonk AG1, Wieland CW, Netea MG, Kullberg BJ.Phagocytosis and intracellular killing of Candida albicansblastoconidia by neutrophils and macrophages: a comparison of different microbiological test systems. *JMicrobiol Methods,* 2002; **49**(1):55-62.
7. Wilkinson PC Neutrophil Leucocytestest. in:Thomas RA editor .techniques in clinical immunology,2nd edition;london: blackwell scientific publication;1981.
8. Ponkshe CA, Indap MM.in vivo and in vitro studies of Immunomodulatory activity of marine animal extract with reference to phagocytosis. *Indian J ExpBiol,* 2002; **40**:1399-402.
9. Lehrer RI,ClineMJ,Leukocytecandidacidal activity and resistance to systemic candidasis in patientswith cancer, 1971; **27**(5),1211-1217.
10. kulkarni SK. Biostatics in experimental Pharmacology. Handbook of ExpPharmacol, Delhi.vallabh prakashan; 1999.
11. KonigP,HordvikNL,Kreutz C.The preventive effect,duration of action of nedocroml sodium and cromolyn sodium on excercise induced asthma inn adults. *J Allergy clinImmunol,* 1987; **79**:64-80.
12. Daniel PS,Abba IT,Tristram GP; Basic and clinical immunology;8th edition ,USA.Appleton and lange;1994.