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Evaluation of potential biological activities of metabolites from endophytic fungi residing in leaves of *Azadirhacta indica*

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Abstract: The objective of the present study is to evaluate anti bacterial activity against *Bacillus* sp, *Pseudomonas aeruginosa* and *Staphylococcus aureus* anti tumour activity against Hep 2 cell line with crude metabolites isolated from endophytic fungi residing in leaves of *Azadirhacta indica*. Endophytic fungi isolated from sterile leaflets of *Azadirhacta indica* and the isolated respective fungi were grown in liquid media, the metabolites were obtained from culture free supernatant of the media after the extraction with ethyl acetate, the concentrated extract used as crude metabolite, the metabolites thus obtained with different concentration evaluated against bacterial strains adopting agar diffusion assay. Minimum inhibitory concentration against the tested bacterial strains was studied adopting broth dilution method. Anti tumour activity was studied against HEP 2 cell line adopting MTT assay..Cytotoxic effect of metabolites on vero cell line adopting MTT assay and human peripheral blood RBC with microscopic examination was also studied. A total of 25 isolates belong to *Alternaria, Cladosporium, Paecilomyces, Rhizopus, Trichoderma sp* were isolated. Among the different fungal extracts, ethyl acetate extract of *Cladosporium sp* showed anti bacterial and anti tumour activity. All the tested bacteria were susceptible to ethyl acetate extract and maximum activity was recorded in *Pseudomonas aeruginosa*.Anti tumour activity against HEP 2 cell line reveals 100 µg concentration inhibited maximum viability of HEP 2 cell line. Distinct cytotoxic effect on vero cell line and RBC was not recorded. The study suggests the possible utilization of endophytic fungal metabolites of *Cladosporium* sp against bacterial strains and cancer.
Keywords. endophytic fungi, *Cladosporium*, metabolites, anti bacterial, anti tumour.

Introduction

An endophyte is a bacterial (including actinomycete) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra cellular healthy tissues of the host plant, typically causing no apparent symptoms of disease[1] (Lugtenberg et al, 2001). Endophytes are being accepted as an important source of novel bioactive secondary metabolites that can be excellent new starting points for the development of novel pharmaceuticals and/or agrochemicals[2] (.Mucciarelli et al,2003). Endophytes are well known as producer of antibiotics and other biologically active substances of higher commercial value, such as vitamins, alkaloids, plant growth factors, enzymes and enzyme inhibitors.They play major role in physiological activities of host plants influencing enhancement of stress, insect, nematode and disease resistance[3,4] (Petrini et al, 1989, Pilnik et al,1989). Endophytes can also accelerate plant growth and nitrogen fixing capabilities of host plants (Many

plants are known to harbour endophytic fungi that are believed to be associated with the production of pharmaceutical products (Fungal endophytes have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities[5] (Ryabushkina, 2005). A recent comprehensive study has indicated that 51% of bioactive substances isolated from endophytic fungi were previously unknown[6] (Schulz and Boyle,2005) Hence, the endophytic fungi are expected to be a potential source for new natural bioactive products[7] (Saikkonen et al,2005). There are many reports and studies on the biological activities of endophytes like antiviral, anticancer and antimicrobial effects.Cytotoxic and antibacterial activities of a total of 300 endophytic fungi were investigated[8] (Van Straten et al,1977). Since the discovery of the world's first billion-dollar anticancer compound– paclitaxel (Taxol) – could be biosynthesized by *Pestalotiopsis microspora*, a fungus that colonizes the Himalayan yew tree, interest in studying such endophytes for their medicinal potential has grown tremendously[9] (Wang et al,2007).In the present study, the endophytic fungi were isolated from sterile leaflet of *Azadirhacta indica* and the crude metabolites obtained from the respective fungi evaluated for the anti bacterial and anti tumour activity.

Experimental

Isolation of endophytic fungi

Endophytic fungi was isolated from surface sterilized leaflets of *Azadirhacta indica* by the modified method of Verma *et al*[10] .In this method, Fresh and health leaves of *Azadirhacta indica* collected from the University campus in sterile polythene bag and brought to the laboratory immediately. The collected leaves were cut into 1mm square surface using sterile blade and then rinsed in sterile distilled water. The cut sections were then consecutively washed in sterile distilled water containing 0.1%HgCl2. Then the cut sections were transferred consecutively to petriplate containing only sterile distilled water retaining in each for one minute. Then the sections were carefully taken one by one with sterile forceps, the excess water was blotted on to a sterile tissue paper and then transferred into the Potato Dextrose Agar medium(PDA). The plates were incubated at 28ºC for 10 days. Daily observation was made to record the fungal growth. The respective fungal growths from the leaves were transferred to PDA plates for colony morphology and further identification. Identification was carried out based on standard methods Identified fungi were maintained on PDA slant.

Crude extraction of bioactive compounds

The fungi grown in PDA slants were flooded with sterile distilled water containing 0.1%Tween 80 and scrapped with surface sterilized glass rod and slurry was filtered through cheese cloth to remove mycerial debris. Then 0.1 ml of spore suspension of the culture was added to 100 ml of sterile Potato Dextrose Broth and kept in a shaker in room temperature at 150 rpm for 7 to 10 days. After the incubation period, the media was filtered and the collected filtrate was extracted with the double the volume of ethyl acetate, The crude extract was vacuum dried at 60ºC and the concentrated extract was used for further studies.

Anti bacterial activity

The antibacterial activity of crude metabolites with 10, 25, 50 and 100 was tested against laboratory stock culture of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus* sp. The strains were maintained on nutrient agar slants. A single colony of the test strains were grown overnight in Mueller Hinton broth on a rotary shaker (200 rpm) at 35°C. The inoculums were prepared by diluting the overnight cultures with 0.9%NaCl to a 0.5 McFarland standard. A lawn of the test organism was made on the Muller Hinton agar (Hi media, Mumbai, India) and the cotton gause loaded with respective concentration of ethyl acetate extract of respective fungal isolate was placed on the petri plate. The plates were incubated at 370C for 24 hours and the plates were observed for zone of inhibition. After the incubation period the diameter of the zone was recorded.

Evaluation of minimum inhibitory concentration (MIC)

Minimum inhibition concentration (MIC) was determined by a turbidometric method. In this method, a series of tubes each containing 2ml broth medium was prepared. The crude metabolites were accurately quantified and dispersed into the broth medium. Briefly, 2mL of nanoparticles added into the tubes separately. After mixing, 2 ml of mixture from these tubes were transferred to the next tubes, and a similar procedure was repeated for the subsequent tubes. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The addition of the metabolites did not affect the pH of the medium. The bacteria suspension was then added to the tubes to achieve a final bacterial concentration of 105 cells/ml. The bacterial concentration was estimated from the optical density of the suspension based on standard calibration

with the assumption that the optical density of 1.0 at 600nm for *Pseudomonas aeruginosa, Enterococcus faecalis* suspension is equivalent to approximately 109 cells/ml and the optical density of 1.0 at 600 nm for *Bacillus* sp suspension is equivalent to approximately 109 cells/ml (22,23).All the tubes were incubated in an orbital shaker with a shaking speed of 200 rpm/min at 37°C for 20 h. The MIC was determined as the minimum concentration at which there is no visible change in the turbidity of the medium.

Anti tumor activity against HEP 2 cell line and non target cytotoxic effect on vero cell line

Cell line Hep2 and Vero were obtained from KINGS institute, Chennai. with passage number 2) were grown in Minimum Eagles Medium (MEM) contained 10% heat inactivated Fetal Calf Serum (FCS) and 100 units/ml Penicillin G and 100 µg/ml Streptomycin at a 370C in a humidified 5% CO2 incubator and the cell lines were treated with different concentration of crude metabolites.. Colorimetric MTT assay was performed to evaluate anti tumour activity. MTT is reduced to purple formazan by mitochondrial succinate dehydrogenase of living cells. Stock MTT (10x), was prepared by dissolving tetrazolium in PBS (phosphate buffer saline) at a concentration of 5 mg/ml and filtering through 0.45 mm filter. The medium of the confluent cells was removed, and then 100 µl of 2X MTT was added to each well. Following incubation at 370c with 5% CO2 for 4 hrs, 100µl of acidic Isopropanol was added and mixed to release the color from the cells. MTT is removed and washed with 2drop of Phosphate Buffer saline. Optical density was measured at 540 nm

Cytotoxicity assay against Human RBC

Using 10ml sterile syringe a peripheral blood was collected in sterile 15 ml centrifuge tube containing 0.1% EDTA and the collected blood was centrifuged at 2500 rpm for 15 min. the supernatant was discarded and the collected RBC was washed with sterile PBS. 0.1 ml of washed cell suspension was incubated with cell supernatants with respective concentration. The mixture was incubated at 37^0Cfor 12-24hrs and RBC count was made using Hemocytometer and microscopic examination with Leishmann stain was done to detect any morphological changes on RBC.

Results

Endophytic fungal isolates

A total of 75 isolates belong to five genera belong to *Alternaria sp, Cladosporium sp,Paecilomyces sp,Rhizopus sp,Tricoderma sp* were isolated (Table 1). Among the different fungal genera, *Cladosporium sp* was found to be dominant (50%) followed by *Paecilomyces sp* (20%), *Rhizopus sp*(15%) and *Tricoderma sp*(10%), *Alternaria* sp (5%). All the fungal isolates were identified based on cultural and morphological characteristics.

Anti bacterial activity

Ethyl acetate extract was prepared from the filtrate of the of the respective fungal organism grown in liquid media and the extract thus obtained was evaluated against anti bacterial and anti tumour activity with different concentration. Anti bacterial activity was recorded in ethyl acetate extract of *Cladosporium sp* and anti bacterial activity was not reported in other fungal extracts (Table 2). All the tested bacteria was susceptible to all the tested concentration. Among the three bacterial species, maximum zone of inhibition was recorded in *Bacillus sp* with 27,29,31 and 31mm at respective concentration followed by 23, 25,26 and 27mm in the case of *Pseudomonas* sp (Figure 1). 22,24,26 and 26 mm of zone of inhibition was recorded in *Enterococcus faecalis*. Minimum inhibitory concentration (MIC) for *Bacillus* sp, *Enterococcus faecalis* and *Pseudomonas* sp was found to be 24-27 µg, 26-29 µg and 31-34 µg (Table 2).

Anti tumour activity and non target cytotoxic effect on vero cell line and human red blood cells

Among the different fungal filtrate ethyl acetate extracts ,*Cladosporium* sp shows anti tumour activity against tested HEP 2 cell line. Different concentration of metabolites was prepared. Hep2 cells grown in 96 well plates were incubated and the viability in respective concentration was determined by MTT assay which reveals that all concentration if metabolites reduced the viability than the control. Effective high cytotoxic effect was recorded at 100µg/ml followed by 50, 25 and 10µg/ml and the percentage of viability at respective concentration was 90.0, 87.5, 65.1 and 51.2 % respectively (Figure 2). Cytotoxicity study with vero cell line revealed that all the tested concentration of metabolite induced less cytotoxic effect with 23.5,12.5 7.0 and 0.0 % (Figure 3).The cytotoxic assay using human blood cells mainly RBC reveals no distinct cytotoxicity in all

tested concentrations. Moreover no lysis, structural changes and reduction was observed. RBC count was found to be stable as in control (Table 4).

Table 1.Generic composition (%) of endophytic fungi

Endophytic fungi	**Generic composition(%)**
Cladosporium	50
Paecilomyces	20
Rhizopus	15
Tricoderma	10
Alternaria	5

Table 2. Antibacterial activity of metabolites as measured by Zone of inhibition (mm) against tested bacteria

	Zone of inhibition (mm)			
Tested bacteria	10	25	50	100 (µg)
Bacillus sp	27	29	31	31
Pseudomonas aeruginosa	23	25	26	27
Enterococcus faecalis	22	24	26	26

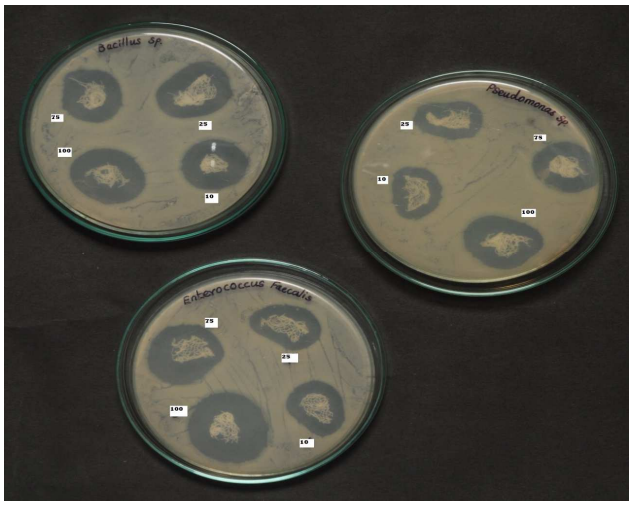

Figure 1. Anti bacterial activity of metabolites against bacteria showing zone of inhibition

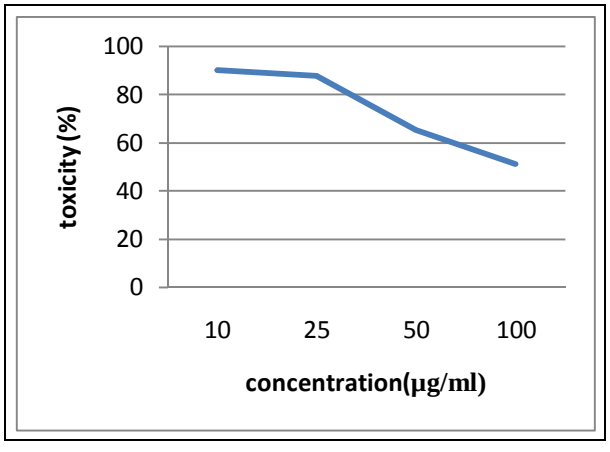

Figure 2. Viability (%) of metabolites against HEP 2 cell line

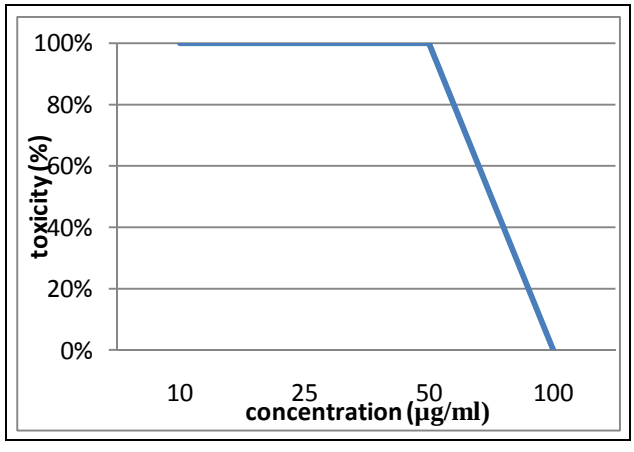

Figure 3.Viability (%) of metabolites against vero cell line

Table 3.Minimum inhibition concentration (MIC) of metabolites against the tested bacteria

Tested bacteria	**MIC (µg)**
Bacillus sp	24-27
Pseudomonas aeruginosa	26-29
Enterococcus faecalis	31-34

Table 4. Effect of metabolites on RBC count

Concentration (µg)	RBC count (10^6)
10	2.1
25	2.1
50	2.1
100	2.1
Control	2.1

Discussion

Since the discovery of endophytes in Darnel, Germany in 1904[11] (Tan and Zou, 2001), various investigators have defined endophytes in different ways which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. Bacon and White give an inclusive and widely accepted definition of endophytes-"Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. Endophytes well known as producer of antibiotics and other biologically active substances of higher commercial value, such as vitamins,alkaloids, plant growth factors, enzymes, and enzyme inhibitors. In the present study, five fungal genera were isolated. Biological activities –anti microbial and anti tumour activity was recorded in ethyl acetate extract of *Cladosporium sp*. All the tested bacteria were found to susceptible to extracts. Thyagarajan and Namasivayam[12] (2011) isolated seven genera of endophytic fungi residing in *Vitex negundo* and mycelium extract of *Penicillium* sp and *Fusarium* sp revealed anti bacterial activity against human pathogenic bacteria. Namasivayam *et al*[13] reported anti fungal activity of butanol cell free extract of endophytic actinomycetes *Streptomyces griseoaureofaciens* against *Candida albicans* isolated from HIV positive patients. Anti-Candida metabolites from endophytic fungi were obtained from the submerged cultures of some 1500 Ascomycota and Basidiomycota ,isolated from their fruit-bodies or as soil-borne[14] (Bhilabutra et al,2007). Endophytic fungi were screened for activity against Candida albicans and arange of other pathogenic and saprotrophic fungi. From five species of endophytes, six bioactive compounds were isolated and identified,viz. cerulenin, arundifungin, sphaeropsidin A, 5-(1,3-butadiene-1-yl)-3-(propene-1-yl)-2-(5H)-furanone, ascosteroside A (formerly called ascosteroside (and a derivative of 5, ascosteroside B. Wang *et.al* [10] conducted a study in which among 67 endophytic fungi isolated from *Quercus variabilis*, 53.7% of endophytic fungal fermentation broths displayed growth inhibition on atleast one test microorganism, such as pathogenic fungi (*Trichophyton rubrum, Candida albicans, Aspergillus niger, Epidermophyton floccosum, Microsporumcanis*) and bacteria (*Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens*). The most active antifungal strain I(R) 9-2, *Cladosporium* sp was isolated from *Q. variabilis*. In the present study, anti bacterial activity was reported against all the tested bacteria in all the tested concentration. Anti tumour activity was also reported in ethyl acetate extract of *Cladosporium* sp with dose dependent manner. Moreover, cytotoxic toxic effect was not observed in vero cell line and RBCs. Further study will helpful to identify the bioactive compound in the ethyl acetate extract of *Cladosporium* sp and lead to development of active pharmacological product.In conclusion, the present study suggests the possible utilization of endophytic fungal metabolites *Cladosporium* sp as a novel anti bacterial and anti tumour drug after the further purification, structural analysis and clinical trails.

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