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Phytochemical analysis and enzyme analysis of endophytic fungi from *Centella asiatica*

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ABSTRACT

Objective: To evaluate the phytochemical properties, enzyme properties of an endophytic fungi from *Centella asiatica* plant. **Methods:** Endophyte was isolated by using potato dextrose agar medium from *Centella asiatica* plant. The qualitative chemicals screening were performed for the ethyl acetate extracts of fungi. Then the phytochemicals like phenols and flavonoids were quantitatively estimated. Then the ability of the fungal culture to produce different enzymes was analysed. Finally, High performance thin layer Chromatography was performed for alkaloid and flavonoid profiles of fungal extract. **Results:** A *Penicillium* sp. was isolated from *Centella asiatica* plant and the fungus was identified by 18S rRNA analysis. The sequence of the fungus was submitted in NCBI with accession number (HM068965) and name *Penicillium* sp.nirjan22. The qualitative chemicals analysis of the fungus showed positive results for alkaloids, phenols, flavonoids, tannin and glycosides. It was found that the fungal crude extract contained 2.76g of phenol and 0.275 g of flavonoid. The *Penicillium* sp.nirjan22 produced cellulase enzyme. The HPTLC analysis of the ethyl acetate extract of fungi inferred that it contain both alkaloid and flavonoid. **Conclusions:** The present study inferred that *Penicillium* sp. nirjan22 contain many bioactive compounds which can further isolate and check in vitro and in vivo models against different human ailments.

1. Introduction

The antibiotic resistance by many pathogens are rapidly increasing for the available therapeutic drugs. So there is indisputable need for new bioactive compounds to control human diseases caused by pathogenic organisms[1]. Endophytic fungi are of biotechnological interest due to their potential use as genetic vectors, metabolites and biological control agents[2]. There are many reports and studies on the biological activities of endophytes like antiviral, anticancer and antimicrobial effects[3]. Endophytes have mutualistic relations to their hosts, often protecting plants against herbivores, insect attack or tissue invading pathogens[4]. Endophytes have been found in every plant species studied, and it is approximated to be around a million or more endophytic fungi in nature[5]. Hence the present study deals with the phytochemical analysis, enzyme analysis and HPTLC analysis of the fungi isolated from *Centella asiatica* medicinal plant.

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2. Materials and Methods

2.1. Source of endophytic fungi

The plant material of *Centella asiatica* was collected in and around Nammakal District Tamilnadu, India. The plant was taxonomically identified and authenticated by Botanical Survey of India, Tamilnadu Agricultural University, Coimbatore. The voucher Specimen was deposited there with register number BSI/SRC/5/23/2011-12/Tech.- 932. The leaves samples from plant were randomLy cut off with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to laboratory within 12 h and stored at 4 ℃ until isolation procedures were completed.

2.2. Isolation and identification of endophytic fungi

The isolation of endophytic fungi from *Centella asiatica* was carried out by using standard method[6,7]. The fungi was then identified by 18S rRNA analysis.

2.3. Fermentation and extraction

Two or three pieces of the grown culture cut from the culture plate were inoculated into 1 000 mL Erlenmeyer flask containing 300 mL potato dextrose broth for 15

days at 25 ℃, 120 r/min. The fungal culture was filtered to remove mycelium. The filtrate was extracted with ethyl acetate (1:1 ratio) three times. The organic phase was evaporated to dryness and stored at 4 ℃ for further usage. The crude extract was dissolved in DMSO to obtain different concentrations.

2.4. Preliminary qualitative phytochemicals screening

The ethyl acetate extract of *Penicillium* sp. checked for the presence of the following secondary metabolites such as Alkaloid, Phenols, Flavonoids, Saponins, Steriods, Cardiac glycosides, and Tannins by standard procedures[8,9].

2.4.1. Alkaloids

The fungal crude extract was evaporated to dryness in a boiling water bath. The residue was dissolved in 2 N HCl. The mixture was filtered and the filtrate was divided into 3 equal portions. One portion was treated with a few drops of Mayers reagent; one portion was treated with equal amount of Dragondroffs reagent and the other portion was treated with equal amount of Wagners reagent. The creamish precipitate, orange precipitate and brown precipitate, indicated the presence of respective alkaloids.

2.4.2. Flavonoids

In a test tube containing 0.5 mL of crude extract, 5-10 drops of diluted HCL and small piece of zinc or magnesium were added and the solution was boiled for few minutes. In the presence of flavonoids, reddish pink or dirty brown colour was produced.

2.4.5. Phenols

The extract is dissolved in 5 mL of distilled water. To this few drops of neutral 5% ferric chloride solution are added. A dark green color indicates the presence of phenolic compounds.

2.4.6. Tannins

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

2.4.7. Cardiac glycosides

Keller-kiliani test was performed to assess the presence of cardiac glycosides. The crude dry powder of fungal extract was treated with 1 mL 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.

2.4.8. Steroids

Liebermann-Burchard reaction was performed to assess the presence of steroids. A chloroform solution of the crude dry powder of fungal extract was treated with acetic anhydride and a few drops of concentrated H_2SO_4 were added down the sides of the test tube. A blue green ring indicated the presence of terpenoids.

2.4.9. Saponins

The presence of saponins was determined by Frothing test. The crude dry powder of fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. No froth indicates absence of saponins and stable froth more than 1.5 cm indicated the presence of saponins.

2.5. Quantitative phytochemicals screening

2.5.1. Determination of total phenol content

Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method described by Cai *et al*[10] with minor modification. Briefly, the appropriate dilutions of the samples (0.2 mL) were oxidized with 0.5 N Folin-Ciocalteu reagents for 4 min at room temperature. Then the reaction was neutralized with saturated sodium carbonate (75 g/L). The absorbance of the resulting blue color was measured at 760 nm with the spectrophotometer after incubation for 2 h at room temperature in dark. Quantification was done on the basis of the standard curve of gallic acid. Results were expressed as gallic acid equivalent (GAE), *i.e.*, g gallic acid/100 g DW or mg gallic acid/100 mL culture.

2.5.2. Total flavonoid content

Total flavonoid content was determined by a colorimetric method reported by Chang *et al*[11]. Extract samples (0.25 mL) at a concentration of 1 mg extract mL-1 were diluted with deionized water (1.25 mL). A sodium nitrite solution at 5% (0.75 mL) was added and samples were incubated for 6 min at room temperature. Aluminum chloride at 10% (0.15 mL) was aggregated and the mixture was incubated (5 min). Finally, 0.50 mL of sodium hydroxide (1 M) was added. The mixture was brought to 2.50 mL with distilled water and incubated at 25 ℃ for 30 min. Absorbance was measured at 510 nm. A standard curve (5-100 μg mL-1) of catechin (Sigma-Aldrich Chemical) was used to calculate the flavonoid content. All evaluations were performed in triplicate.

2.6. Screening for enzymes

Extracellular enzymes assay were 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℃. The diameter of the clear zone was used as a measurement of the amount of enzyme production[12].

2.6.1. Amylase

Starch agar was prepared and autoclaved. The endophytic fungus was inoculated onto the agar plates and incubated for 3–5 days. After incubation the plates were flooded with iodine. The clear zone formed surrounding the colony was considered positive for amylase production.

2.6.2 Cellulase

Yeast extract peptone agar medium supplemented with Na-carboxymethyl cellulose were 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 min. The clear zone around the colony indicate the cellulase activity.

2.6.3. Laccase

GYP agar medium amended with 1-napthol, 0.005% at pH 6 were prepared and fungus was inoculated and kept for incubation. On oxidation of 1-napthol by laccase, the medium change from clear to blue.

2.6.4. Lipase

The fungus was grown on peptone agar medium supplemented with Tween 20. A clear zone around the colony indicates lipase positive fungus.

2.6.5. Protease

The fungus was grown on GYP agar medium amemded with 0.4% phenol and adjusted with pH 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested phenol were precipitated with ammonium sulphate and digested area around the colony was clear.

2.7. HPTLC Profile

The high performance thin layer chromatography analysis was performed using Hamilton syringe and CAMAG LINOMAT 5 instrument, CAMAG REPROSTAR 3 photo-documentation chamber and CAMAG TLC SCANNER 3. The fungal ethyl acetate extract was checked for alkaloid and flavonoids profile in HPTLC analysis. Ethyl acetate-methanol-water (10 : 1.3 : 1) used as mobile phase for Alkaloid and Ethyl acetate-formic acid-acetic acid-water (10 : 1.1 : 1.1 : 2.6) for flavonoid. The peak table, peak display and peak densitogram were noted.

3. Results

3.1. Isolation and identification of endophytic fungi

A fungi was isolated from the leaves of *Centella asiatica* plant and it was identified as *Penicillium* sp. The fungi sequence was submitted in National centre for biotechnology information with accession number.

3.2. Biochemical analysis

The results of qualitative phytochemicals analysis inferred that the fungal ethyl acetate extract contain alkaloids, phenols, flavonoids, tannin and glycosides. But saponins was absent in fungal extract.

3.3. Total phenol and flavonoid content

It was found that the fungal crude extract contained 2.760 g of phenol /100g of gallic acid equivalent. The total flavonoid content of the fungi was 0.275 g.

3.4. Enzymes screening assays

The fungal culture has the ability to produce cellulase enzyme (Figure 1). But the results for other enzymes were unable to produce other enzymes.

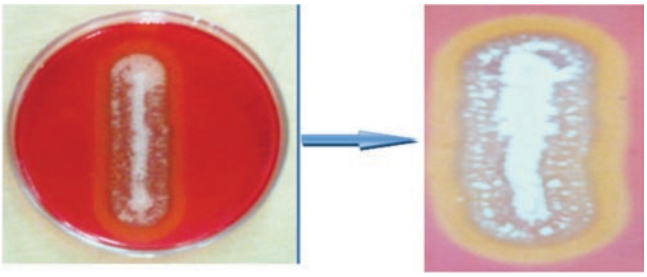

Figure 1. Cellulase enzyme production by *Penicillium* sp.nirjan22.

3.5. HPTLC analysis

The ethyl acetate extract of fungal culture filtrate checked for alkaloid and flavonoid profile by HPTLC. Emetine was used as standard for alkaloid analysis and Rutin was used as standard for flavonoid profile. Orange-brown coloured zone at Daylight mode and black coloured quenching zone at UV 254 nm mode present in the standard and sample tracks observed in the chromatogram after derivatization, which confirmed the presence of alkaloid in the given standard and may be in the samples (Table 1, Figure 2&3).

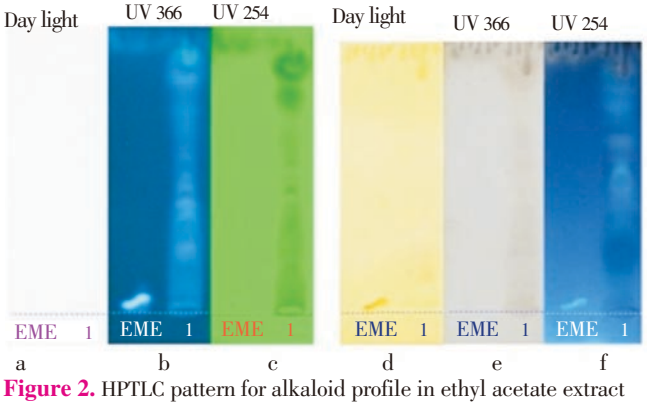

Figure 2. HPTLC pattern for alkaloid profile in ethyl acetate extract of *Penicillium* sp.nirjan22.
a-c: alkaloid chromatogram of extract and standard before dervitatization under day light, UV 366 nm and UV 254 nm; d-f: alkaloid chromatogram of extract and standard after dervitatization; EME: Emetine.

Table 1
HPTLC peak table for alkaloid profile.

Track	Peak	Rf	Height	Area	Assigned substance
Emetine	1	0.04	45.4	1140.0	Emetine standard
Sample	1	0.02	172.8	2294.1	Unknown
Sample	2	0.05	101.2	1788.6	Unknown
Sample	3	0.10	16.3	358.8	Unknown
Sample	4	0.15	90.1	3071.0	Alkaloid
Sample	5	0.29	97.5	3188.6	Unknown
Sample	6	0.31	97.0	2490.3	Alkaloid
Sample	7	0.38	62.0	1580.0	Unknown
Sample	8	0.42	62.3	1641.1	Unknown
Sample	9	0.52	122.4	5961.7	Unknown
Sample	10	0.63	88.4	3559.8	Unknown
Sample	11	0.68	123.4	4068.7	Unknown
Sample	12	0.72	154.1	5047.6	Unknown
Sample	13	0.81	156.1	4033.1	Unknown
Sample	14	0.84	155.9	3905.4	Unknown
Sample	15	0.90	165.1	8702.3	Unknown

Blue or yellow colored fluorescent zone at UV 366 nm present in the standard and sample tracks observed in the chromatogram after derivatization, which confirmed the presence of flavonoids in the given standard and may be in the samples (Table 2, Figure 4&5).

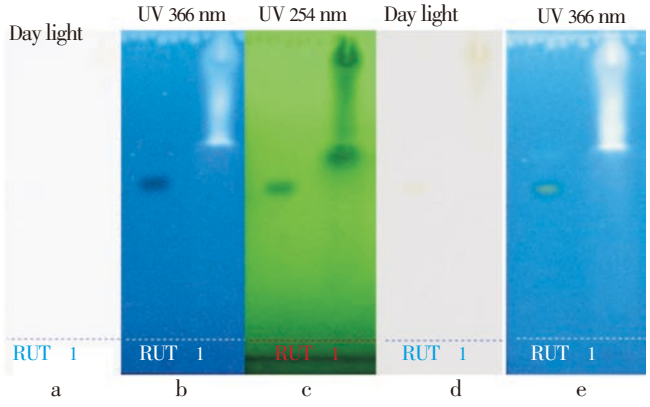

Figure 3. HPTLC pattern for alkaloid profile in ethyl acetate extract of *Penicillium* sp.nirjan22.
a–c: alkaloid chromatogram of extract and standard before derivatization under day light, UV 366nm and UV 254 nm. d, e: alkaloid chromatogram of extract and standard after derivatization; RUT: Rutin.

Table 2
HPTLC peak table for flavonoid profile.

Track	Peak	Rf	Height	Area	Assigned substance
Rutin	1	0.49	338.8	13193.1	Rutin standard
Sample	1	0.35	24.6	164.1	Unknown
Sample	2	0.63	149.1	3502.7	Flavonoid
Sample	3	0.70	119.8	5012.6	Unknown
Sample	4	0.82	196.9	12218.9	Unknown
Sample	5	0.90	329.7	24233.3	Flavonoid
Sample	6	0.98	114.6	1272.3	Unknown

4. Discussions

In India medicinal plants have long been used for the treatment of various diseases. Medicinal plants provide a special environment for endophytes. Many previous reported endophytic fungi with novel and bioactive natural products are obtained from medicinal plants[13–15].

In this study, endophytes were isolated and characterised from *Centella asiatica*. *Centella asiatica* are widely distributed in the Indian environment and have been used traditionally for treatment of various diseases[16]. Most of the endophytes isolated in this study belong to the phylum Deuteromycota and ascomycetes. This result supports the Rakotoniriana *et al*[17] findings that fungal endophytes mainly belong to the ascomycetes. *Penicillium* sp. were selected as the result shown was good. Molecular techniques have been successfully used for identifying endophytic fungi in recent studies[18]. The present study fungi identified as *Penicillium* sp. by 18S rRNA analysis.

The phytochemical analysis was carried out for the identified *Penicillium* sp. The *Penicillium* sp. showed the presence of different phytochemicals, phenolic compounds[19], steroids[20], cardiac glycosides[21], tannins[22], alkaloids and flavonoids[23].

Choi *et al*[24] described the endophytes, as potential enzyme producer. Similar to this, *Penicillium* sp. produced cellulase enzyme. There are many bioactive compounds were observed in the *Penicillium* sp. Our results similar to many already available reports[25]. Major natural products of secondary metabolism in plants and fungi are phenolic compounds. Phenol and flavonoid compounds have been reported to possess different bioactivies[26]. In our bioactive compound analysis also we observed and estimated the phenols and flavonoids level.

Penicillium sp. nirjan22 has ability to produce various secondary metabolites which will be useful for various human ailments. So it could be recommended as an organism of pharmaceutical importance. However isolation of individual secondary metabolite constituents and subjecting it to biological activity will definitely give fruitful results. However, further studies will need to be undertaken to ascertain fully its bioactivity, toxicity profile effect on the ecosystem and agricultural products.

Conflict of interest statement

We declare that we have no conflict of interest

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