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In-vitro Antimicrobial and Cytotoxic ativity of Methanolic extract of *Osbeckia wynaadensis*

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Abstract

In- vitro antibacterial and antifungal activities of methanolic extract of osbeckia wynaadensis was evaluated in the present study for the first time by disc diffusion method using five bacterial strains (S. pneumonia, B.cereus, A. hydrophila , V. cholera and MRSA) and five fungal strains (Candida albicans, Aspergillus niger, Streptomysis Greusis, M. purpureus and Aspergillus fumigate) respectively. The zone of inhibition and minimum inhibitory concentration were measured. Ampicillin (30 µg/disc) and clotrimazole (20 µg/disc) were used as standard for antibacterial and antifungal activity respectively. In-vitro cytotoxic activity of Osbeckia wynaadensis was evaluated against the human cervical adenocarcinoma cell line (HeLa) and murine embryonic fibroblasts cell line (NIH 3T3) by MTT assay and the IC_{50} value found to be 220.3 µg/mL and 93. 25 µg/mL. It is concluded that Osbeckia wynaadensis exhibited significant antibacterial, antifungal & in-vitro cytotoxic activity.

Key words: *Osbeckia wynaadensis*, *in-virto* cytotoxicity, MTT assay, antibacterial activity, antifungal activity.

Introduction

All over the world, people depended on the herbs for the treatment of various ailments before the advent of modern medicine. Medicinal plants constituents an arsenal of chemicals that could be exploited by human to prevent microbial invasion. They have been a major source for drug development. Plant extracts and products are used in the treatment of bacterial, fungal and viral infection[1]. Plants play a vital role in our lives more than animals mainly due to their extraordinary array of diverse class of biochemicals with a variety of biological activities[2]. Plants have been used for the treatment of disease all over the world before the advent of modern clinical drugs. Natural phytochemicals are known to contain substance that can be used for therapeutic purposes or as precursor for the synthesis of novel useful drugs. The natural products play an important role in drug development in pharmaceutical industry. Use of plant as a source of medicine has been inherited and is an important component of the health care system[3].

Plants have a long history of use in the treatment of cancer. Drug discovery from plants is a multi-disciplinary approach which combines various botanical, ethno-botanicals, photochemicals and biological and chemical separation techniques[4]. However, despite these observations, it is significant that over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and micro-organisms[5,6].

Aromatic and medicinal plants are known to produce certain bioactive molecules which react with other organisms in the environment, inhibiting bacterial or fungal growth[7,8]. The antimicrobial activity have been screened because of their great medicinal relevance with the recent years, infections have increased to a great extent and resistant against antibiotics, becomes an ever increasing therapeutic problem[9]. Natural products of higher plants may give a new source of antimicrobial agents. There are many research groups that are now engaged in medicinal plants research[10-13].

Osbeckia wynaadensis (Melastomatacea), a herb, is distributed wild in Western Ghats, along river banks. The genus *Osbeckia* contains about 12 species. The whole plants of this genus are used as traditional medicine with the function of heat-clearing and detoxicating, hematischesis and astrigence. And those roots are used to treat dysentery and gonorrhea[14]. Chemical constituents were reported from *O.crinita, O.aspera, O.chinensis* showed the presence of flavanoids[15-17], organic acids[18] and steroids[19]. The biological activities of the *Osbeckia* genus are given in the table-1. Antioxidant and immunomodulatory effects were investigated with *O.aspera* and *O.octandra* was studied for its hepatoprotective activity. Further *O.octandra*, *O.chinensis* and *O.nepalensis* were showed to exhibit antidiabetic activity. Altogether so far six species has been investigated and all other species was yet to explore for their phytoconstituents and biological activities. This promoted us to investigate the remaining osbeckia species for its activities and phytoconstituents. To start with *O.wynaadensis* was selected for our present work. It is a slender erect under shrub with purple flowers in sub- terminal corymbs, large long-petioled leaves and very characteristic comb-like scales on the calyx-tube. Plant pacifies vitiated pitta, inflammation, urinary tract infection, hemorrhage, menorrhagia, hemorrhoids and leucorrhea. Presence of this plant is an indication of pure underground water. This plant is on the verge of extinction due to water pollution and manmade destructive-activities.

As far as our literature survey no work is reported on this plant. The present work is to evaluate the antibacterial, antifungal and cytotoxic activity of the methanolic extract of *O.wynaadensis.*

Material and Methods

Collection and identification of plants: The whole plant of *O.wynaadensis* is collected during March-April 2009, from Wayanad, Kerala, India. The plant was authetificated from BSI, Coimbatore and the specimen no is BSI/SRC/5/23/2010-11/Tec-2098.

Preparation of extract: The collected plants were washed with water and dried in shade. The air dried plant is powdered and 1.5 kg of this powdered material was soaked in 70% methanol for 72 hours and the extract was collected and concentrated to yield a residue (50 mg).

Microorganisms: The following bacterial strains were employed in the screening: Gram positive *Streptococcus pneumonia* and *Bacillus cerus and* the Gram negative *Aeromonas hydrophila* and *Vibrio cholera* and *Methicilin-resistant staphylococcus aureus* (MRSA). In the antifungal screening the following fungi were tested: *Candida albicans, Aspergillus niger, Streptomysis Greusis (mm), M.purpureus, Aspergillus fumigate*.

Antibacterial screening: Disc diffusion method: The bacterial strains (*Streptococcus* sp*., B.cereus, A. hydrophila V. cholerae* were inoculated in the nutrient broth under aspectic condition and incubated at 37 ^{0}C for 18 hours. After the incubation period, the test bacterial was swabbed on the nutrient agar plate using sterile cotton swab. In each of these plates, wells (10 mm) were cutout using sterile cork borer. The methanol extract was dissolved in the solvent. Controls were maintained by loading same quantity of Ampicillin into the wells. Then the petri dishes were incubated at 37^{0}C for 14 hours. The anti microbial activity was evaluated by measuring the zone of inhibition in diameter. The zone of inhibition in diameter was observed and recorded in millimeter. The same method was carried out for MRSA using Ampicillin as the positive control.

Minimum Inhibitory concentration (MIC): The Minimum inhibitory concentration (MIC) was determined through the dilution method[20]. Bacteria were grown in nutrient broth (NA) for 6 hrs. After this, 20 µL of 106 cells/mL were inoculated in tubes with nutrient broth supplemented with 5 different concentrations (25 µL, 50 µL, 100 µL, 150 µL, 200 µL) of the oils. After 24 hrs at 37°C, the MIC of each sample was measured through optical density in the spectrophotometer (620nm) through the comparison of the sample readout with the known inoculated nutrient broth[21] and the results are enlisted in tables, Ampicillin was used as a standard substance, DMSO as the negative control. The same method was carried out for MRSA using Ampicillin as the positive control, DMSO as the negative control.

Antifungal screening: The inoculums for the experiment were prepared in fresh sabouraud's broth from preserved slant culture. The inoculum was standardized by adjusting the turbidity of the culture to that of McFarland standards. The turbidity of the culture may be adjusted by the addition of sterile saline or broth (if excessive or by further incubationg to get required turbidity (Leonard Jarrett et.al.). Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or dry heat (only for wooden swabs) by packing the swabs in culture tubes, papers or tins etc. The standardized inoculums is inoculated in the plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing by pressing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed.

Each Petri dish is divided into 2 parts, in 2 parts extract discs such as osw (250mcg) discs, (discs are soaked overnight in extract solution) and one quadrant for Std clotrimazole 10mcg, are placed in each quadrant with the help of sterile forceps. Then Petri dishes are placed in the refrigerator at 4ºC or at room temperature for 1 hour for diffusion. Incubate at room temperature for 24 - 48 hours. Observe the zone of inhibition produced by different Antibiotics. Measure it using a scale or divider or venire calipers and record the average of two diameters of each zone of inhibition.

Cytotoxic Activity: The human cervical adenocarcinoma cell line (HeLa) and murine embryonic fibroblasts cell line (NIH 3T3) were obtained from National Centre for Cell Science (NCCS), Pune. HeLa was grown in Eagles Minimum Essential Medium (EMEM) and NIH 3T3 was grown in Dulbeccos Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cells were maintained at 37^0 C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10^5 cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^{0}C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethylsulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2 fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl

of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addiction the plates were incubated for an additional 48 h at 37^0 C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations[22, 23].

MTT assay: MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.
% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC50 was determined using GraphPad Prism software.

Results and Discussion

Antibacterial and antifungal Screening: Antimicrobial activity was conducted against a food borne pathogenic microorganisms including Gram positive and Gram negative bacteria and fungi.

The antibacterial activity and antifungal activity of the extracts of *O.wynaadensis* at different concentrations was screened by disc diffusion technique and the zone of inhibition was measured in mm diameter. The results are given in the table 2 and 3 respectively.

The antimicrobial activity of the *O.wynaadensis* against gram (+ve) and gram (-ve) bacteria shown in table 2. *O.wynaadensis* exhibited inhibitory activity against *S. pneumonia*, *B.cereus, A. hydrophila, V. cholera* with narrow inhibition zones of 16.0, 12.0, 12.0 and 14.0 mm and MIC value of 2.5, 2.0, 2.5, 2.0 mg/ml and for MRSA an inhibition zone of 14 mm and MIC value of 2.5 mg/ml.

The methanol extract exhibited significant antifungal activity against most of the tested fungi species with zones of inhibition between 11-25 mm at the tested concentration. The antifungal activity of *O.wynaadensis* against *Candida albicans, Aspergillus niger, Streptomysis Greusis, M.purpureus* and *Aspergillus fumigate* with narrow inhibition zone 11.0, 12.0, 16.0, 11.0 and 25.0 mm and MIC value of 2.5, 2.5, 1.25, 2.5, 1.25 mg/ml and the results are comparable with the standard substance.

Anti MRSA activity: Methicillin-resistant Staphylococcus aureus (MRSA) has become endemic in most hospitals and health care facilities. The MRSA strains are broadly resistant to β-lactam and macrolide/azalide antimicrobials but responsive to certain non-β-lactam antibiotics[24,25]. However, resistance rates are increasing and there are other limitations in the use of those drugs. Thus given the widespread dissemination and morality caused by MRSA, the synthesis and development of new drug is imperative.

In the present study methanolic extract of *O.wynaadensis* is too led for its MRSA activity. Our drug showed moderate activity against the Methicillin-resistant Staphylococcus aureus. It showed zone of inhibition 14 mm, MIC at 2.5 mg. The standard compound Ampicillin showed 17 mm but the MIC at 8.0 mg. This is the first report on the antimicrobial activity of the *O.wynaadensis* and the *Osbeckia* genus. The results are given in table 4.

Overall the results suggest that the methanolic extract of *O.wynaadensis* may be potential use in the treatment of MRSA and other bacterial infections.

Cytotoxic activity (MTT assay): *O.wynaadensis* showed cytotoxic activity against HeLa and NH3T3 and the IC50 value found 220.3 µg/mL and 92.35 µg/mL respectively. The results are given in the table 5.

In the present study, In-vitro cytotoxic effect of methanolic extract of *O.wynaadensis* against human cervical cancer cell line (**HeLa**), mouse embryonic fibroblasts cell line (**NIH 3T3**) cell lines were determined by MTT assay. The results indicate that *O.wynaadensis* showed good activity against murine embryonic fibroblasts cell line (**NIH 3T3**), IC_{50} 93.2 µg/mLcompared to human cervical cancer line IC_{50} 220 µg/mL. This is the first report of its kind on the *in-vitro* cytotoxic activity against HeLa and NIH3T3 cancer cell lines.

Conclusion

In the present study we confirm the *in-vitro* cytotoxicity activity and antimicrobial activity of methanolic extract of *O.wynaadensis* was carried out for the first time.

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Table-1
Biological activities reported in the *Osbeckia* genus

S. No	Plant Name	Activity
1	*O. aspera*	1. Protection by *O.aspera* against carbon tetrachloride-mediated alterations in microsomal drug metabolizing enzyme activity[26]. 2. Antioxidant activity of *O.aspera*[27]. 3. Phenolic and Terpenoid Constituents from the Sri Lankan Medicinal Plant *O.aspera*[28]. 4. In vitro studies on the immunomodulatory effects of extracts of *O.aspera*[29].
2	O.crinita	1. *The chemical Constituents of* O.crinita[30].
3	*O. octandra*	1. Protective effects of *O.octandra* against paracetamol-induced liver injury[31]. 2. Protective effects of *O.octandra* against galactosamine and *tert*-butyl hydroperoxide induced hepatocyte damage[32]. 3. A comparative study of the efficacy of Pavetta indica and *O.octandra* in the treatment of liver dysfunction[33]. 4. A comparative study of the beneficial effects of *O.octandra* and *O. aspera* in liver dysfunction in rats[34]. 5. Hypoglycemic activity of three plant treatments for diabetes mellitus: artocarpus heterophyllus, *O.ctandra* and asteracantha longifolia[35]. 6. An evaluation of the potency of *O.octandra* and Melothria maderaspatana as antihepatotoxic agents[36].
4	*O.opipara*	1. Chemical constituents in roots of *O.opipara*[37].
5	*O.chinensis*	1. Chemical constituents of *O.chinensis*[38]. 2. Tannin antioxidants from *O.chinensis*[39]. 3. Study of the Traditionally Used Medicinal Plant *O.chinensis* For Hypoglycemic and Anti-hyperglycemic Effects in Mice[40].
6	*O.nepalensis*	1. Antihyperglycemic effect of Aqueous and Ethanol extract of Aerial part of *O.nepalensis* Hook in Alloxan induced Diabetic rats[41].

Table-2
In-vitro* antibacterial activity of methanolic extract of *O.wynaadensis

Bacteria	**Zone of inhibition (mm)**		**MIC (mg)**	
Gram positive	*OSW**	Ampicillin	*OSW*	Ampicillin
S. pneumonia	16.0	-	2.5	-
B. cereus	14.0	15.0	2.0	5.0
Gram negative	*OSW*	Ampicillin	*OSW*	Ampicillin
A. hydrophila	14.0	-	2.5	-
V. cholera	12.0	16.0	2.0	5.0

*OSW**: Methanolic extract of *O.wynaadensis*

Table-3
In- vitro* antifungal activity of methanolic extract of *O.wynaadensis

Fungi	**Zone of inhibition (mm)**		**MIC (mg)**
	OSW	**Clotrimazole**	***OSW***
Candida albicans	11.0	12	2.5
Aspergillus niger	12.0	11.0	2.5
Streptomysis Greusis(mm)	16.0	13.0	1.25
M.purpureus	11.0	15.0	2.5
Aspergillus fumigate	25.0	18.0	1.25

*OSW**: Methanolic extract of *O.wynaadensis*

Table 4
In-vitro* anti MRSA activity against methanolic extract of *O.wynaadensis

Bacteria	**Zone of inhibition (mm)**		**MIC (mg)**	
	OSW	**Ampicillin**	***OSW***	**Ampicillin**
MRSA	14.0	17.0	2.5	8.0

*OSW**: Methanolic extract of *O.wynaadensis*

Table-5
cytotoxic properties of methanolic extract of *O.wynaadensis* in human cervical cancer cell line (HeLa) and Murine Embryonic Fibroblasts Cell Line (NH3T3) by MTT assay

S. No	**Cell lines**	**IC_{50} in µg/mL**
1	HeLa	220.3
2	NIH 3T3	92.35

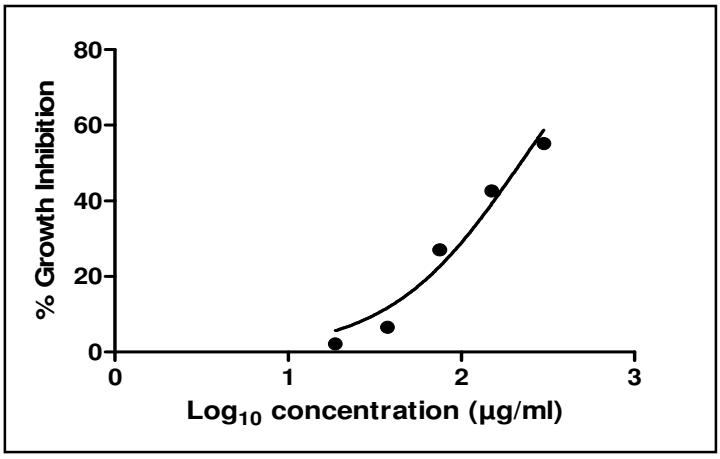

Figure-1
MTT assay of O.wynaadensis on HeLa cell line

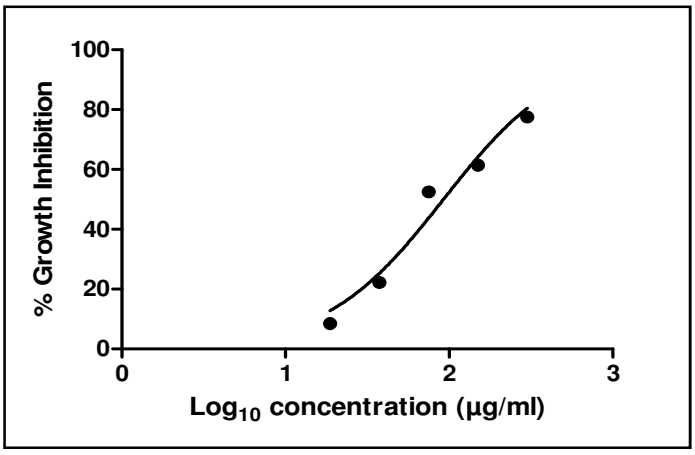

Figure-2
MTT assay of O.wynaadensis on NH3T3 cell line