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Evaluation of antimicrobial activities of endophytic fungal metabolites against clinical importance microbes

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Abstract
Antimicrobial activity of crude fungal metabolites of endophytic fungi isolated from leaves of *Avicennia marina.* Four different concentrations (25, 50, 75 and 100µl) of endophytic fungal metabolities act against microorganisms such as *B. subtilis, E. aerogenes, K. pneumoniae, P. aeroginosa, S. typhi, A. niger, A. ochraceous, C. geniculata, F. moniliforme* and *T. viride* was carried out by agar well diffusion method. The maximum zone inhibitions were measured in 100µl of *Aspergillus flavus* and *Penicillium citrinum* fungal metabolities against microbes. But, no zone of inhibition detected in *T. viride* against *Aspergillus flavus* fungal metabolites Findings from the research indicated that the studies could also pave a way for new therapeutic agents who can be used as potential drugs against the selected microorganisms.

Keywords: *Avicennia marina*, endophytic fungi, secondary metabolities

Introduction
Endophytic fungi are omni potent on plants as well as earth. They reside in the host plant tissues are established either symbiotic or pathogenic relationships. These are reported to produce a plethora of biomolecules involved in promoting plant growth or providing plant protection (Strobel *et al*., 2004) [19]. Endophytes are microorganisms that live in the intercellular spaces of healthy host tissues without causing obvious symptoms (Strobel and Daisy, 2003) [17]. Exploitation of novel classes of antimicrobial metabolites is increasingly noticeable over recent years. A considerable research has investigated the diversity, ecological role, secondary metabolites and bioactivity of the endophytic fungi isolated from various medicinal plants (Ni *et al*., 2008) [12].

Natural products from microbial origin have been a consistent source of novel lead molecules and recently several endophytes have been shown to possess the capacity to synthesize bioactive compounds that have found great use for novel drug discovery (Okoye *et al*., 2005) [14]. A single endophytic fungi could produce several novel bioactive compounds, hence they have been receiving increased considerations in recent times (Katoch *et al*., 2014 and Kalyanasundaram *et al*., 2015) [6, 5].

Microorganisms are important sources of bioactive natural products with enormous potential for the discovery of new molecules for drug discovery, industrial use and agricultural applications (Titilope *et al*., 2012 and Mothana *et al*., 2009). In comparison to other natural sources like plants, microorganisms are highly diverse but narrowly explored. The analysis of microbial populations has revealed that only about 1% of bacteria and 5% of fungi have been characterized and the rest remain unexplored for their contribution to the human welfare. In addition, more than 60% of the anticancer and 70% of the antimicrobial drugs currently in clinical use are natural products or natural product derivatives (Igoli *et al*., 2005).

Endophytic fungi can stimulate plant growth, increase resistance towards disease causing pathogens, suppressed the weed, increased the tolerance to abiotic and biotic stresses (Sturz *et al*., 2000) [20]. In addition, they also have potential to produce vast bioactive secondary metabolites with pharmaceutical importance (Tan and Zou, 2001 and Demain, 2014) [22, 3]. Many endophytic fungi provide protection to the host by inducing defense mechanisms in plants against a broad range of pathogens. The endophytes are known to produce an antibiotic substance which inhibits the pathogen growth, or may compete with pathogen for space and nutrition.

Material and Methods

Isolation of endophytic fungi, fermentation and extraction of secondary metabolites (Arnold *et al*., 2000) [1]

The *Aspergillus flavus* and *Penicillium citrinum* endophytic fungi were isolated from *Avicennia marina* healthy leaf and collected from Muthukuda Mangrove environs of Pudukkottai District and prepared potato dextrose broth in 1L Erlenmeyer flasks and were separately inoculated with 3 mm diameter agar blocks containing the two endophytic fungi then incubated at 27 °C for 21 days. At the completion of fermentation, the secondary metabolites were extracted in ethyl acetate and then concentrated under vacuum at 40 °C using a rotary evaporator.

Antimicrobial Assay of fungal metabolites (Onyegbule *et al*. 2014) [15]

The antimicrobial screening of fungal extracts was carried out using the agar well diffusion method against *B. subtilits, E. aerogenes, K. Pneumoniae, P. aeroginosa* and *S. typhi* and fungi *Aspergillus niger, A. ochraceous, Cunnigamala geniculata, Fusarium moniliforme* and *T. viride* were investigated. The endophytic fungi were cultured in PDA seven days for room temperature. To prepared Nutrient Agar (NA) and Potato Dextrose Agar (PDA) were poured into sterile Petri plates and allowed to solidification. The overnight bacterial and fungal cultures were swabbed aseptically on the agar plates and well (6 mm) were made in the agar plates using a sterile metal cork-borer. The different concentrations of seven days old endophytic curde secondary metabolites (25, 50, 75 and 100µl) were put in each well respectively under aseptic condition. The NA plates were then incubated at 37 °C for 24 hours and the PDA plates were incubated at 30°C for 2 days. The inhibition zone diameters were measured and recorded.

Results and Discussion

Discovery of new and potential drugs molecules can be focused on the production of bioactive compounds by plants, microbial and marine organisms. Endophytic microorganisms isolated from plants constitute a source of search for novel secondary metabolites (Firakova *et al*., 2007), since a single endophyte may be able to produce a variety of bioactive metabolites (Ramasamy *et al*., 2010).

Endophytic fungal species are now considered as exciting novel sources for obtaining new bioactive compounds and have been reported from several hosts (Verma *et al*., 2009) [24]. In the present study, two endophytic fungi of *Aspergillus flavus* and *Penicillium citrinum* were isolated from *Avicennia marina* leaves. These endophytic fungal metabolites studied in antimicrobial activity against microbes.

The increasing use of chemical products in order to implant and maintain healthy crops and high productivity has been caused negative effects for the biotic complex of nature, affecting animals, humans and plants (Mochi *et al.,* 2005).

In the current work, four different concentrations (25, 50, 75 and 100µl) of endophytic fungal metabolities were used in antimicrobial activites. The maximum zones of inhibition were observed in 100µl of *Penicillium citrinum* as *B. subtilis* (11.00±3.67mm), *E. aerogenes* (7.00±2.34mm), *K. pneumoniae* (8.00±2.67mm), *P. aeroginosa* (10.00±3.34mm) and *S. typhi* (8.00±2.67mm) recorded respectively. Whereas, 100µl of *Aspergillus flavus* fungal metabolities recorded in 10.00±3.34, 4.34±1.45, 4.67±1.56, 4.67±1.56 and 3.67±1.23 mm zone of inhibition of *B. substilis, E. aerogenes, K. pneumoniae, P. aeroginosa* and *S. typhi* than compared to other concentration respectively (Table 1).

Similarly, Bernardi-Wenzel (2008) and Rhoden *et al*. (2012) observed minor efficiency of endophytic secondary metabolites extracted with methanol regarding antimicrobial were analysed. Bernardi-Wenzel (2008) studied that the antimicrobial activity of fungal secondary metabolites produced by endophytes from *Luehea divaricata* against the human pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. None extracts showed antagonistic activity against *S. aureus*, while some extracts inhibited the *E. coli* growth.

In the present antifungal activity study, the maximum zone of inhibition recorded in 100µl concentration of both endophytic fungi extract. *Aspergillus niger* (2.0±0.67mm), *A. ochraceous* (3.0±1.0mm), *C. geniculata* (4.0±1.34mm), *F. moniliforme* (4.0±1.34mm) zone recorded in *Aspergillus flavus* extract and no zone detected in *T. viride*. While, *Penicillium citrinum* extract was inhibit the zone as 2.00±0.67, 3.40±1.46, 5.34±1.85, 5.0±1.25 and 6.34±2.12 mm zone observed in *A. niger, A. ochraceous, C. geniculata, F. moniliforme* and *T. viride* (Table 2). These results correlated with the findings of other reports where they reported the antimicrobial activity of endophytes (Verma *et al*., 2009) [24].

Sutjaritvorakul *et al*. (2011) studied that the used the paper disk susceptibility test to evaluate the antimicrobial activity of metabolites produced by fungal endophytes against five human pathogenic microorganisms (S. *aureus, B. subtilis, Pseudomonas aeruginosa, E. coli* and *C. albicans*). The fungal metabolites inhibited two or more pathogens and the growth of Gram positive bacteria were more inhibited than those Gram negative, with inhibition halos up to 20.1 mm which was produced by extract of *Pestalotiopsis* sp. against *B. subtilis*.

Most of the fungal extracts were active against *Staphylococcus aureus* and *B. subtilis*, however among the isolated strains *Alternaria* sp., showed highest zone of inhibition against *Bacillus subtilis and Staphylococcus aureus* where the inhibition was in the range 12mm. The crude extract of *Pestalotiopsis* sp. was active against *Staphylococcus aureus, Vibrio cholera* and *Salmonella typhi* with 18 mm of zone of inhibition. At the same time, *Colletotrichum* sp. showed activity against *Staphylococcus aureus, Vibrio cholera* and *Bacillus subtilis* with zone of inhibition about 15 mm (Kuralarasi and Lingakumar, 2018) [10].

Previous studies have shown that several extracts from endophytic fungi exhibited antimicrobial activity (Subbulakshmi *et al*., 2012) [21]. The antimicrobial activities of crude extract of the secondary metabolite isolated from the endophytic fungi showed a board spectrum and effective antibacterial and antifungal activity. The crude extracts of five endophytic fungi exhibited a wide variety of antimicrobial activities against six tested microorganisms. Each of the endophytic fungi produced bioactive compounds that exhibited antimicrobial activity against at least one test microorganisms were used (Nwakanma *et al*., 2016) [13].

It is concluded that the study, the *Aspergillus flavus* and *Penicillium citrinum* endophytic fungi isolated from *Avicennia marina* leaf sample. The endophytic endophytic fungal secondary metabolites were studied the antimicrobial activity. The secondary metabolites are high potential in medicinal properties and they can be used in pharmaceutical industries.

Table 1: Effect of antibacterial activities of endophytic fungi

Name of the bacteria	Zone of inhibition (mm)							
	Aspergillus flavus				*Penicillium citrinum*			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
B.subtilis	6.00±2.00	6.00±2.00	8.00±2.67	10.00±3.34	6.67±1.89	8.00±2.67	10.00±3.34	11.00±3.67
E. aerogenes	3.00±1.00	4.00±1.34	3.34±1.12	04.34±1.45	3.00±1.00	5.67±1.89	05.68±1.89	07.00±2.34
K. pnemoniae	2.34±0.78	3.00±1.00	3.67±1.23	04.67±1.56	4.00±1.34	5.67±1.89	07.00±2.34	08.00±2.67
P.aeroginosa	2.00±0.67	3.00±1.00	3.34±1.12	04.67±1.56	4.02±1.32	5.34±1.78	06.34±2.12	10.00±3.34
S. typhi	3.00±0.67	2.00±0.67	4.00±1.34	03.67±1.23	3.00±2.00	0.06±2.00	07.00±2.34	08.00±2.67

± Standard deviation

Table 2: Effect of antifungal activities of endophytic fungi

Name of the fungi	Zone of inhibition (mm)							
	Aspergillus flavus				*Penicillium citrinum*			
	25µl	**50µl**	**75µl**	**100µl**	**25µl**	**50µl**	**75µl**	**100µl**
Aspergillus niger	1.34±0.45	1.67±0.56	2.00±0.67	2.00±0.67	-	-	2.00±0.67	2.00±0.67
A. ochraceous	2.00±0.67	2.00±0.67	3.66±1.22	3.00±1.00	2.00±0.67	3.00±1.34	3.34±1.12	3.40±1.46
C. geniculata	2.34±1.12	2.00±1.34	3.00±1.00	4.00±1.34	3.67. ±1.23	4.00±1.34	4. 67 ±1.56	5.34±1.85
F. moniliforme	3.67±1.23	3.34±1.12	3.34±1.12	4.00±1.34	3.67±1.23	4.00±1.34	4.00±0.34	5.00±1.25
T. viride	-	-	-	-	3.67±1.23	4.00±1.34	4.67±1.56	6.34±2.12

± Standard deviation

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