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Evaluation of antimicrobial compounds synthesized by endophytic fungi isolated from five Indian medicinal plants

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Abstract: Antibiotic resistance in microbes has grown into a global concern, hence the exploration of new antibacterial agents from natural sources is an emerging area of research. Endophytic fungi were isolated from five medicinal plants in southern Tamil Nadu and were explored for their antimicrobial potential. Endophytic fungui isolated from the leaves of the medicinal plants were grown in potato dextrose broth for 21 days. The culture filtrate was extracted with ethyl acetate. The antimicrobial activity was evaluated against bacterial pathogens such as *Escherichia coli, Corynebacterium diphtheriae, Methicillin Resistant Staphylococcus aureus* (MRSA), *Proteus mirablis* and *Salmonella typhi*. The extract from endophytic fungi VN-1 and OB-1 exhibited an effective antibacterial activity against all the pathogens. JJ-5 fungal extract showed moderate activity against the pathogens. The extracts from JJ-1, JJ-4, CS-3 and GP-1 exhibited very less activity against the pathogens. This study shows that the extracts from endophytic fungi isolated from medicinal plants will serve as a potential source for the production of effective antibacterial compounds.

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

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Introduction

Antibiotic resistance among pathogenic microorganisms has increased in recent years, and concerns have been raised that cross resistance might develop due to frequent antibiotic exposure (Russel *et al*., 1999). Since the olden days, Medicinal plants with curative properties have been used to treat various diseases. Endophytes are microorganisms which grow symptomless within spaces of stems, petioles, roots and leaves of plants that show no obvious sign of infection or disease (Strobel and Long 1998; Hallmann *et al*., 1997). The screening of antimicrobial compounds from endophytes is a promising way to meet the increasing threat of drug -resistant strains of human and plant pathogens (Bills and Polishook, 1991). Fungi have long been known as a rich source of biologically active secondary metabolites. After the discovery of penicillin by Alexander Fleming in 1928, Fungi have become an important source of drugs for the treatment of a variety of infectious and non-infectious diseases (Kjer *et al*., 2010). Fungal endophytes have also been recognized as a .

warehouse of novel secondary metabolites, some of which have valuable biological activities (Bills and Polishook., 1991). It has been stated that 51% of all important biologically active metabolites have been isolated from the fungal endophytes (Strobel *et al*., 1999). Since a single endophyte may be able to produce a variety of bioactive metabolites (Ramasamy *et al*., 2010). Some species of endophytic fungi have been identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds (Saikkonen *et al*., 2004). Compounds extracted from traditional medicinal plants have potent therapeutic properties (Petrini *et al*., 1993). The need for new antimicrobial compounds to treat infectious human diseases is ever growing. It is important to understand the rationale methods used to provide the best opportunities to isolate new endophytic microorganisms provides novel bioactive compounds. Objective of this study is to evaluate the antibacterial activity of the secondary metabolites extracted from the endophytic fungi isolated from five medicinal plants.

2. Materials and Methods

2.1 Collection of Plant Samples

The young and mature leaves of five medicinal plants such as *Vitex negundo* L. (VN), *Justicia jenderusa* L. (JJ), *Ocimum basalicum* L. (OB), *Costus spictus* L. (CS) and *Glycosmis pentaphylla* (GP) 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 the laboratory in air-tight bags and further processed to isolate the endophytic fungi.

2.2 Isolation of endophytic fungi

Collected leaves were washed with the running tap water and surface sterilized by consecutive immersion in 75% ethanol for 1 min, in 3% Sodium hypochlorite solution for 2 min, in 75% ethanol for 30 sec and finally washed with distilled water for 30 sec (Petrini *et al*., 1982). The surface sterilized leaves were dried with sterile paper towels. Approximately 0.5 cm long discs were cut from the upper, middle and lower portion of surface sterilized young and mature leaves. A set of fours discs were then evenly placed in each 90 mm Petri dish containing potato dextrose agar (PDA). PDA was supplemented with 1mg/ml streptomycin to avoid bacterial contamination in the plates. Inoculated plates were incubated at 26^0C (Taylor *et al.,* 1999). Rapidly growing fungal strains were repeatedly cultured on PDA plates and transferred to fresh agar slants and the master plates were incubated further to allow the growth of slow growers (Bills and Polishook, 1992).

2.3 Extraction of fungal metabolites

The fungal isolates were grown in potato dextrose broth (PDB) for 21 days. After incubation, the culture filtrate was filtered using Whatmann No. 1 filter paper and extracted three times with equal amount of ethyl acetate using separating funnel (Pavithra *et al*., 2012). Solvent phase was collected and condensed using rotary vacuum evaporator. The dry weight of the residue was calculated and re-dissolved with the known volume of ethyl acetate for further analysis.

2.4Assessment of antimicrobial activity

Antimicrobial activity of the extracts was assessed using Agar well diffusion method reported by Boyanova et al., in 2005. 10mg/mL concentration of crude extracts were prepared. Streptomycin was used as positive control and pure solvent was used as negative control. In this assay, antibacterial activity of the crude extracts was tested against five human pathogens (MRSA*, E. coli, P. mirabilis, C. diphtheriae,* and *S. typhi*).

2.5 Morphological characterization of positive isolates

The positive isolates were selected for the morphological characterization using microscopical observation of hyphae and spore structures. The mycelia and spore were stained with lacto-phenol cotton blue staining and observed under microscope.

3. Results and Discussion

3.1 Collection of Medicinal plants

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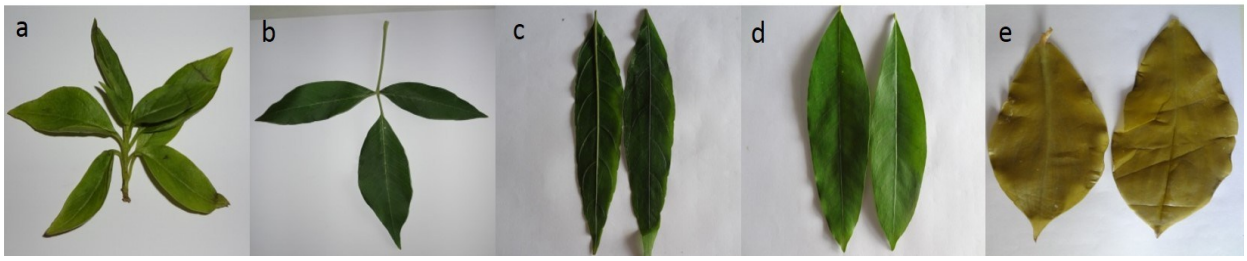

Figure 1: Five different medicinal plants (a) *Oscimum basalicum*, (b) *Vitex negundo*, (c) *Justicia jenderusa*, (d) *Glycosmis pentaphylla*, (e) *Costus spictus*.

A total of five medicinal plants were collected from the Botanical garden, Madurai Kamaraj University, Madurai, Tamil Nadu (8°04'46.15'N 77°33'05.45'E). Surface sterilized leaves were cut into small discs and discs were placed on petri dish with PDA medium amended with antibiotic (Figure 1) to isolate the fungal endophytes.

3.2 Isolation of endophytes

A total of 22 endophytic fungi were isolated from the middle part of the leaves with veins of the medicinal plants. Endophytes growing out of the host tissues were transferred and sub-cultured on PDA agar plates. Maximum number of isolates (7) were obtained from the medicinal plant *Justicia jenderusa* which followed by 6 isolates from *Vitex negundo*. Fungal isolates obtained from the master plate were frequently cultured on PDA plates to obtain pure strain and stored in PDA slants at 4°C for further studies.

Table -1: Number of fungal isolates from medicinal plants.

Medicinal plant	No. of fungal isolates
Justicia jenderusa	7
Vitex negundo	6
Ocimum basalicum	3
Costus spictus	4
Glycosmis pentaphylla	2

3.3 Extraction of secondary metabolites from culture filtrate

All the 22 fungal isolates obtained from the medicinal plants were selected for extraction of secondary metabolites. Fungi were grown on 500 ml PDB in Erlynmeyer flasks for 21 days. Metabolites synthesized by the fungi in the culture filtrate were extracted by adding of equal amount of ethyl acetate for three times. Culture filtrate solvent mixtures were transferred to separating funnel and kept for 24hrs for extraction of metabolites, intermediate shaking enhances the better extraction of compounds. Metabolites extracted in the ethyl acetate were condensed in rotary vacuum evaporator and the dry weight of the compound was weighed. Extracts were dissolved with the known volume of solvent to obtain the concentration of 1mg/ml.

3.4 Antibacterial activity

Antibacterial activity of the extracts from 7 fungal species evaluated against five bacterial pathogens viz: *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Corynebacterium diphtheria, and Proteus vulgaris*. The zone of inhibition exhibited by different fungal extracts against tested bacterial pathogens is shown in Figure 2. The zone of inhibition values were ranging between 10-35 mm (Table 2). The experiment was carried out in triplicates. The crude extract from fungal isolate VN-1 was very effective (Zone of inhibition diameter > 20 mm) against all the five bacterial strains. In addition, isolate VN-1 exhibited a highest antibacterial activity against *Salmonella typhi* (35mm) (Figure 1) with comparison to other isolates. Isolate named OB-1 exhibited moderate antibacterial activity against all the five bacterial pathogens. All the seven isolates inhibited *Escherichia coli* pathogenic strain compared to other tested pathogenic strains. Positive control (Streptomycin 1mg/ml) showed a zone of inhibition of 25-35 mm.

3.5 Morphological characterization of positive isolates

Extracts from 7 fungal isolates exhibited high antibacterial activity. These positive fungal strains was selected

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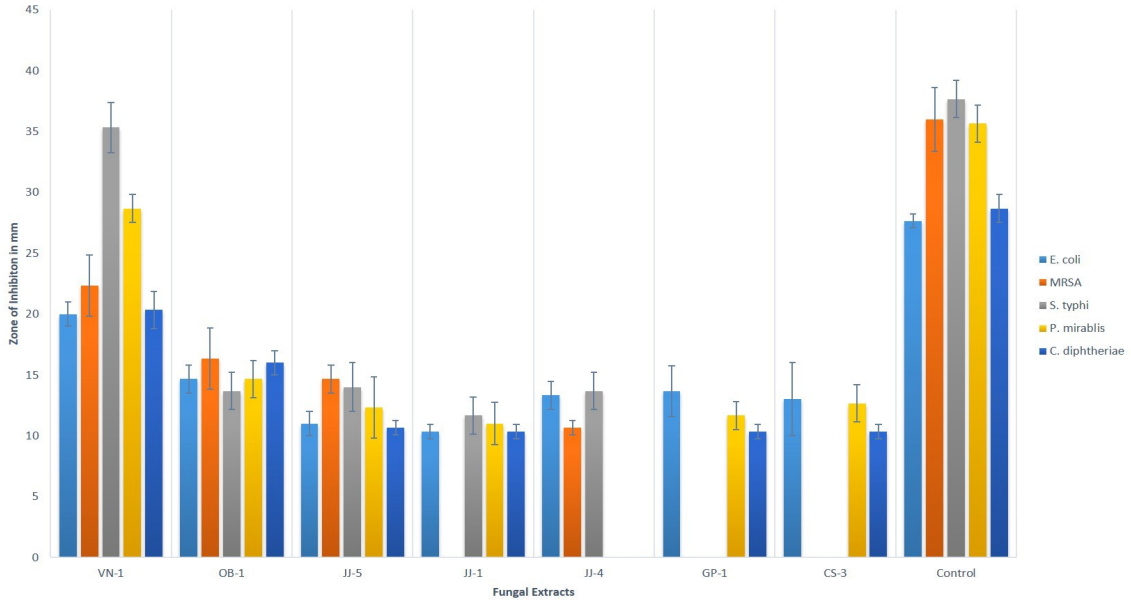

Figure 2: Graphical representation of zone of inhibition exhibited by fungal extracts

Table 2: Antibacterial activity of endophytic fungal extracts (1mg/ml) against bacterial species tested by well-diffusion assay

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

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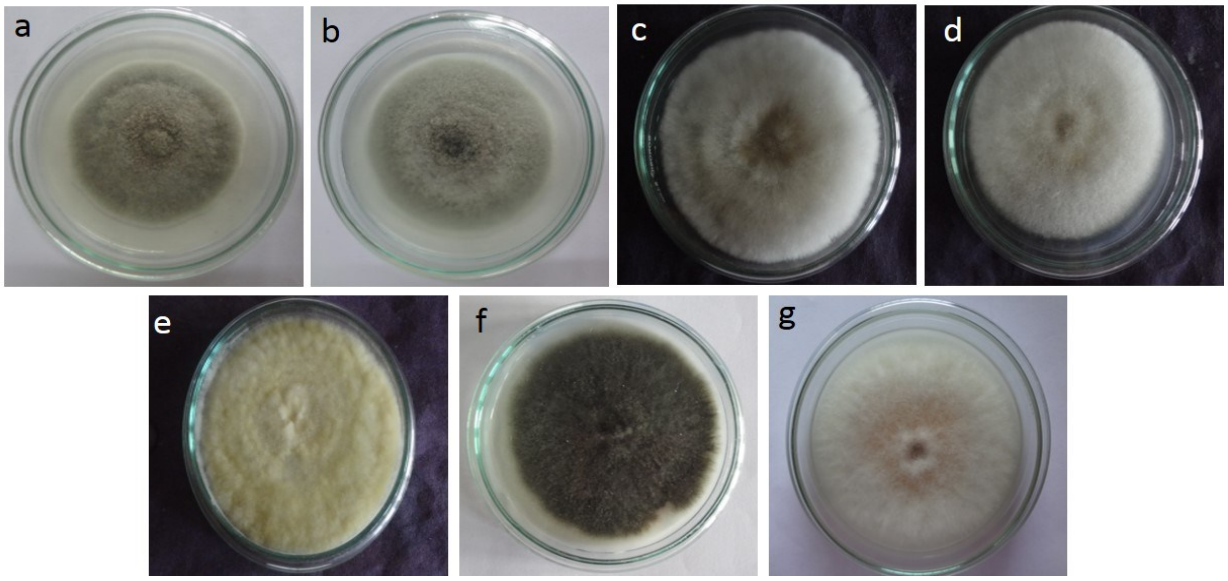

Figure 3: Positive endophytic fungal strains on PDA plates (a) VN-1, (b) OB-1, (c) JJ-5, (d) JJ-1, (e) JJ-4, (f) GP-1, (g) CS-3

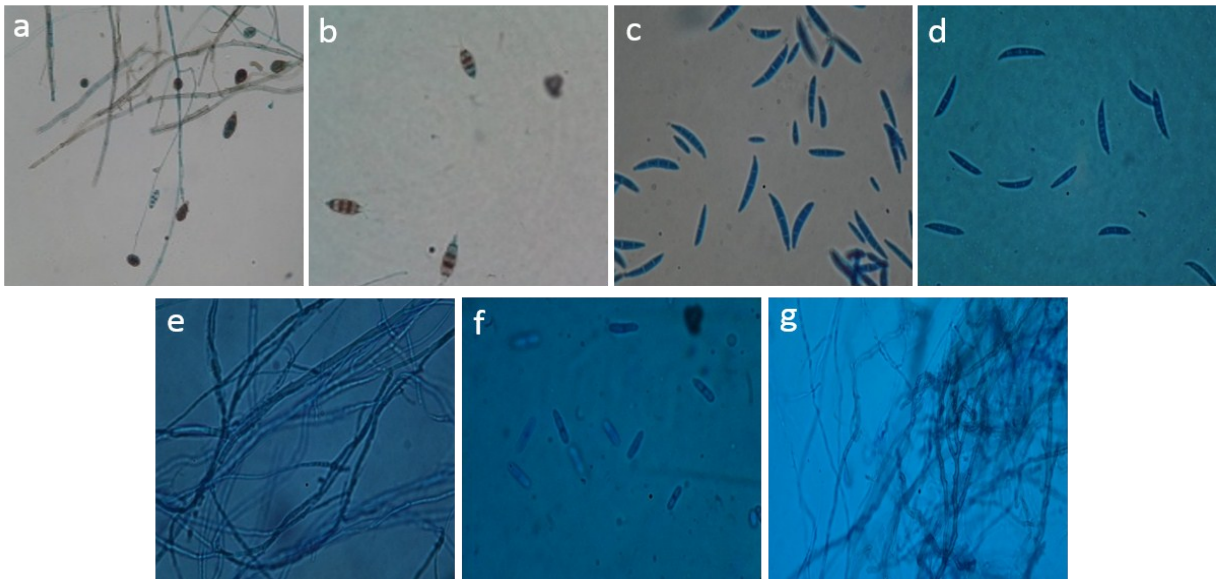

Figure 4: Spore structure observation for positive isolates, (a) VN-1, (b) OB-1, (c) JJ-5, (d) JJ-1, (e) JJ-4, (f) GP-1, (g) CS-3.

for morphological characterization. The morphology of the selected fungi was determined (Figure 3). The spore morphology of the selected fungi were observed and identified as *Pestolopsis sp*., two strains of *Fusarium sp*., *Alternaria sp*., and two fungi without spore formation (Figure 4).

3.6 Conclusion

In this study, ethyl acetate extracts of the endophytic fungi isolated from the medicinal plant leaves of VN-1 and OB-1 showed promising antibacterial activity against the tested five human pathogens. Purified form of 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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