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PHYTOCHEMICAL SCREENING, ENZYME AND ANTIBACTERIAL ACTIVITY ANALYSIS OF ENDOPHYTIC FUNGI *BOTRYTIS SP* ISOLATED FROM *FICUS BENGHALENSIS* (L.)

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Abstract: The present study deals with phytochemical analysis, enzyme properties and antibacterial activity analysis of endophytic fungi *Botrytis sp* isolated from aerial root of *Ficus benghalensis*. An endophytic fungus was isolated by using PDA (Potato Dextrose Agar) medium. Qualitative analysis of the phytochemical was performed following generally accepted laboratory techniques; so also the different enzymes produced from the fungus were analyzed. The antibacterial activity of isolated endophytic fungi was tested against one gram positive bacteria namely *Staphylococcus aureus* and two gram negative bacteria namely *Escherichia coli* and *Klebsiella sp* adopting well diffusion method. The qualitative phytochemical analysis of the endophytic fungi showed positive results for alkaloids, flavonoids, terpenoids, steroids, alkaloids and saponins. (Except phenols, cardiac glycosides). The fungal culture has the ability to produce amylase and laccase enzymes. (Except cellulase, lipase and protease). The crude fungal extract showed inhibition only of *Escherichia coli* (13mm) and *Klebsiella sp* (15 mm). Streptomycin used as a control showed inhibition zone range of 25 to 30 mm. The present study recorded that endophytic fungi *Botrytis sp* produce bioactive compounds. 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: Antibacterial activity, Enzyme activity, Endophytic fungi, *Ficus benghalensis*, phytochemical analysis.

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INTRODUCTION

Endophytes are symbiotic microorganisms of living plants and potential sources of biologically active natural products useful in medical, agricultural and industrial applications[1]. They have been found in all plant families and it is estimated that there may be atleast one million species of endophytic fungi alone. Recently endophytes are viewed as outstanding source of secondary metabolites and bioactive antimicrobial natural products[2]. Endophytes are known to produce metabolites such as alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols, phenolic acids, and peptides[3].Endophyte have been most extensively studied for their ability to produce antibacterial, anticancer, antioxidants, antidiabetic and immunosuppressive compounds[4]. The present study deals with phytochemical analysis, enzyme properties and antibacterial activity of the endophytic fungi isolated from aerial root of Ficus benghalensis.

MATERIALS AND METHODS

Source of endophytic fungi

The plant material of *Ficus benghalensis* was collected during the month of September 2012 from different places of Madurai district, Tamilnadu, India. The aerial root samples were washed in running tap water for 10 minutes to remove soil particles and adhered debris. The root samples were cut longitudinally before inoculation. Then the root samples were washed thoroughly with sterile distilled water and air dried under laminar air chamber; Samples were immersed in 10 mL distilled water 3 minutes; then the samples were placed in 1 % aqueous solution of $HgCl_2$ for 3 minutes. Later the samples were rinsed in distilled water for 1 minute. The samples were utilized within 6 hours of collection.

Identification of the Endophytic fungi

Based on their microscopic characters and their external morphology, it was identified as *Botrytis sp.*

Fermentation and extraction

Two or three pieces of grown culture cut from mother culture was inoculated into 500 mL Erlenmeyer flask containing 300 mL potato dextrose broth for 21 days. After the fungal broth culture was filtered to remove mycelium. The filtrate was extracted with ethyl acetate (1:1 ratio) two times. The organic phase was evaporated to dryness and stored at 4°C for further usage. The crude extract was dissolved in DMSO to obtain different concentrations.

Phytochemical analyses

The ethyl acetate extract of endophytic fungi checked for the presence of the following secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, steroids, cardiac glycosides, phenols, tannins by standard procedures[5, 6].

Alkaloids

The fungal crude extract was dissolved in 2 N HCL solutions. The mixture was treated with a few drops of Mayer's reagent (3 ml of potassium iodide solution mixed with 2 ml mercuric chloride solution). The creamish precipitate indicates the presence of alkaloids.

Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The crude dry powder of fungal extract was treated with 1mL of $FeCl_3$ reagent (mixture of 1 volume of 5% $FeCl_3$ solution and 99 volumes of glacial acetic acid). To this solution a few drops of concentrated H_2SO_4 was added. Appearance of greenish blue color within a few minutes indicated the presence of cardiac glycosides.

Flavonoids

In the test tube containing 1 ml of fungal crude extract was added with a few drops of 20 % NaOH solution. A change to yellow color which on addition of acid changed to colorless solution depicted the presence of flavonoids.

Phenols

The fungal extract was dissolved in 5 ml of distilled water. To this few drops of neutral 5 % ferric chloride solution was added. A dark green color indicated the presence of phenolic compounds.

Saponins

The presence of saponins was determined by frothing test. The fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 minute. (Formation of a fairly stable emulsion indicated the presence of saponins).

Steroids

Libermann-Burchard reaction was performed to assess the presence of steroids. The crude fungal extract was added with 1 ml of chloroform solution the mixture was treated with acetic anhydride and few drops of concentrated H_2SO_4 were added. A blue green ring indicated the presence of steroids.

Tannins

The fungal crude extract was treated with alcoholic $FeCl_3$ reagent. A bluish black color, which disappears on addiction of a little dilute H_2SO_4, was followed by the formation of yellowish brown precipitate indicated the presence of tannins.

Terpenoids

1 mL of fungal crude extract was mixed in 2 ml of chloroform. 3 ml of concentrated H_2SO_4 was then added to form a layer. A reddish-brown precipitate coloration at the interface formed indicated the presence of terpenoids.

Enzyme assay

Extracellular enzymes assay was conducted to investigate the production of enzymes by the endophytic fungi. It was assessed by

digestion of suspended or dissolved substrate in agar plates after inoculation with 3 mm mycelia plugs and incubation for 3-5 days at 37°C. The diameter of the clear zone was used as a measurement of the amount of enzyme production[3, 5].

Amylase

Amylase enzyme activity was assessed by growing the fungi on glucose yeast extract peptone agar medium (GYP) (glucose-1g, yeast extract 0.1g, peptone 0.5g agar 16g, distilled water 1000mL and pH 6) containing 1% soluble starch. After 5 days incubation, the plates with fungal colony were flooded with1% iodine in 2% potassium iodide. The appearance of clear zone surrounding the colony was considered positive for amylase enzyme.

Cellulase

Yeast extract peptone agar medium (yeast extract 3g, peptone 5g, agar 15g, distilled water 1000mL and pH 6) supplemented with Na-carboxymethyl cellulase was used. The endophytic fungus was cultured and kept for incubation. After incubation, the plates were flooded with 2 % aqueous Congo red and destained with 1 M Nacl for 15 minutes. The clear zone around the colony indicated the cellulase activity.

Laccase

Glucose Yeast extract peptone agar (GYP) (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16g, distilled water1000 mL and pH 6) medium amended with 1-napthol, 0.005% were prepared and fungus was inoculated and kept for incubation. On oxidation of 1-napthol by laccase, the medium changes from clear to blue.

Lipase

The fungus was grown on peptone (peptone 10.g, sodium chloride 5g, agar 14g, distilled water 1000 mL and pH 6) agar medium supplemented with Tween 20. A clear zone around the colony indicated the presence of lipase enzyme.

Protease

The fungus was grown on Glucose Yeast extract peptone (GYP) (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16, distilled water1000mL and pH 6) agar medium (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16 g, distilled water 1000mL and pH 6) amended with 0.4% phenol and adjusted with pH 6. After 3 days of incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested phenols were precipitated with ammonium sulphate and digested area around the colony would appear a clear zone.

Antibacterial assay

Nutrient Agar medium was prepared according to the manufacturer's instructions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure and was used for tests. Sterile nutrient agar medium was poured aseptically into sterile petridishes (15 ml

each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification 1 ml of bacterial culture *Escherichia coli*, *Klebsiella sp*, and *Staphylococcus aureus* was placed on the medium. It was then spreaded over the surface of agar using 'L 'rod. Four wells were bored on the agar surface using a cork borer. One well was filled with streptomycin (25 µg/ml) was used as a control and remaining three wells were filled with 200µL of endophyte culture filtrates respectively. The next day the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were read by the presence or absence of zone of inhibition with slight modifications[7].

RESULTS

Ficus benghalensis, Indian banyan tree this plant belongs to the family Moraceae. It produces propagating roots which grow downwards as aerial roots. Once these roots reach the ground, they grow into woody trunks that can become indistinguishable from the main trunk (Figure 1).

Isolation of endophytic fungi

Endophytic fungus was isolated from the aerial root of *Ficus benghalensis* plant and their morphological and fungal spore microscopic view characteristics it was identified as *Botrytis sp*. (Figure 2).

Phytochemical analysis

The result of endophytic fungi was screened for phytochemical constituent analysis inferred that the crude fungal extracts contain saponins, alkaloids, steroids, flavonoids and terpenoids. However alkaloids, phenols, cardiac glycosides were absent in fungal crude extract (Table 1).

Enzyme assay

The fungal culture has the ability to produce Amylase and laccase enzymes. But the results endophytic fungus was unable to produce other enzymes such as cellulase, lipase, and protease (Figure 3).

Antibacterial activity

The antibacterial activity of isolated endophytic fungi tested against gram positive bacteria namely Staphylococcus aureus and gram negative bacteria namely *Escherichia coli*, *Klebsilla sp* using for well diffusion methods are reported (Table 2).

Bioactive compounds of endophytic fungal isolate showed inhibition of Escherichia coli (13 mm), Klebsiella sp (15 mm). The fungal isolates could not inhibit the *Staphylococcus aureus*. Streptomycin used as the control showed a zone of inhibition of 25 to 30 mm. The isolate fungal crude extract showed highest zone of inhibition against *Klebsiella sp* (15mm) (Figure 4).

DISCUSSIONS

In this study endophytic fungus was isolated from aerial root of *Ficus benghalensis*, based on their microscopic characters and their external morphology, it was identified

as *Botrytis sp.* Our results similar to Mitosporic fungi and several sterile forms were isolated as endophytes from the leaf tissues and aerial roots of *Ficus benghalensis* (Moraceae). The endophytic fungi more densely colonized the petiole, the speices composition and colonization frequency of the endophytes were more for aerial roots entering the soil, when compared those growing in the air since the roots recruited some endophytes from the soil. The endophytes assemblages of the leaf and the aerial root growing in the air and soil showed little overlap suggesting that the nature of the host tissue as well as environment determine the endophyte of a host[8].

Aerial root of *Ficus benghalensis* shows anthelmintic activity. It is used in Ayurveda for treatment of diarrhea, piles, teeth and skin disorders. The bark is used in inflammation, swelling at neck, gonorrhea, scabies mouthwash for tooth ache, and for strengthening gums, and steeped freshly burnt bark has been said to cure cases of obstinate hiccup[9].

In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic anticancer activities have been successfully discovered from the endophytic fungi. These bioactive compounds could be classified as alkaloids, terpenoids, steroids, quinones, lignans, phenols and lactones[10]. Similar to this the phytochemical analysis was carried out of the endophytic fungi showed the presence of saponins, steroids, flavonoids, tannins, and terpenoids.

Endophytes a potential source of enzyme producer[3]. Similar to this the endophytic fungi ability to produce Amylase and laccase enzymes.

The isolate fungal crude extract showed highest zone of inhibition against *Klebsiella sp* (15mm) diameter. The endophytic fungal isolate (P13T5) showed highest zone of inhibition against *Pseudomonas aeroginosa* and fungal isolates (P13T5) showed inhibition of *Salmonella typhimurium* up to (16mm) diameter inhibition zone[2]. Our results similar to bioactive compounds of endophytic fungal isolates showed inhibition of Escherichia coli (13 mm), *Klebsiella sp* (15 mm). The fungal isolates could not inhibit the *Staphylococcus aureus*. The isolate fungal bioactive compound showed highest zone of inhibition against *Klebsiella sp* (15mm). The fungal bioactive active compound revealed good antibacterial activity.

Endophytic fungi produce various bioactive compounds, 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.

CONCLUSIONS

The present study showed that the phytochemical analysis, enzyme properties and antibacterial activity analysis of

endophytic fungi *Botrytis sp* isolated from aerial root of *Ficus benghalensis.* The qualitative phytochemical analysis of the endophytic fungi showed positive results for alkaloids, flavonoids, terpenoids, steroids and saponins. (except phenols, cardiac glycosides, alkaloids). The fungal culture has the ability to produce amylase and laccase enzymes. (except cellulase, lipase and protease). The fungal bioactive compound showed inhibition only of *Escherichia coli* (13mm) and *Klebsiella sp* (15 mm). Endophytic fungi could not inhibit against *Staphylococcus aureus.*

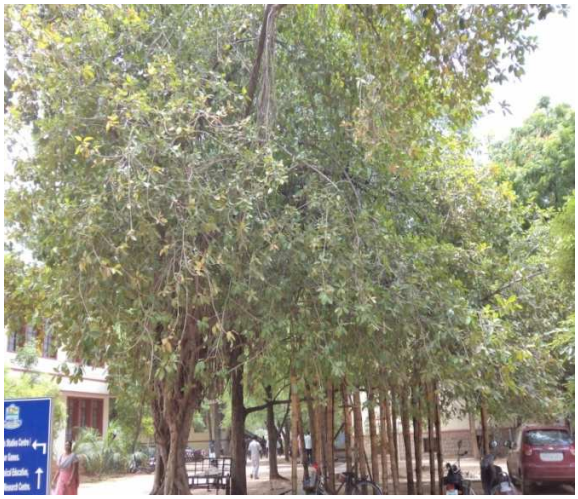
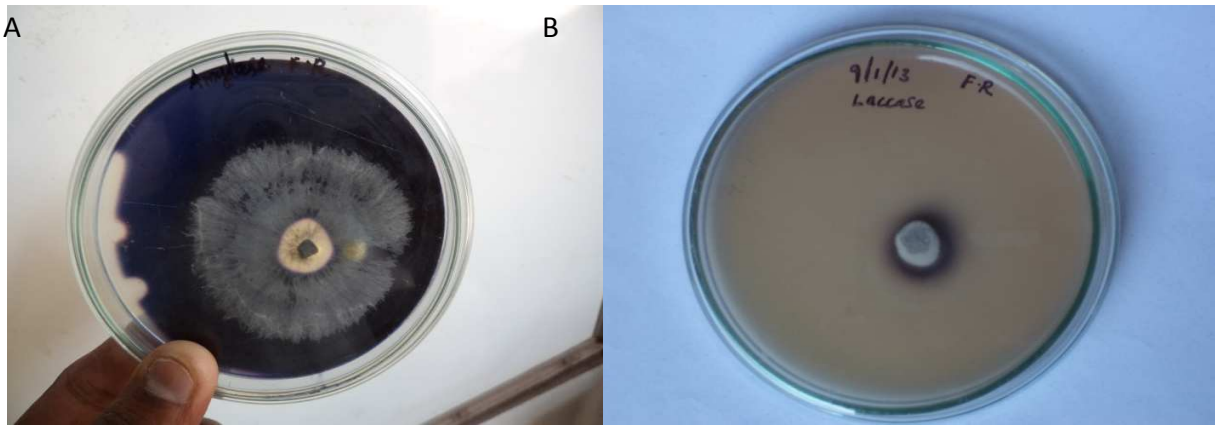


Figure No: 1 *Ficus benghalensis* (L.)

Figure No: 2 *Botrytis sp* isolated from *Ficus benghalensis* plant



A. Amylase enzyme production by *Botrytis sp* B. Laccase enzyme production by *Botrytis sp*

Figure No: 3 Production of enzymes by *Botrytis sp*

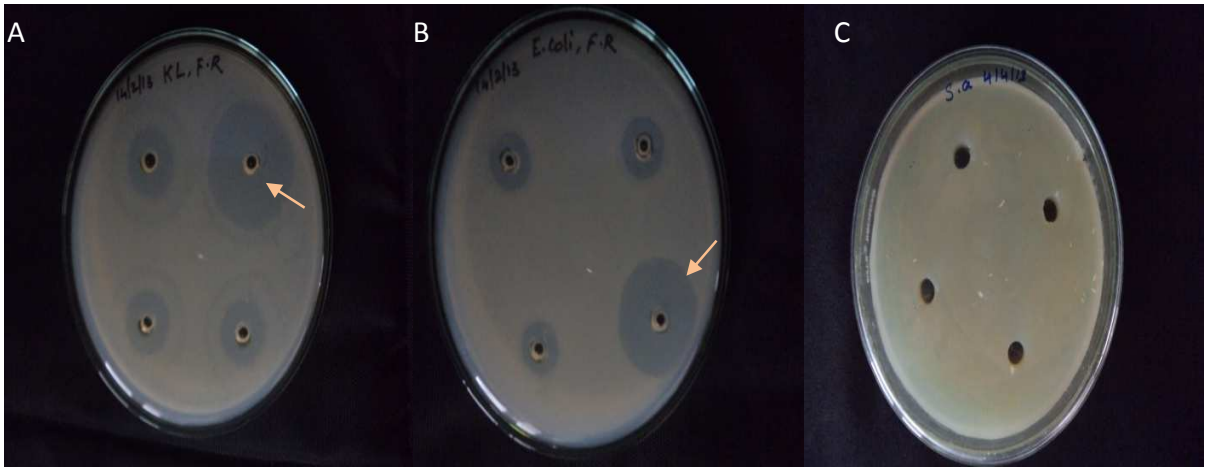


Figure No: 4 Inhibition zone of Endophytic fungal bioactive compound against three test organisms

A. Inhibition of fungal bioactive compound against *Klebsilla sp.*

B. Inhibition of fungal bioactive compound against *Escherichia coli*.

C. No inhibition of fungal bioactive compound against *Staphylococcus aureus*

Arrow mark indicates Streptomycin used as a control (plate A and B)

Table 1.

Phytochemical compounds of the Endophytic fungi isolated from Ficus benghalensis

Phytochemcial compounds	Presence or Absence
Alkaloids	+
Cardiac glycosides	-
Flavonoids	+
Phenols	-
Saponins	+
Tannins	-
Steroids	+
Terpenoids	+

+ = Indicates the presence, - = Indicates the absence

Table 2.

Antibacterial activity of *Botrytis sp* isolated from *Ficus benghalensis*

Test organisms	Streptomycin control	Zone of inhibition (mm)
Escherichia coli	30	13
Klebsiella sp	25	15
Staphylococcus aureus	-	-

- = Indicates absence, mm-indicates millimeter

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