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Research Article

IN-VITRO PHYTOCHEMICAL ANALYSIS, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI *PESTALOTIOPSIS* SP. ISOLATED FROM *ACALYPHA INDICA* (LINN)

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ABSTRACT

Objective: This study deals with phytochemical analysis, high-performance liquid chromatography (HPLC), antimicrobial activity analysis of endophytic fungi *Pestalotiopsis* sp. isolated from midrib and lamina region of *Acalypha indica* leaves.

Methods: An endophytic fungus isolated from potato dextrose agar medium. Ethyl acetate extracts of *Pestalotiopsis* sp. were investigated for their HPLC, and a qualitative analysis of the phytochemical analysis was performed. The antibacterial activity of isolated endophytic fungi was tested against two Gram-positive bacteria (*Staphylococcus aureus and Streptococcus pneumoniae*) and two Gram-negative bacteria (*Salmonella typhi and Escherichia coli*) by disk diffusion method.

Results: The crude extract of *Pestalotiopsis* sp. HPLC retention time of 5.76 shows the maximum area of 78.15%. The qualitative analysis of endophytic fungi showed positive results for terpenoid and negative results for alkaloid, flavanoid, tannin, and carbohydrate. Crude extracts could inhibit Gram-positive bacteria (*S. aureus* and *S. pneumoniae*) and Gram-negative bacteria (*S. typhi* and *E. coli*).

Conclusion: This study recorded that *Pestalotiopsis* sp. produce bioactive compound. Further studies on characterization, *in-vitro* and *in-vivo* studies, would reveal the antibacterial activity can be performed to see the activity of this endophytic fungus in clinical trials against different human ailments.

Keywords: *Pestalotiopsis* sp., Phytochemical analysis, Antibacterial activity, *Acalypha indica*.

INTRODUCTION

Endophytic fungi live asymptomatically within the living tissue of the host plant and form mutualistic symbiosis. There could be more than one type of endophytic fungi found within one plant [1,2] demonstrated endophytic fungi caused no harmful effects to the living host plant and they can be isolated from various parts of the plant with a careful selection and screening process. The isolation method may affect the species composition of the endophyte collection within a given host plant. A large number of isolates can be obtained from host species, however, only a few host specific strains are dominant [1].

Pestalotiopsis microspora, as endophytic from the inner bark of *Taxus wallichiana,* which produced taxol in mycelial culture. The culture amendments, *viz*., lowering of phosphate and the addition of sodium benzoate increased the taxol production. The taxol was assayed by competitive enzyme immunoassay [3]. Jestrone and hydroxyl-jestrone isolated from *Pestalotiopsis jesteri* from a medicinal tree *Terminalia morobensis* is having anti-oomycetic activity [4].

In Malaysia, extract from many types of local plants is used in the traditional manner for treatments of various ailments [5]. The question is whether they are produced by the plant itself or as a consequence of a mutualistic relationship with beneficial organisms in their tissue. Many reports showed that in a plant-microbe relationship, endophytes contribute substances that possess various types of bioactivity such as antibacterial and antifungal. Therefore in the this study, deals with phytochemical analysis, high-performance liquid chromatography (HPLC), antimicrobial activity analysis of endophytic fungi *Pestalotiopsis* sp. isolated from midrib, and lamina region of *Acalypha indica* leaves.

METHODS

Collection of leaves samples

The *A. indica* leave's samples were collected from Anna Herbal Garden, Tamil Nadu. The samples were transported in closed sterile polythene bags and processed within 24 hrs collection.

Test bacterial strains

The pathogenic bacterial strains such as *Salmonella typhi, Escherichia coli, Staphylococcus aureus,* and *Streptococcus pneumoniae* were received from Department of Microbiology, Madras University.

Surface sterilization

The leaves were thoroughly washed in running tap water. Then, the leaf segments were surface sterilized by immersion in 70% ethanol for 5 seconds, followed by treatment in 4% sodium hypochlorite for 90 seconds and finally rinsed in sterile distilled water for 10 seconds [6].

Isolation of endophytic fungi

The surface sterilized leaf segments were evenly spaced in Petri dishes containing potato dextrose agar (PDA) medium amended with 10 mg of chloramphenicol. The Petri dishes incubated at 26±1°C in a light chamber [7] and monitored every day for the growth of endophytic fungal colonies from leaf segments. The hyphal tips, which grew out from leaf segments, were isolated and brought into pure culture. The isolated endophytic fungi were identified down to species level using standard manuals and monographs [8,9].

Extraction of bioactive compounds

The selected endophytic fungi *Pestalotiopsis* sp. were grown in Czapek's broth for 48 hrs and incubated for 21 days at 120 rpm. The extract was separated using filtration procedure (Whatman No 1). Ethyl acetate

(EA) was added in culture filtrate, and the compounds were separated using separating flask and concentrated in a rotary vacuum evaporator. The dry semi-solid residue was redissolved in EA for further use [10].

HPLC

The EA extract fungal samples were subjected to HPLC. The sample was analyzed by HPLC (Shimadzu 9A) model used. The mobile phase components were filtered through 0.2 µ membrane filter before use and pumped from the solvent reservoir at a flow rate of 0.5 ml/minutes, which yielded column backup, the pressure of 160-170 kgf/cm^2 the column was maintained at 27 deg. syringe (Bonaduz Scheweiz, Hamilton) was used for injection 20 µl of respective samples [11].

Phytochemical analysis

Alkaloids (Dragendorff's test)

A few mg of the substance is treated with 2 drops of dragendorff's reagent (Bismuth nitrate and tartaric acid - Solution A and potassium iodide - Solution B). Solution A and B mixed together in equal proportion red or orange precipitate indicates the presence of alkaloids.

Flavonoids

About 10% $AlCl_3$ in alcohol is treated with few mg of the substance and heated at 150°C and see under ultraviolet (UV) at 366 nm. Fluorescence color shows the presence of flavonoids.

Terpenoids

A few mg of the substance is treated with Puncal reagent (ammonium heptamolybdate, ceric sulfate in conc. sulfuric acid) and heated at 150°C blue color shows the presence of terpenoids.

Tannin

A few mg of the substance is treated with a saturated solution of ferric chloride; blue color indicates the presence of tannin.

Carbohydrates

A few mg of the substance was added to the conc. sulfuric acid in alcohol Heated gently if necessary, charring or black color shows the presence of sugar.

Antibacterial activity

The concentrate crude extract *Pestalotiopsis* sp. was then impregnated (80 µl/disc) onto sterile Whatman 6 mm diameter disc and the antibacterial activity was assayed against *S. typhi, E. coli, S. aureus,* and *S. pneumoniae* following the disc diffusion assay [12]. The assay was carried out in triplicate. Control plates with solvents were also maintained separately. The zone of inhibition was measured from the edge of the disc to the clear zone in millimeter.

Column chromatography purification

The active *Pestalotiopsis* sp. extracts were then column fractionated using normal phase silica gel chromatography employing a step gradient (hexane 100%; hexane 75%: EA 25%; hexane 50%: EA 50%; hexane 25%; EA 75%; EA 100). The fractions were concentrated and used for antibacterial assay was carried out to find out the active fraction [10].

RESULTS

The surface sterilized leaf segments were plated on PDA medium amended with chloramphenicol and incubated at 30±1°C for 2 weeks (Fig. 1a). The fungi grow out from tissues were brought into pure culture and identified (Fig. 1b). The HPLC profile of *Pestalotiopsis* sp. shows four peaks with a retention time (RT) of 4.38, 5.76, 6.47, and 9.78. The RT of 5.76 shows the maximum area of 78.15%. The results are presented in Fig. 2. The *Pestalotiopsis* sp. was screened for phytochemical constituent analysis inferred that the crude extracts contain terpenoids. However, alkaloids, tannin, flavanoids, and carbohydrates were absent in fungal extract (Table 1). The *Pestalotiopsis* sp. crude extracts could inhibit the bacteria. 24 µg/ml inhibited Gram-positive bacteria (*S. aureus* and *S. pneumoniae*), 75 µg/ml inhibited Gram-negative bacteria (*S. typhi* and *E. coli*). The column purified different fraction of *Pestalotiopsis* sp. showed antibacterial activity. The *E. coli* showed maximum activity (3.9±0.18) in (75% hexane: 25% EA) than *S. typhi* (3.1±0.17), *S. aureus* (2.8±0.15) and *S. pneumoniae* (2.5±0.22) (Table 2).

DISCUSSION

The endophytic fungi *Aspergillus* niger and *Aspergillus flavus* are common in all plants *Pestalotiopsis* sp. is dominant in all host species studied. *Aspergillus* genus and *Pestalotiopsis* occurs as endophyte in a wide range of plant species [13,14]. *Aspergillus* sp., *Alternaria* sp., and *Curvularia* sp. occur as phylloplane fungi but are capable of penetrating superficial layers of leaf; when they do so they survive the vigorous surface sterilization steps during isolation and grow out as colonies in plates [15] suggesting that phylloplane fungi too resort to an endophytic mode of life to overcome drastic environmental conditions such as compelion and desiccation. The surface sterilized leaf segments were plated on PDA medium amended with chloramphenicol and incubated at 30±1°C for 2 weeks. The fungi grow out from tissues were brought into pure culture and identified. Pestalotiopsis sp. are dominant in midrib region than the lamina region of Acalypha indica leaf. The more intensive samplings

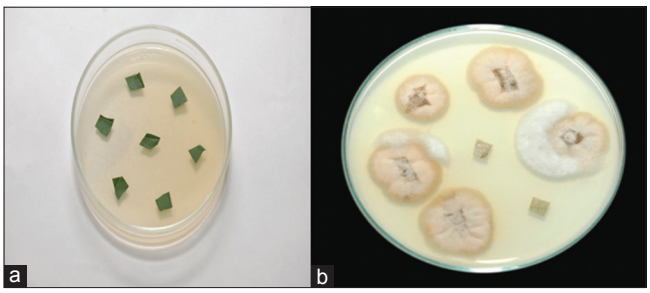

Fig. 1: Endophytic fungus from *Acalypha indica* leaf samples. (a) At the time of inoculation, (b) after incubation

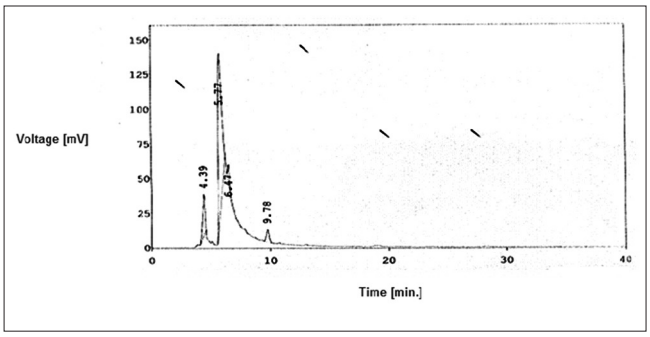

Fig. 2: High-performance liquid chromatography analysis of crude extracts of *Pestalotiopsis* sp.

Table 1: Phytochemical compounds of the endophytic fungi isolated from *Pestalotiopsis* sp.

Serial number	Phytochemical compounds	Presence or absence
1	Alkaloid	–
2	Flavanoid	–
3	Tannin	–
4	Terpenoid	+
5	Carbohydrate	–

+: Presence, –: Absence

Table 2: Antibacterial activity of *Pestalotiopsis* sp. column purified different fractions

Serial number	Pathogenic bacterial strains	Zone of inhibition (mm) (mean±SD) *n*=5 experiments				
		H 100%	H:EA 75%:25%	H:EA 50%:50%	H:EA 25%:75%	EA 100%
1	*E. coli*	-	3.9±0.18	2.0±0.22	1.8±0.13	1.4±0.10
2	*S. typhi*	-	3.1±0.17	1.3±0.13	1.5±0.53	1.2±0.12
3	*S. aureus*	-	2.8±0.15	1.5±0.52	1.3±0.12	1.1±0.12
4	*S. pneumonia*	-	2.5±0.22	1.1±0.11	T	2.0±0.10

EA: Ethyl acetate, H: Hexane, T: Trace, SD: Standard deviation, *E. coli: Escherichia coli, S. typhi: Salmonella typhi, S. aureus: Staphylococcus aureus, S. pneumonia: Streptococcus pneumonia*

are necessary to clarify the fungal assemblages of the leaves and branches, as in traditional practice, the local population used mostly the extract from the leaves of the plants [5].

Two different coelomycetous fungi were screened for the production of taxol, an anticancer drug. The taxol production was confirmed by the following methods: UV spectroscopic analysis, infra-red analysis, HPLC and liquid chromatography mass spectrum, and the taxol compared with authentic taxol. The fungal taxol was identical to authentic taxol. The taxol produced by the above fungi was tested against cancer A549 cell line [16]. The HPLC profile of *Pestalotiopsis* sp. shows four peaks with a RT of 4.38, 5.76, 6.47, 9.78. The RT of 5.76 shows the maximum area of 78.15%.

In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, and cytotoxic anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, quinines, lignans, phenols, and lactones [17]. The endophytic fungi were screened for phytochemical constituent analysis inferred that the crude extracts contain terpenoids. However, alkaloids, tannin, flavanoids, and carbohydrates were absent in fungal extract.

Crude ethyl extracts derived from submerged fermentation of four endophytes showing positive results were further evaluated. *Aspergillus* sp. and *Pestalotiopsis* sp. inhibited many bacteria and *Candida albicans,* while inhibition by *Aspergillus* sp. showed high antimicrobial activity. One of the fractions of *Pestalotiopsis* sp. showed considerable inhibition of *Bacillus subtilis*, *S. aureus,* and *C. albicans*. Seven endophytic fungi were assessed for the production of extracellular enzymes (amylase, cellulose, chitinase, laccase, lipase, protease and tyrosinase) by culture plate method. Cellulase and lipase activity were in all fungi, while amylase and protease in a few endophytes [18]. In this study, *Pestalotiopsis* sp. demonstrated a selective antibacterial activity against Gram-positive bacteria (*S. aureus* and *S. pneumoniae*) and Gram-negative bacteria (*S. typhi* and *E. coli*). The *Pestalotiopsis* sp. crude extracts could inhibit the bacteria. 26 µg/ml inhibited Gram-positive bacteria (*S. aureus* and *S. pneumoniae*), 76 µg/ml inhibited Gram-negative bacteria (*S. typhi* and *E. coli*). The *Pestalotiopsis* sp. crude extracts could inhibit the bacteria. 24 µg/ml inhibited Gram-positive bacteria (*S. aureus* and *S. pneumoniae*), 75 µg/ml inhibited Gram-negative bacteria (*S. typhi* and *E. coli*). The *E. coli* showed higher antibacterial activity on EA crude extracts of *Pestalotiopsis* sp. against column purified compound. The column purified different fraction of *Pestalotiopsis* sp. showed anti-bacterial activity.

CONCLUSION

This study showed that *in-vitro* phytochemical analysis, HPLC, and antibacterial activity analysis of endophytic fungi *Pestalotiopsis* sp. isolated from midrib and lamina region of *A. indica* leaves. The qualitative phytochemical analysis of the endophytic fungi showed a positive result for terpenoid. The HPLC analysis of the endophytic fungi showed RT of 5.76 shows the maximum area of 78.15%. The endophytic fungi showed promising antibacterial activity against the Gram-positive and Gram-negative bacteria. The isolation of endophytic fungi from medicinal plants for any bioactive compound may facilitate the product discovery process.

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