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In vitro antioxidant and antibacterial activity of endophytic fungi isolated from *Mussaenda luteola*

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ABSTRACT

Diverse group of endophytic fungi exist within every plant tissues which constitute major and novel natural therapeutic compounds. Hence we investigated five fungal endophytes associated with the medicinal plant *Mussaenda luteola* L. (Rubiaceae) for the presence of phytochemicals, total phenolic, total flavonoid content, antioxidant and antibacterial activity. The results revealed that the ethyl acetate extract of *Alternaria sp.* (ML4) showed highest total phenolic and flavonoid content of 108.65±0.12 mg of GAE/g and 56.45±0.10 mg of RE/g of extract respectively. It also had DPPH scavenging activity of 85.20% at the concentration of 300 µg/ml and high reducing power activity. The remaining fungal extracts exhibited significant level of total phenolic and flavonoid content, antioxidant and antibacterial activity. This study shows that the endophytic fungi isolated from *M. luteola* can be a potential antioxidant and antibacterial resource.

INTRODUCTION

Endophytic fungi reside within most tissues of the living plants which are known to possess different rare and novel secondary metabolites (Ibrahim *et al*., 2015). It proved to be one of the most important and diverse resources available for active natural products with significant biological activities (Peng *et al*., 2013). There are large number of bioactive compounds that have been isolated and identified from endophytic fungi which has various biological activities such as antioxidant, anticancer, antiviral, immunomodulatory, antitubercular, insecticidal and antiparasitic activities. So these bioactive natural products have a wide application in pharmaceuticals and agrochemical industries (Hussain *et al*., 2014; Chomcheon *et al*., 2009). Free radicals are reactive oxygen species often generated by various biological reactions in our body or from exogenous factors. These free radicals cause oxidative stress leading to damage membrane lipids, proteins, enzymes and DNA. It is responsible for many degenerative human diseases like diabetes mellitus, Alzheimer's, Parkinson's disease, ageing, atherosclerosis and inflammatory diseases etc. (Jayanthi *et al*., 2011; Yadav *et al*., 2014). Apart from dietary intake, sufficient amounts of exogenous antioxidants would prevent the pathological conditions induced by free radicals (Bharathidasan *et al*., 2012). There are many antioxidant compounds, for example pestacin and isopestacin, have been obtained from the culture of *Pestalotiopsis microspora*, an endophytic fungi isolated from the medicinal plant *Terminalia morobensis* (Strobel and Daisy, 2003). These endophytic microorganisms can also be used as antimicrobial agents since it produces wide structural classes of secondary metabolites such as alkaloids, peptides, steroids, terpenoids, phenols, quinones and flavonoids. The development of new antibiotics is important to overcome the increasing threat of drug resistant human pathogenic strains (Garcia *et al*., 2012; Sahu *et al*., 2014). The genus *Mussaenda* belongs to Rubiaceae family which is native to West Africa through the Indian sub-continent, South East Asia and Southern China.

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Some important species were widely distributed in Central Nepal, India and Srilanka. It has been reported to possess many medicinal properties. Their medicinal activities include cytotoxicity, anti-inflammatory, antiviral, antioxidant and antibacterial properties (Vidyalakshmi *et al*., 2008). Hence we investigated the total phenolic content, total flavonoid content, antioxidant capacities and antibacterial activity of endophytic fungi isolated from the medicinal plant *M. luteola.*

MATERIALS AND METHODS

Sample collection

Fresh leaf, stem and root samples of *M. luteola* were collected from Vellore district, Tamilnadu in the month of June 2016. The collected samples were placed in sterile plastic bags and brought to the laboratory on the same day.

Isolation of endophytic fungi

The samples were surface sterilized according to the method described by Schulz *et al*. 1993. The small segments of surface sterilized samples were placed on Potato Dextrose Agar (PDA) medium supplemented with 150 mg/L streptomycin to prevent bacterial growth. Plates were incubated at 26º-30ºC for a week (Dhankhar *et al*., 2012). The emerging endophytic fungi were isolated, purified and maintained by continuous subculturing (Jayanthi *et al*., 2011).

Identification of endophytic fungi:

Isolated fungi were identified on the basis of their colony or hyphal morphology, pigmentation, aerial mycelium, surface texture and characteristics of the spores using various standard manuals. Also microscopic studies were done by lactophenol cotton blue staining (Sadananda *et al*., 2014).

Extraction of crude secondary metabolic compounds

The fungal endophytes were cultivated on 500 ml Potato Dextrose Broth (Himedia) and were incubated as static culture at 26 ± 2°C for 1 Week. After the incubation period, mycelium was separated from the fermented liquid broth by filtration and the filtrate was extracted by solvent extraction using ethyl acetate. All the solvent was evaporated and the resultant compound was dried and the crude extract was obtained (Madki *et al*., 2010; Sadananda *et al*., 2011).

Preliminary phytochemical screening of fungal extract

The crude ethyl acetate extract was used for phytochemical screening to analyse the presence of various secondary metabolites such as alkaloids, phenols, flavonoids, steroids, terpenoids, saponins, tannins (Bhardwaj *et al*., 2015).

Determination of total phenolic content

Total phenol content of ethyl acetate extract obtained from five different endophytic fungi was estimated using Folin-Ciocalteau reagent based assay using gallic acid as standard. Each extract (1 mg/mL) of 100 µl was added with 500 µL of 1N Folin - Ciocalteau reagent. Then 1.5 mL of 20% of $NaCO_3$ was added to the mixture. Final volume was made 5 mL by adding distilled water. The mixture was incubated for 30 min at room temperature and the absorbance of the developed colour was recorded at 765 nm using UV-vis spectrophotometer. The same procedure was done with 1 mL aliquot of 5, 10 to 50 µg/mL gallic acid were used as standard for calibration curve (Jayanthi *et al*., 2011).

Determination of total flavonoid content:

Total flavonoid content of ethyl acetate extract obtained from five different endophytic fungi was estimated using Aluminium chloride colorimetric method (Saravanan and Parimelazhagan, 2014). The extract (1 mg/mL) of 100µl was mixed with 2 mL of distilled water and 0.15 mL of 5% $NaNO_2$ solution. Then after 6 min, 0.15 mL of 10% $AlCl_3$ of was added and the mixture was allowed to stand for another 6 min, then 2 mL of 4% NaOH solution was added.

The final volume was made up to 5 mL by adding distilled water immediately and the mixture was allowed to stand for 15 min at room temperature. Absorbance was recorded at 510 nm using UV-vis spectrophotometer. Rutin was used as a standard compound for quantification of flavonoid and the results were expressed as rutin equivalents (RE).

Antioxidant assays

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Assay:

For DPPH assay (Asker *et al*., 2013), aliquots of extract at different concentration were added to 2 ml of (DPPH) methanolic solution (0.1mM). The reaction mixture was shaken well and incubated at room temperature for 10 mins. The absorbance of the resulting solution was read at 517nm against a blank. The radical scavenging activity was calculated using the following equation:

Scavenging ability (%) = [(ΔA517 of control – ΔA517 of sample) / ΔA517 of control] × 100

Reducing power assay

The reducing power assay was performed for each sample extract (1 mg/mL) at different concentrations and first mixed with 2.5 mL 0.2 M phosphate buffer, pH 6.6 and 2.5mL 1% potassium ferricyanide. After incubation for 20 min at 50°C, 2.5 mL 10% trichloro acetic acid was added to the mixture and then centrifugation was done at 3000 rpm for 10 min. Subsequently, 2.5 mL of the supernatant was added to 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride, and the absorbance of the resulting solution was read at 700 nm against a blank. Ascorbic acid was used as positive controls (Zeng *et al*., 2011).

Antibacterial assay

The antibacterial assay was carried out by agar well diffusion technique. The Mueller Hinton Agar plates were swabbed with 0.1 ml culture of different bacterial clinical isolates obtained from microbiology department VIT University, Vellore

(*Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa*). The respective wells were poured with each sample extract of 30µL/ well (1mg/ml concentration). In other wells, DMSO was used as a negative control and standard antibiotic chloramphenicol was used as positive control. The experiment was carried out in triplicate. The plates were incubated at 37ºC for 24 hours and results were recorded as zone of inhibition in mm. (Desale *et al*., 2013).

Statistical analysis

All results are reported as means ± standard deviation. The statistical analysis was done using one way ANOVA. The 'P value' found to be <0.05, was considered as significant.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

The healthy leaf, stem and root of the plant *M. luteola* were subjected to isolate the endophytic fungi and the identification was based on colony morphology and microscopic studies. The colony morphology and frequency of occurrence of each endophytic fungus was noticed. The results are presented in Table 1.

Table 1: Frequency of occurrence and colony morphology of endophytic fungi in different parts of the plant.

Endophytic fungi	Colony morphology	Frequency of occurrence		
		Leaves	Stem	Root
Aspergillus sp. (ML1)	Carbon black mycelia, brownish on reverse	3	-	-
Xylaria sp. (ML2)	White cottony mycelium, golden blonde on reverse	2	-	-
Penicillim sp. (ML3)	White to pale brown mycelium, reddish brown on reverse	1	1	-
Alternaria sp. (ML4)	Pale grayish hyphae, black on reverse	2	1	-
Unknown sp. (ML5)	White mycelium, pale yellow on reverse	2	2	-

Phytochemical screening

The ethyl acetate extract of all endophytic fungus were subjected to phytochemical analysis to check for the presence of phenols, alkaloids, flavonoids, steroids, saponins, terpenoids, tannins and the results are presented in **Table 2.** These chemical constituents are responsible for different medicinal properties of extracts. The yield of ethyl acetate extract of all fungus was found to be 1.0 - 1.8g/ 500 ml

Table 2: Phytochemicals present in ethyl acetate extract of endophytic fungi

Phytochemical	*Aspergillus sp. (ML1)*	*Xylaria sp. (ML2)*	*Penicillium sp. (ML3)*	*Alternaria sp. (ML4)*	*Unknown sp. (ML5)*
Phenol	+	+	-	+	-
Flavaonoid	+	-	+	+	-
Alkaloid	-	-	-	-	-
Terpenoid	-	-	-	-	-
Steroid	-	-	-	-	-
Saponin	+	+	-	+	+
Tannin	+	-	-	+	-

Note: + means Presence; - means not present.

Determination of Total phenolic and flavonoid content

There was a wide range of total phenolic and flavonoid concentrations were found in different endophytic fungal extracts. The highest concentration of phenol (108.65±0.12 mg of GAE/g of extract) and flavonoid (56.45±0.10) was observed in ethyl acetate extract of ML4. The results were presented in **Table 3.**

Table 3: Total phenolic and flavonoid content of ethyl acetate extract from endophytic fungus

Endophytic fungi	Total phenolic content (mg of GAE/g of extract)	Total flavonoid content (mg of RE/g of extract)
Alternaria Sp (MLL1)	32.77±0.04^{b}	18.62±0.02^{b}
Xylaria Sp (MLL2)	28.15±0.02^{c}	15.85±0.08^{c}
Penicillim Sp (MLL4)	24.38±0.03^{d}	15.62±0.05^{d}
Penicillim Sp (MLL6)	108.65±0.12^{a}	56.45±0.10^{a}
Unknown (MLS1)	9.59±0.04^{e}	5.01±0.03^{e}

Note: Values are expressed as mean ± standard deviation (n = 3). Mean values followed by different superscript letters indicate significant statistical difference (P < 0.05).

Antioxidant assay

In present study, ethyl acetate extract of five different endophytic fungi were investigated for antioxidant potential by using two different assays. All extract of five different endophytic fungi showed antioxidant activity up to varying extent.

DPPH radical scavenging activity

DPPH, a stable free radical was used to study the radical scavenging effects of the fungal extract. As antioxidant donate proton to this radical, this decreases the absorption. The reaction was visible as a colour change from purple to yellow. The EA extract of *Alternaria sp.* (ML4) showed a high scavenging activity of 85.20% whereas the *Unknown sp*. (ML5) showed the least antioxidant activity of 33.95%. Ascorbic acid was taken as standard showing 96.91% antioxidant activity. Percentage of DPPH radical scavenging activity of endophytic fungi and ascorbic acid is shown in **Figure 2**

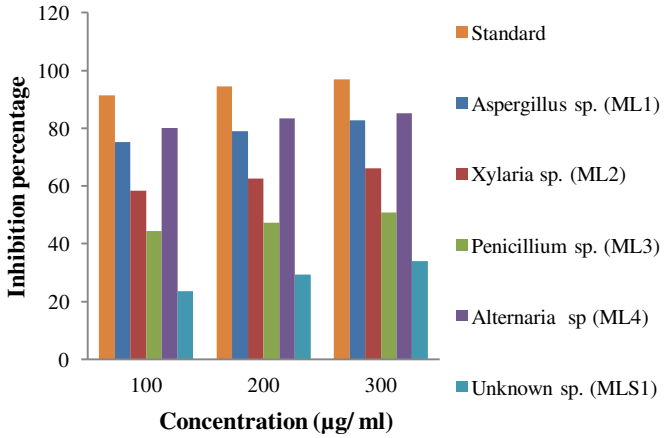

Fig. 2: DPPH free radical scavenging activity of ethyl acetate of all endophytic fungus.

Reducing power assay

In reducing power assay reducing ability was measured by reaction change of Fe3+ to Fe2+. The EA extract of ML4 had

high absorbance values more than standard that indicated higher reductive potential and electron donor ability for stabilizing free radicals. Reducing power of all endophytic fungal extract and ascorbic acid with respect to their absorbance values are shown in **Figure 3.**

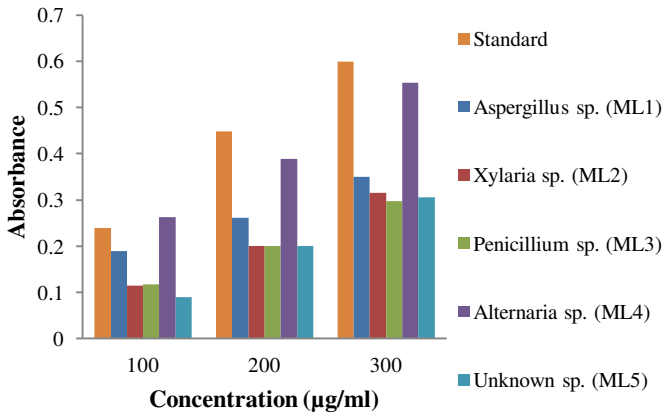

Fig. 3: Reducing power activity of ethyl acetate extract from all endophytic fungus.

Antibacterial activity

The crude extract obtained from five endophytic fungi was subjected for its anti-microbial activity against two gram positive bacteria *(B. subtilis, S. aureus*) and two gram negative bacteria (*E. coli, P. aeruginosa*). The results revealed that the *Alternaria sp*. (ML4) showed more antibacterial activity against all organisms and *Aspergillus sp.* (ML1), *Penicillium sp.* (ML3) shows significant activity against *E.coli* and *P.aeroginosa.* The standard antibiotic chloramphenicol was used as a positive control. The diameter of zone of inhibition was measured in millimeters and presented in **Table 4**.

Table 4: Antimicrobial activity of endophytic fungus on Test organisms (determined by diameter of inhibition zones).

Endophytic fungus	Zone of inhibition (mm)			
	E. coli	***P. aeruginosa***	***B. subtilis***	***S. aureus***
Aspergillus sp. (ML1)	10	9.2	-	9.0
Xylaria sp. (ML2)	-	-	5.6	-
Penicillium sp. (ML3)	8.9	8.6	-	-
Alternaria sp. (ML4)	11.5	10.0	9.3	10.7
Unknown sp. (ML5)	-	-	-	-
Chloramphenicol	14	15	14	13

DISCUSSION

Medicinal plants provide a special environment for endophytic organisms. Many previous studies reported endophytic fungi isolated from medicinal plant have novel and bioactive natural products. These fungi have been isolated from the leaf, stem or inner bark tissues of the plant (Gurupavithra and Jayachitra, 2013). Medicinal plants usually harbour endophytes with similar secondary metabolites and pharmacological activities as host plant (Yadav *et al*., 2014). The genus *Mussaenda* is an important source of pharmacologically active natural products, particularly iridoids, triterpenes and flavonoids (Vidyalakshmi *et al*., 2008). In this study, endophytic fungi were isolated from *M. luteola* was investigated for the presence of various phytochemicals, total phenolic content, antioxidant and antibacterial activity.

Ethyl acetate extraction is most efficient method of isolating secondary metabolites from fungal species (Yadav *et al*., 2014), so all assays were carried out in ethyl acetate extract. The phytochemical analysis confirms the presence of phenols, flavonoids, alkaloids, terpenoids and saponins to varying concentration. Phenolic and flavonoid compounds seem to have an important role in reducing lipid peroxidation and hence act as primary and secondary antioxidants (Pawle *et al*., 2014).

Among different antioxidant assays, DPPH free radical scavenging assay is the most widely used assay and it is considered to be the most accurate screening method used to evaluate the antioxidant activity. Also DPPH is not affected by metals or enzyme inhibition (Yadav *et al*., 2014).

In reducing power assay, the compound exhibit antioxidant activity by breaking the free radical chain through donation of a hydrogen atom (Ravindran *et al*., 2012).

The increase in radical scavenging ability may have been due to increase in the concentration of total phenol compounds (Govindappa *et al*., 2013). In this study, all fungal extract had a wide range of scavenging ability. Among that, ethyl acetate extract of *Alternaria sp.* (ML4) showed 85.20 % inhibition activity against DPPH which is nearly equivalent to ascorbic acid with the inhibition activity of 96.91%, also it shows high reducing power activity compared to standard ascorbic acid with the highest total phenolic and flavonoid concentration of 108.65±0.12 mg of GAE/g and 56.45±0.10 mg of RE/g of extract respectively. Remaining fungal extracts showed significant level of antioxidant activity. A good correlation was found between the level of total phenol, flavonoid contents and antioxidant ability for all the extracts. The fungal extract with high scavenging and reducing power activity had a significant amount of total phenol concentration. In antibacterial assay, *Alternaria sp.* (ML4) showed high activity against both gram positive and gram negative bacteria. *Aspergillus sp.* (ML1) and *Penicillium sp.* (ML3) showed significant activity against *E. coli, P. aeroginosa*. The different sensitivity between Gram positive and Gram negative bacteria could be due to cell wall morphological differences between these microorganisms. (Sadrati *et al*., 2013). Our study demonstrated that the endophytic fungus isolated from *M. luteola* plant had significant range of biological activities and presence of phytochemicals which on further purification the active compounds can be obtained.

CONCLUSION

The present study concludes that the endophytic fungal extract exhibited antioxidant and antimicrobial activity due to the presence of bioactive natural compounds. Furthermore, active crude extracts are being subjected to purification process for identification of active compounds which may provide a better

source for developing new pharmacological agents. These active natural compounds have a potential application as antioxidants and antibacterial agents in many pharmaceutical products.

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