Research Article

Ethyl acetate Extraction of Antibacterial Compounds of Endophytic Fungi Isolated from Medicinal Plants

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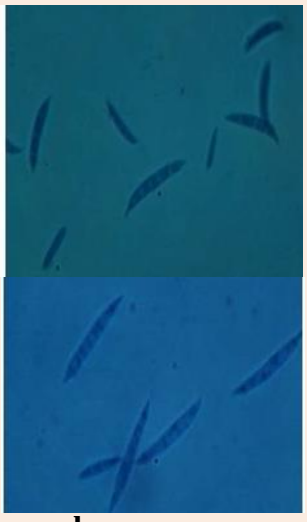
Abstract

Drug resistance in microorganisms has become a global concern and explore for new antibacterial agents is urging and ongoing. Endophytic fungi from five medicinal plants in southern Tamilnadu were explored for their ability to produce antimicrobial compounds. Endophytic fungus isolated from the leaves of *Vitex negundo* (VN), *Justicia jenderusa* (JJ), *Ocimum basalicum* (OB), *Costus spictus* (CS) and *Glycosmis pentophylla* (GP) screened for its antimicrobial activity. The isolated fungi were grown in potato dextrose broth for 21 days, and the compounds were extracted with ethyl acetate. The fungal extract showed potent inhibitory activity against the following pathogens (*Escherichia coli, Corynebacterium diphtheriae, Methicillin Resistant Staphylococcus aureus* (MRSA), *Proteus mirablis, Salmonella typhi*). The endophytic fungi VN-1 and OB-1 showed effective antibacterial activity against all the five human pathogens. JJ-5 showed moderate activity and JJ-1, JJ-4, CS-3, GP-1 showed less activity against the pathogens.

Keywords: Medicinal plants, Endophytic fungi, Antibacterial assay, Ethyl acetate.

This study reveals that the endophytic fungi serve as a potential source for the production of effective bioactive compounds.



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Introduction

Since ancient times, people have been exploring the nature particularly plants in search of new drugs. This has resulted in use of large numbers of medicinal plants with curative properties to treat various diseases. According to World Health Organization, 80% of the people living in rural areas depend on medicinal plants as primary health care system. Natural products from plants have been valuable sources for cancer drug discovery [1]. Endophytes are microorganisms found to be growing in the inner part of the healthy plants [2, 3]. They associate with their host plant, often produce metabolites that could help to protect the host plants from insect and other pathogenic microorganisms and enhance the growth of their host plant. It has been stated that 51% of all important biologically active metabolites have been isolated from the fungal endophytes [4]. Endophytic fungi are biotechnological important source for novel compounds such as antibiotics, chemotherapeutic agents and agrochemicals to human welfare. Some useful features of endophytic fungal metabolites can be found by further exploration due to highly effective, posses low toxicity and will have a minor environmental impact. Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compound [5, 6]. Endophytic fungi have been recognized as useful sources of bioactive secondary metabolites. Secondary metabolites act as a safeguard for plants against pathogens, pests and herbivores [7]. Compounds extracted from traditional medicinal plants have potent therapeutic properties [8]. Secondary metabolites from endophytic fungi are exploited in pharmaceutical and agricultural industries [9]. The need for new and useful compounds to provide assistance and relief in all aspects of

the human condition is ever growing. It is important to understand the methods and rationale used to provide the best opportunities to isolate new endophytic microorganisms as well as the ones making novel bioactive compounds. Objective of this study is to screen the antibacterial activity of the secondary metabolites extracted from the endophytic fungi isolated from medicinal plants.

Materials and methods

Collection of Plant Samples

All medicinal plants were collected from the Botanical garden, Madurai Kamaraj University, Madurai, Tamil Nadu (8°04'46.15'N 77°33'05.45'E). The collected plant samples were transferred to laboratory in air-tight paper bags and processed to isolate the endophytic fungi.

Isolation of endophytic fungi

Plant samples which contain young and mature leaves were collected from different part of the plants. Collected plant materials were washed in the tap water to remove the dust particles present in the surface of the plants. Plant materials were surface sterilized by consecutive immersion for 1min in 75% ethanol, Further Sterilized sequentially in 3% Sodium hypochlorite Solution for 2 min and 30sec in 75% ethanol [10]. From the surface sterilized leaf, approximately 0.5 cm long pieces were cut from the upper, middle and lower portion of young and mature leaves respectively. The leaves were surface sterilized and dried with sterile paper towels. Sets of fours discs were then evenly placed in each 90 mm Petri dish containing PDA supplemented with 1mg/ml Streptomycin. In order to isolate, slow growing fungi and incubated at 26^0C under a 12hour light 12hour dark cycle [11]. Fungal colonies growing in the petri dishes were transferred to fresh agar slants. To prevent rapidly growing isolates from inhibiting slow growing species, the former were removed to fresh slants [12].

Extraction of fungal metabolites

Isolates were grown in potato dextrose broth for 21 days. After the incubation period, all the culture filtrate was treated with ethyl acetate using separating funnel. The solvent phase and aqueous phase was separated in the funnel. In this separation, solvent extract the fungal metabolites from the culture filtrate. Solvent phase was collected and condensed using rotary vacuum evaporator. The dry residue was re-dissolved in Ethyl acetate for further research activities.

Assessment of antimicrobial activity of fungal extracts

Antimicrobial activity was assessed by Agar well diffusion method [13] in preliminary stage. Condensed solvent mixtures were prepared in 10mg/mL concentration. Streptomycin was used as positive control and solvent used as negative control. In this assay, antibacterial activity was tested against five human pathogens (MRSA, *E. coli*, *P. mirabilis*, *C. diphtheriae*, and *S. typhi*). Based on this results high potential fungi were chosen for further work.

Results and Discussion

Collection of Medicinal plants

A total of five medicinal plants named as *Vitex negundo* L. (VN), *Justicia jenderusa* L. (JJ), *Ocimum basalicum* L. (OB), *Costus spictus* L. (CS) and *Glycosmis pentaphylla* (GP). Collected medicinal plants were cut into small segments and surface sterilized. Sterilized segments were placed on the PDA medium amended with antibiotic to isolate the fungal endophytes.

Isolation plant endophytes

A total of 22 endophytic fungi were isolated from the middle part of the leaves with veins and from midrib of the above said medicinal plants. Endophytes growing out of the host tissues were subculture on PDA agar. Maximum of 7 isolates obtained from the medicinal plant *Justicia jenderusa* followed by 6 isolates from *Vitex negundo* respectively. Fungal isolates obtain from the host culture were frequently cultured on PDA plates to obtain pure and store at 4°C for further studies.

Extraction of fungal metabolites from culture filtrate

All the 22 fungi isolates obtained from the medicinal plants were selected for extraction of secondary metabolites. Extraction of secondary metabolites, fungi were grown on 500ml of Erlynmeyer flask containing potato dextrose broth for 21 days. Presence of metabolites in the culture filtrate synthesized by the fungi was extracted by addition of equal amount of ethyl acetate [14]. Culture filtrates with the ethyl acetate were transferred to separating funnel and then it was kept for 24hrs for extraction of metabolites, intermediate shaking of the culture filtrate with solvent enhances the better extraction of compounds. Metabolites present in the ethyl acetate were condensed in rotary evaporator and the concentration of the compound was weighed.

Antibacterial activity

Antibacterial screening was carried out with the extracts of the 7 fungal species against the five standard bacterial pathogens (*Escherichia coli, Salmonella typhi, Staphylococcus aureus, Corynebacterium diphtheria, and Proteus vulgaris*) given in table 1. The antibacterial effect of the fungal extracts is shown in figure 2. The inhibition zone values were highly variable, ranging between 10-37 mm. The extracts from fungal isolate VN1 was very effective (I. Z. D. > 20 mm) against all the five bacterial strains.

Further, isolate VN 1 exhibited a highest antibacterial activity against *Salmonella typhi* (37mm) (Figure 1) compared to other isolates. Isolate OB1 showed moderate antibacterial activity against all the five bacterial strains. All the seven isolates inhibited *Escherichia coli* pathogenic strain significantly high compared to other tested microbes. Streptomycin used as positive control for bacteria showed a zone of inhibition of 25 mm.

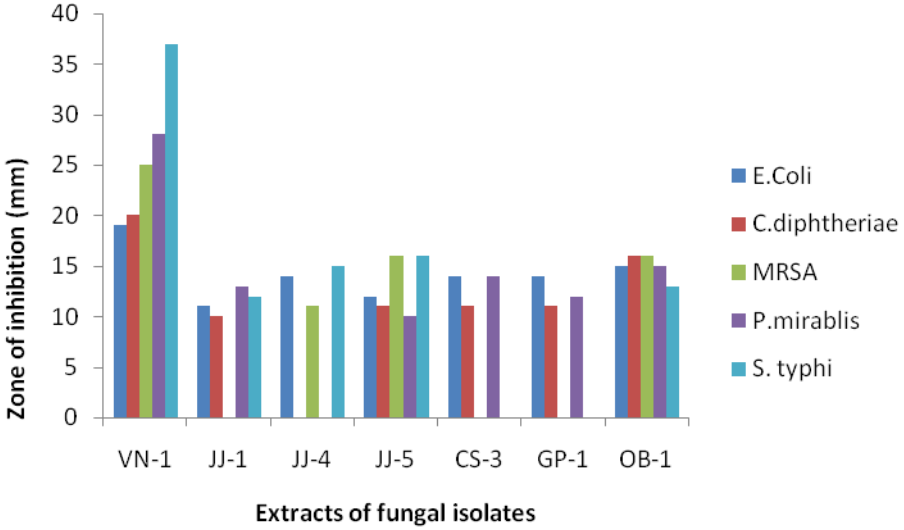

Figure 1 Graphical representation of zone of inhibition of 7 different fungal extracts

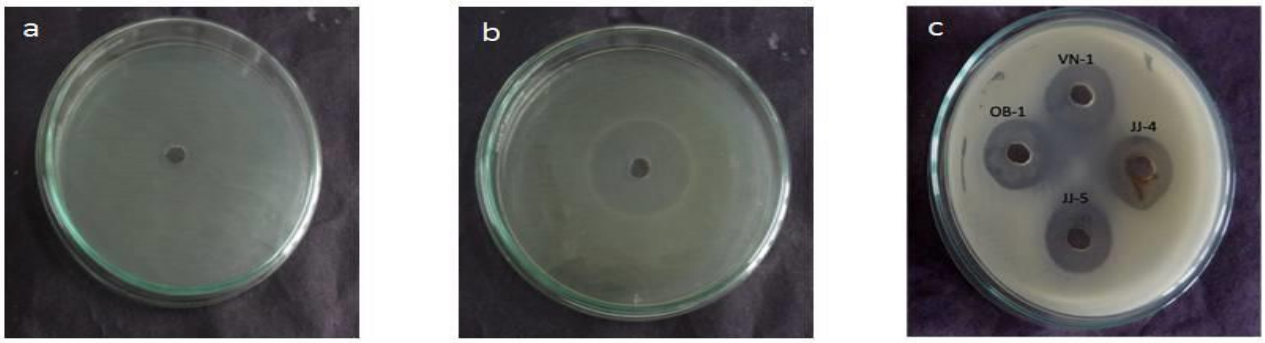

Figure 2 Antibacterial activity of four fungal extracts, (a) negative control (b) Positive control and (c) antibacterial activity of four fungal extracts.

Table 1 Antibacterial activity of endophytic fungal ethyl acetate extracts (10 mg/ml) against bacterial species tested by well-diffusion assay

Strain	Test pathogens				
	E. coli	*C. diphtheriae*	MRSA	*P. mirablis*	*S. typhi*
VN-1	++	++	++	+++	+++
JJ-1	+	+	-	+	+
JJ-4	+	-	+	-	++
JJ-5	+	+	++	+	+
CS-3	+	+	-	+	-
GP-1	++	+	-	+	-
OB-1	++	++	++	++	+

+++ Superior activity; ++ Moderate activity; + Less activity

Morphological characterization of positive isolates

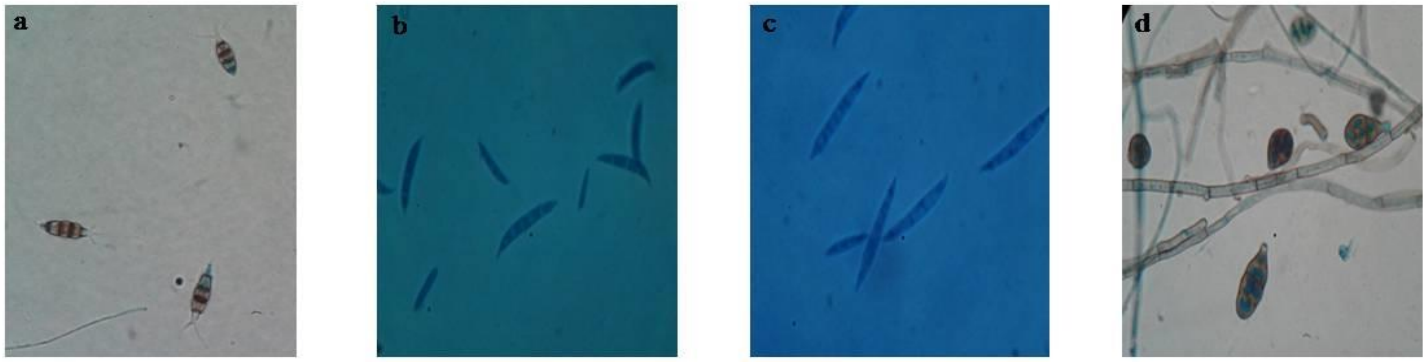

Figure 3 morphological observations of positive isolates, (a) *Pestolopsis sps*, (b) *Fusarium sps*, (c) *Fusarium sps* and (d) *Alternaria sps*.

Extracts from 4 medicinal plants endophytic fungus showed antibacterial effect was selected for morphological characterization. Through microscopically observation, spore morphology of the selected fungi was identified to be *Pestolopsis sps*, *Fusarium sps*, *Fusarium sps* and *Alternaria sps*

Conclusion

In this study, crude extracts of the endophytic fungi isolated from the medicinal plant leaves of VN-1 and OB-1 showed promising antibacterial activity against all the tested five human pathogens. Secondary metabolites extracted from the endophytic fungi could be a great source for antimicrobial compounds and these compounds can be further checked in *in-vivo* models as antimicrobial agents.

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