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Isolation and Identification of Endophytic Fungi from *Piper hymenophyllum* Miq. and their Antioxidant Activity and Anti-bacterial Activity Against MDR Bacteria

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ABSTRACT

Bacteria or fungi that live inside plant tissues or organs without posing a threat are known as endophytic microorganisms. Apart from generating biotechnologically significant compounds, they have potential to protect plants from diseases and insect attacks. In India, *Piper hymenophyllum* is known as “Kattukurumulaku”. Research objective was to examine biotechnological potential of fungal endophyte’s crude extracts, isolated from *P. hymenophyllum*, *Neofusicoccum parvum* (PQ001944.1) and *Pestalotiopsis sp.* (PP849886.1). The study assesses the secondary metabolites, *in vitro* antioxidant, and MDR pathogen activities of ethyl acetate from *P. hymenophyllum*. The results demonstrated the high levels of total phenols (760.86mg GAE/g), tannin (760.31mg GAE/g), along with flavonoids (48.84mg RE/g) in the *Pestalotiopsis sp.* ethyl acetate extract. *Pestalotiopsis sp.* ethyl acetate extract exhibited good resistance to phosphomolybdenum (106.02 mg AAE/g extract), superoxide (38.78%), DPPH (26.48 µg/L), and ABTS (74097.2µM TE/g extracts). Furthermore, *Pestalotiopsis sp* ethyl acetate extract demonstrated strong antibacterial activity at dosage of 10mg/ml against *Staphylococcus aureus* (11mm), *Enterococcus faecalis* (12mm),*Enterobacter cloacae* (12mm) and *Pseudomonas aeruginosa* (12mm). According to these findings, *P. hymenophyllum* leaves of endophytic fungi *Pestalotiopsis sp.*,particularly ethyl acetate extract, are a significant source of antioxidants and have a high potential for containing components of MDR pathogen activity.

Keywords: *P. hymenophyllum, Neofusicoccum parvum, Pestalotiopsis sp.*and MDR pathogen

INTRODUCTION

Since we haven't sampled or described the diversity of fungal species, we don't know much about their roles in ecosystems. With increased knowledge of endophytes, the study of their ecology, life history, and phylogeny has exploded in the last three decades. The term "endophyte" has become nearly synonymous with mutualism, despite some disagreement regarding its etymology. Even though endophytes in woody plants may significantly affect plant populations and communities, very little study have been done on this topic. They are essential for plants to repel pests, nematodes, and insects. They also assist plants resist environmental illnesses[1]. These enhance the nitrogen fixation process and promote plant development. In an effort to create or uncover new chemicals, endophyte research has lately shifted from documenting species to evaluating dynamics of endophyte-plant interactions, with focus on medicinal plant-associated fungi[2].

Endophytic microorganisms include bacteria and fungi that, at least once during their life cycle, invade intracellular or intercellular areas of plant tissues[3]. Since they don't seem to harm host plants[4] and live asymptomatically[5], their relationship with them is frequently stable.

Researchers extracted a range of compounds from plant endophytes[3]. Accordingly, there is rising number of investigations focused on extraction and/or application of endophytes from medicinal plants[6]. A range of biotechnologically intriguing compounds, including secondary metabolites with pharmacological uses[7] and antimicrobials that prevent growth of pathogens[8], can be produced by the interaction of endophytes and plants.

One of the many flowering plant families is the Piperaceae, which includes Piper. Thirteen genera under the family Piperaceae are known to include around 3,600 species. Both Piper and Peperomia are major genera in the Piperaceae family, with over 2,000 species between them. Black and long peppers contain the alkaloidal component piperine (1-piperoyl piperidine), which is used all over the world[9]. The bitter, peppery, and harsh-tasting root and fruits of the Piper nigrum plant are used medicinally. Suffering from digestive issues, aphrodisiac, lumbago, palsy, gout, stomachic, emmenagogue, abortifacient, or liver tonics, you might want to give one a try. The tongue becomes numb and saliva production increases when pepper is overdone. According to Ganesh *et al.*[10], the fruits and roots can be administered topically to alleviate muscle soreness, treat respiratory tract disorders such as asthma, bronchitis, and cough, and function as a counter-irritant. Toothaches, headaches, stomachaches, coughs, rheumatism, colds, and bronchitis are just some of the many ailments that Piper peepuloides is believed to alleviate. Therefore, secondary metabolites, antioxidant activity and antimicrobial activityare for the current investigation.

MATERIALS AND METHODS

Plant sample collection

The climbing Piper hymenophyllum plants were randomly sampled in Western Ghats region (Nilgiris, Tamil Nadu, India) for their mature, healthy, symptom-free leaves. A specimen of *P. hymenophyllum* was sampled from the foothills of Coonoor in the Nilgiris region of Tamil Nadu, India (BSI/SRC/5/23/2023/Tech-837). The plant material an icebox to transport the sterile polythene bags containing the leaf samples to the lab. Endophytic fungi were isolated from samples kept at 4ºC within 24hrs of collection

Endophytic strains

Garcia *et al.*[11] molecularly identified endophytic strains that were chosen to obtain crude extracts from leaves of a *Piper hymenophyllum Miq.* Sequences of *Neofusicoccum parvum* isolates (PQ001944.1) and *Pestalotiopsis sp.* isolates (PP849886.1) were deposited in NCBI.

Obtainment of crude extracts

Crude extracts were produced using modified version of process outlined[12]. In accordance with Smith and Onions [13] and modification by Pamphile *et al.*[14], endophytic fungus was incubated in culture media using Potato Dextrose (PD) for 10 days at 25ºC. Centrifuged resulting fermented medium 3,600rpm for 10min. Equal volume of crude ethyl acetate was added to a separatory funnel containing the supernatant. The phases were separated by polarity differences after the funnel was vigorously shaken. This procedure was

carried out twice. Ethyl acetate solution with fungal metabolites was 98% concentrated using Büchi R-3000 Rotavapor at 40°C.

Quantification Assays

Quantification of total phenolic contents

Parimelazhagan method[15] was employed for estimating total phenolic content of plant extracts. Phenolics were present in incubated test tubes, indicated by formation of blue color. Absorbance was assessed at 725nm shortly after incubation against reagent blank and reported as gallic acid equivalents per gram of extract (GAE/g Extract).

Quantification of total tannin contents

There are tannins and non-tannin phenolics in the total phenolics. To find amount of phenolics that aren't tannins, we subtracted the tannin content from the total phenolics[15]. We also measured total and free phenolics three times and reported the findings as tannic acid equivalents (TAE). Following these 2 outcomes, following was used to determine tannin concentration of plant samples:

Tannins=Total phenolics-Non tannin phenolics

Quantification of total flavonoid contents

Method described by Parimelazhagan[15] was used to quantify flavonoid contents of extracts. Flavonoids' presence was detected at 510nm via spectrophotometer, which resulted in a pink color. The experiment was conducted in triplicates with rutin as the standard, and outcomes were represented as rutin equivalents (RE).

***In vitro* antioxidant assays**

DPPH% scavenging activity

In accordance with Parimelazhagan technique[(15)], extract's antioxidant activity was assessed by estimating its capacity for donating hydrogen or scavenging radicals by employing stable radical DPPH. Each sample, standard (Rutin and BHT), and control were tested for absorbance at 517nm relative to methanol blank. IC50-value, which indicates sample concentration needed to block 50% DPPH concentration, was employed for expressing samples' radical scavenging activity.

ABTS scavenging activity

ABTS radical cation decolorization test was performed in accordance with Parimelazhagan method[(15)], was utilized to establish overall antioxidant activity of samples. Comparison was made between samples and standards (BHT and Rutin) to determine the ethanol absorbance at 734nm after the incubation process was completed. According to the findings, the amount of extracts that were equivalent to µM Trolox was reported.

Phosphomolybdenum assay

The generation of green phosphomolybdenum complexes was subjected to the Parimelazhagan procedure[(15)], which was utilized to evaluate antioxidant activity of products. Milligrams (mg) of ascorbic acid equivalents per gram (g) of extract were used to report the findings, with ascorbic acid acting as standard of reference.

Superoxide radical scavenging activity

Experiment was designed to determine whether or not various extracts had the capacity for scavenging superoxide radicals that were produced by riboflavin-light-NBT system, hence preventing formation of formazan Parimelazhagan[(15)]. The formula for determining percentage inhibition of superoxide anion generation is as follows: % Inhibition=[(A0-A1)/A0]x100. This formula is based on assumption that A1 indicates absorbance of sample extract or standard while A0 indicates absorbance of control.

Assessment of antimicrobial activity

Disk diffusion technique (cup plate) was used to quantitatively analyze antimicrobial activity in triplicate. *P. aeruginosa, E. faecalis*,*S. aureus* and *E. cloacae* were human MDR pathogenic bacteria that were employed in this test.

Bacteria were grown in liquid Luria Bertani (LB) mediumfor 24hrs,adjusted to 10^6cells/ml, and then distributed (100µl) on Petri dishes having solid LB media to test antibacterial activity. 4 disks of sterile Whatman no.-4 (6mm) filter paper infected with 30µl of extracts were included in each dish, evenly spaced apart.Absolute ethanol and autoclaved distilled water were used to inoculate paper disks as negative controls. 50µg ml^{-1} of tigecycline was employed as a positive control. For 24hrs, plates were incubated at 37°C. State that the creation of inhibitory halos was used to measure the antibacterial activity[16-17].

RESULTS AND DISCUSSION

Quantification assays

Quantification of total phenolics content

Total phenolics content in *P. hymenophyllum*had been assessed by employing Folin-Ciocalteu reagent. Endophytic fungi *Pestalotiopsis sp.*and *Neofusicoccum parvum* leaf ethyl acetate extracts were included in the analysis. With an R^2 value of 0.9919, the gallic acid standard graph was drowned. Gallic acid equivalents per gram of extract (mg GAE/g extract) are units used to measure total phenolic content in the conventional graph equitation method. Findings are demonstrated in Table 1 after statistical analysis. Phenolic content of ethyl acetate extracts of *Pestalotiopsis sp.* and *Neofusicoccum parvum* was observed to be 760.86mg GAE/g extract, with lowest concentration recorded at 586.2mg GAE/g extract, respectively. Aromatic phenols protect plants from stress and boost antioxidant capacity[18-19]. Phenolic chemicals disturb cellular chain oxidation reactions as persistent phenoxyl radicals[20]. These specifically scavenge free radicals[21]. These chemicals boost antioxidant capability, protecting against serious diseases[22], stronger secondary metabolites in plant samples indicate stronger antioxidant potential.

Quantification of total tannins content

Table 1 displays the total tannins content of *P. hymenophyllum*-associated endophytic fungus *Neofusicoccum parvum* and *Pestalotiopsis sp.*The *Pestalotiopsis sp.*ethyl acetate extract showed stronger radical scavenging activity (760.31 mgRE/g extract) than the other extracts. According to Zheleva-Dimitrova*et al.*[(23)], tannins are bitter-tasting organic molecules with potent astringent qualities, including antibacterial, anti-inflammatory, and antioxidant activity that cause protein precipitation. Tannin is a naturally occurring antioxidant that helps the body defend itself when it is present in the plant system.

Quantification of total flavonoid content

In Table 1, we can see the amounts of total flavonoids found in *P. hymenophyllum* leaf extracts, fungus isolates *Neofusicoccum parvum* and *Pestalotiopsis sp.* There was a respectable amount of flavonoid content in the ethyl acetate extracts of *Pestalotiopsis sp.,*and *Neofusicoccum parvum* with the former having the greatest concentration at 40.84mg RE/g extract and the latter at 40mg RE/g extract. Of all the natural phenolics, flavonoids are among the most significant and diverse. Both ethyl acetate extracts have a considerably high flavonoid concentration ($p<0.05$).Flavonoids are the most widespread polyphenolic chemicals. These antioxidant derivatives scavenge free radicals and chelate trace elements[24-26]. Flavonoids are essential for cell cycle regulation[26]. It is crucial for treating cardiovascular illnesses due to its antioxidant and anti-inflammatory characteristics[27].

In vitro antioxidant assays

DPPH scavenging activity

IC_{50} value, which estimates radical scavenging activity against DPPH 2 (2-diphenyl-1-picrylhydrazyl), after 30min of reaction, was determined by finding concentration that removed 50% of the free radical. In comparison to rutin and BHT, the antioxidant activity of *P. hymenophyllum* leaves, *Neofusicoccum parvum*and *Pestalotiopsis sp.*fungus isolates. The ethyl acetate extract of *Pestalotiopsis sp.* had the greatest radical scavenging activity at 26.48µg/mL, while ethyl acetate extract of *Neofusicoccum parvum* had a remarkably low level at 31.26µg/mL. As a positive control in this investigation, rutin exhibited IC_{50} value of 22.24µg/mL, while BHT exhibited IC_{50} value of 17.57µg/mL.Due to its inability to disintegrate in methanol, ethanol, or water, DPPH, organic chemical compound with a constant free radical molecule has

Table 1: Phenolic, Tannin and flavonoids content of Endophytic Fungus

Extracts	Phenolic GAE/g extract	Tannin GAE/g extract	Flavonoids RE/100 g
Pestalotiopsis sp.	760.86 ± 4.16	760.31 ± 3.97	48.84 ± 0.89
Neofusicoccum parvum	586.2 ± 2	585.04 ± 1.6	40 ± 1.02

GAE: Gallic Acid Equivalents Values are mean of triplicate determination (n=3) ± standard deviation

Table 2: DPPH, ABTS scavenging activity, Phosphomolybdenum assayand Superoxide radical of Endophytic Fungus

Samples	Extracts	DPPH IC_{50} (*μ*g/mL) (*μ*M TE/g	ABTS Scavenging Activity extract)	Phosphomolybdenum assay mg AAE/g (extract)	Superoxide radical scavenging activity (%)
P. hymenophyllum	*Pestalotiopsis* sp.	26.48	74097.2 ± 661.54d	106.02 ± 1.1c	38.78 ± 1.32c
	Neofusicoccum parvum	31.26	76458.33 ± 551.19c	53.09 ± 1.55d	33.85 ± 1.72d
Standard	Rutin	22.24	94166.7 ± 416.6b	176.25 ± 2.74b	54.7 ± 0.25a
	BHT	17.57	95347.2 ± 636.4a	270.07 ± 3.64a	53.4 ± 0.1b

Values are mean of triplicate determination (n = 3) ± standard deviation, TE- Trolox Equivalents, AAE - Ascorbic Acid Equivalent, statistically significant at p <0.05 whereas a>b>c>din each column

been widely studied for antioxidant potential. DPPH free radicals can quickly produce stable diamagnetic molecules by receiving electrons or hydrogen from antioxidant molecules[28]. According to Wang *et al*. [(29)], the plant extract reduces stable DPPH radicals, turning purple to yellow.

ABTS scavenging activity

ABTS scavenging activity of *P. hymenophyllum* leaves, *Neofusicoccum parvum*and and *Pestalotiopsis sp.*fungus isolatesand standards have been indicated in Table 2. *Neofusicoccum parvum*ethyl acetate extract demonstrated higher radical scavenging activity (76458.33μM TE/g extract) in comparison to other extracts whereas standards rutin and BHTdemonstrated 94166.7 and 95347.2μM TE/g extract respectively. Plants store antioxidant-rich chemicals. This assay uses potassium persulfate to test the sample's hydrogen-donating ability by watching for discolouration at a relevant wavelength[30-31]. Lowering the sample amount halves the color intensity, indicating increased antioxidant content[32]. In lipophilic and hydrophilic environments, soluble ABTS radical scavenging activity shows hydrogen-donating ability[33]. According to Oliveira-Neto *et al*. [(34)], high-molecular-weight phenolic compounds quench free radicals better.

Phosphomolybdenum assay

Table 2 displays the findings of phosphomolybdenum assay, which was employed for measuring total antioxidant capacity of extracts from *P. hymenophyllum* leaves, *Neofusicoccum parvum*, and *Pestalotiopsis sp*. fungus. Nonetheless, the data are extrapolated from a normal linear curve for ascorbic acid (y=0.0159x +0.0695; R^2=0.9891). With a reducing ability of 106.02±1.1mg AAE/g extract, ethyl acetate extract of *Pestalotiopsis sp*. outperformed other extracts, while the ethyl acetate

Table 3: Anti-bacterial activity of Endophytic Fungus

MDR pathogen	Concentration (10mg/ml)	P. *hymenophyllum*		Positive control (Tigecycline)
		Pestalotiopsis sp.	Neofusicoccum parvum	
Negative Control (Ethyl acetate)	30 µl	-	-	-
Staphylococcus aureus	30 µl	11 ± 0.03	8 ± 0.06	12 ± 0.07
Enterococcus faecalis	30 µl	12 ± 0.03	10 ± 0.02	16 ± 0.04
Enterobacter cloacae	30 µl	12 ± 0.06	10 ± 0.01	15 ± 0.04
Pseudomonas aeruginosa	30 µl	12 ± 0.04	13 ± 0.04	15 ± 0.02

Values are mean of triplicate determination (n = 3) ± standard deviation

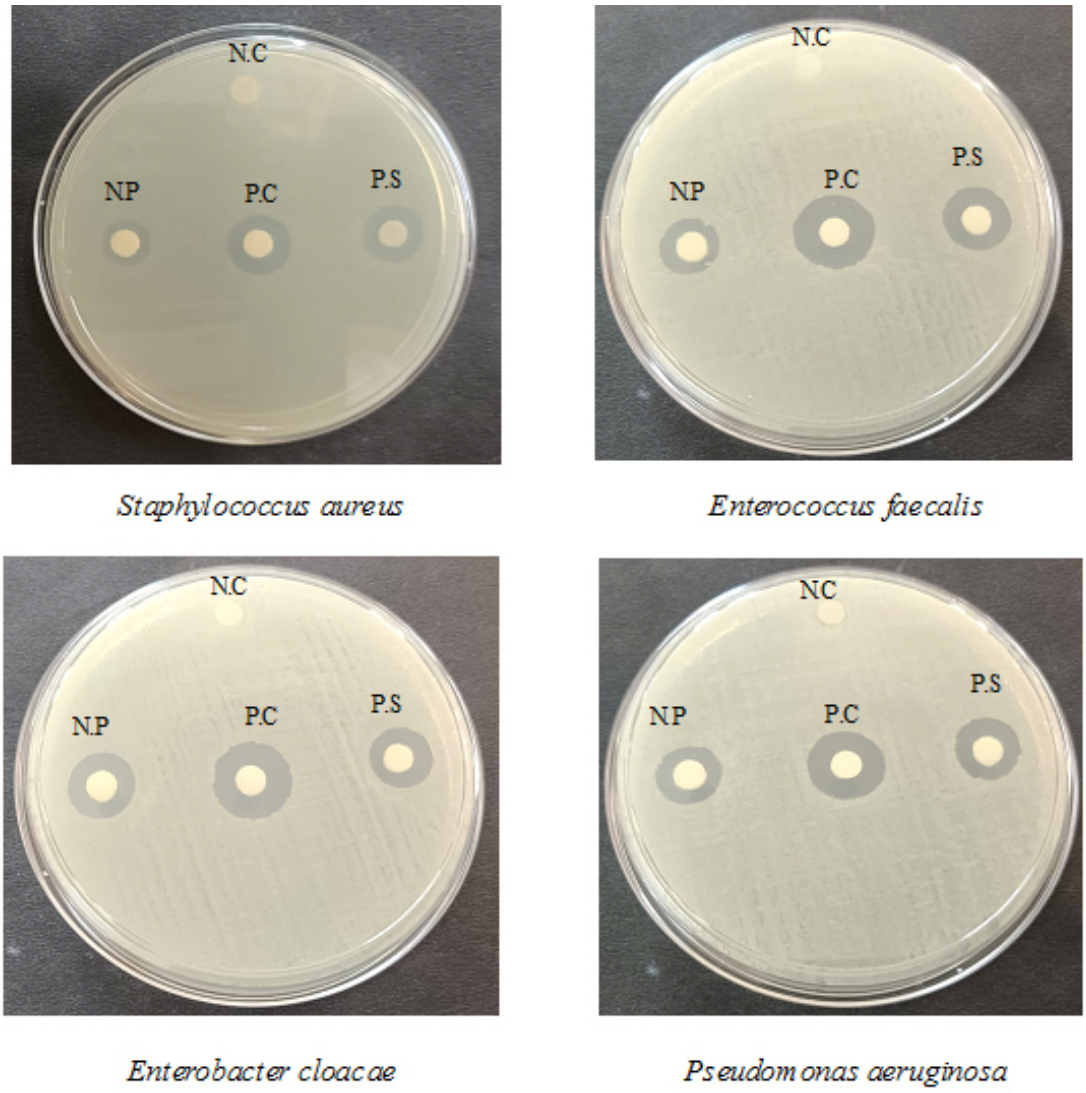

Plate 1. Anti-bacterial activity of Endophytic Fungus

extracts of *Neofusicoccum parvum* leaves also displayed a considerable amount of reduction ability, at 53.09±1.55mg AAE/g extract. *P.hexapetalum* leaf extracts' radical reducing ability along with commercially available antioxidants has following order: *Neofusicoccum parvum*<*Pestalotiopsis sp.*< rutin <BHT.This basic test is inadequate for antioxidant assays. Antioxidant activity is measured in mg/g ascorbic acid equivalents. Antioxidants reduce Mo (VI) to Mo (V) and develop green phosphate/Mo (V) complex with peak absorbance at 695nm Kumaran and Karunakaran[35].

Superoxide radical scavenging activity

Capacity of superoxide radicals produced by dissolved oxygen to diminish NBT is a good indicator of their concentration. The ability of plant extract, reference substance rutin, and BHT for quenching superoxide radicals in reaction mixture is demonstrated by their reduction in absorbance at 560nm. According to Table 2, ethyl acetate extract of *Pestalotiopsis sp.* had considerable inhibitory effect (38.78%), whereas ethyl acetate extract of *Neofusicoccum parvum* had lowest efficiency estimate (33.85%). In comparison, rutin and BHT

were determined to have scavenging activities of 54.7 and 53.4%, respectively.

Superoxide anions occur when mitochondrial electron transport system reduces molecular oxygen. Lipid peroxidation generates superoxides, which create ROS like hydroxyl radicals and damage lipids, proteins, and DNA. Superoxide anions are pioneers of active free radicals that can combine with biological molecules to harm tissue and induce other significant illnesses[36]. Phenolic hydroxyl groups in aromatic amino acids donate electrons to scavenge free radicals[37].

Anti-bacterial Activity

Antibacterial activity of *P. hymenophyllum* leaves, *Pestalotiopsis sp*. and *Neofusicoccum parvum*, fungus ethyl acetate extract was checked against Four MDR pathogens. Antibacterial activity results are indicated in Table 3 and Plate 1. Ethyl acetate extract of endophytic fungi *Pestalotiopsis sp*. demonstrated better zone of inhibition for *Staphylococcus aureus* (11mm), *Enterococcus faecalis* (12mm), *Enterobacter cloacae*(12mm), *Pseudomonas aeruginosa* (12mm) at concentration of 10mg/ml. and *Neofusicoccum parvum*demonstrated significant zone of inhibition for *Staphylococcus aureus* (8mm), *Enterococcus faecalis* (10mm), *Enterobacter cloacae*(10mm), *Pseudomonas aeruginosa* (13mm) at concentration of 10mg/ml. and Results revealed that *Neofusicoccum parvum* ethyl acetate extracts showed promising antibacterial activity.Since fungi isolate more often, increasing the possibility of finding an antibacterial chemical from a range of species, Radi'c and Strukelj [38] state that fungi develop more secondary metabolites than any other endophyte. A lot of phenolic chemicals and flavonoids are made by the endophytic fungus Colletotrichum gloeosporioides[39].

CONCLUSION

The current study concludes that endophytic fungi isolated from *P. hymenophyllum*, a medicinal plant, exhibited antioxidant and antibacterial activity in vitro, as well as the presence of powerful secondary metabolites. Endophytes derived from *P. hymenophyllum* are exceptional since they represent a naturally occurring source of metabolites with biological activity. An exciting new avenue for cancer prevention, care, and progression reduction has opened up thanks to the bioactive compounds discovered in *P. hymenophyllum*. Molecular characterization of the medicinal components of *P. hymenophyllum* and elucidation of their unique mechanisms of medicine interaction require further investigations employing advanced databases and multidisciplinary approaches.

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Conflict of interest

Authors reported no conflicts of interest.

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