

Phytochemical and Antioxidant Studies of Some Isolated Endophytic Fungi

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Abstract

The present study was designed to analyze the phytochemicals and antioxidant activity of isolated endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The ethyl acetate extracts of culture filtrate and mycelial mat of selected endophytic fungi were prepared and used for this study. The qualitative phytochemical analysis showed the presence of carbohydrates, proteins, steroids, terpenoids, phenolic compounds, flavonoids, alkaloids, tannins, saponins and anthroquinones in culture filtrate and mycelial mat of endophytic fungi. The content of total phenolic compounds and flavonoids were also determined. The maximum amount of phenolic compounds 24.14 ± 0.46 mg/g in gallic acid equivalents (GAE) and flavonoids 7.54 ± 0.98 mg/g in quercetin equivalents (QE) was found in mycelial mat of *Penicillium janthinellum*. The antioxidant activity of culture filtrate, mycelial mat and the mixture of culture filtrate and mycelial mat (1:1) was analysed by Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay. The extracts of mixture of culture filtrate and mycelial mat (1:1) of *Penicillium janthinellum* showed the maximum radical scavenging activity with the IC_{50} value of 238.64 ± 10.36 μ g/ml by DPPH assay. The extracts of mixture of culture filtrate and mycelial mat (1:1) of *Penicillium janthinellum* showed maximum percentage of ABTS absorption inhibition activity $90.86 \pm 3.46\%$. So, the present study confirmed that the presence of phytochemicals and *in vitro* antioxidant activity of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*

Keywords: Antioxidants, Endophytic fungi, Phytochemicals, *Chaetomium globosum*, *Cladosporium tenuissimum*, *Penicillium janthinellum*

1. Introduction

Medicinal plants have long been used for the treatment of various diseases and they provide a special environment for endophytes. In a microbe plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antibacterial, antifungal, antibiotic, antitumor, antioxidant and anti-inflammatory. Fungal endophytes associated with higher plants appear to be a good source of novel antioxidants as well as plants. Based on the medicinal importance and availability, the medicinal plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda* were selected for the isolation of endophytic fungi. The leaves of selected medicinal plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda* were collected from Narthamalai hills at Pudukkottai District, Tamilnadu, India for the isolation and identification of endophytic fungi. The selected plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda* were found colonized with endophytic fungi. Altogether 56 fungal endophytes belonging to 24 genera were isolated from the leaves of *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda*. Among the 56 fungal endophytes, the *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* were selected and isolated from the leaves of medicinal plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda*, respectively.

Endophytic fungi are microorganisms which live within the plant tissues without causing any noticeable symptoms of disease [1]. Endophytic fungi have a mutualistic relationship with the host, protecting the host against pathogen and in some cases may be an opportunistic pathogen [2]. Endophytic fungi that are residing asymptotically in internal tissues of all higher plants are of growing interest as promising sources of biologically active agents. Endophytic fungi are one of the most creative groups of secondary metabolite producers that play important biological

roles for human life. It also considers and their medicinal applications especially in the production of anticancer, antimicrobial, antioxidant, and antiviral compounds. So, the endophytic fungi from medicinal plants have received much attention in recent years as they are excellent source of biologically active compounds. The compounds isolated and characterized from endophytic fungi have potential for use in modern medicine. The presence of novel secondary metabolites in endophytic fungi and some of which possess biological activities were reported [3].

Endophytic fungi have been found in nearly all plant families examined to date. A large number of secondary metabolites have been extracted, isolated and characterized from endophytic microbes [4]. Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of the medicines used in industrialized countries is derived directly or indirectly from plants [5].

Free radicals are generated by metabolic pathways within body tissues. They introduced by external sources, like food, drugs and environmental pollution etc. Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide and peroxy nitrite radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases [6]. In old age, the rate of production of free radicals is more dominant than its removal, leading to peroxidation of all macromolecules including lipids, proteins and nucleic acids. Many metabolic disorders like diabetes, atherosclerosis, arthritis, nervous diseases etc are free radical mediated diseases. Oxidative stress or excessive production of reactive oxygen species (ROS) is being implicated in many diseases such as cancer, atherosclerosis, ageing and diabetics [6]. Free radicals are often generated as by-products of biological reactions or from exogenous factors. A potent scavenger of free radicals may serve as a possible preventive intervention for the diseases [7]. Antioxidants may protect the body against ROS toxicity either by reducing the formation of ROS, by converting them to less reactive molecules or by scavenging the reactive metabolites [8]. The natural antioxidants were characterized from endophytic fungi.

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. The number of studies on the antioxidant properties of specific plant foods and their phenolic constituents has become very impressive [9]. Antioxidants are radical scavengers which protect the human body against free radicals that may cause pathological

conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neuro-degeneration, parkinson's diseases, mongolism, ageing process and perhaps dementias [10]. In the last few years, there has been a growing interest in providing natural antioxidants.

The importance of compounds bearing antioxidant activity lies in the fact that they are highly effective against damage caused by reactive oxygen species (ROS) and oxygen derived free radicals, which contribute to a variety of pathological effects such as DNA damages, carcinogenesis, and cellular degeneration [11]. Antioxidants have been considered promising therapy for prevention and treatment of ROS linked diseases as cancer, cardiovascular disease, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative diseases (Alzheimer and Parkinson diseases), rheumatoid arthritis, and ageing [12].

Phenolics have potential health benefits mainly due to their antioxidant properties such as reactive oxygen species (ROS) scavenging and inhibition, electrophile scavenging and metal chelation [13]. Epidemiological studies support a relationship between the consumption of phenolic rich food products and a low incidence of coronary diseases [14]. The chemistry and nutritional properties of phenolic compounds have been extensively reviewed [15]. Flavonoids have numerous medicinal effects, including antioxidant, vasoprotective, anti-inflammatory, antiviral, antibacterial, and antitumor [16]. Several investigations have determined correlations between flavonoid structural features and antioxidative free radical scavenging activities [17, 18]. Flavonoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties.

Endophytes are the chemical synthesizers inside plants [19]. Many of them are capable of synthesizing bioactive compounds that can be used as potential sources of pharmaceutical leads. Endophytic fungi have been proven useful for novel drug discovery as suggested by the chemical diversity of their secondary metabolites. The researchers reviewed the comprehensive information on compounds from endophytic fungi, together with the botany, phytochemistry, pharmacology and toxicology, and discussed the possible trends and the scope for future research on endophytic fungi [20, 21]. It is well established that compounds isolated and characterized from endophytic fungi have potential for use in modern medicine, agriculture and industry. In traditional medicine, the plants are assumed to have some healing power that may be due to unknown bioactive compounds within the plant tissues [22].

So, the present study has been designed to investigate the phytochemicals and *in vitro* antioxidant potential of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*.

2. Materials and Methods

2.1 Chemicals

Gallic acid, Quercetin dihydrate, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and 2,2'-Azinobis (3-ethylbenzo thiazoline - 6- sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich Chemicals Co. St Louis, Mo, USA. All other chemicals and reagents were of analytical grade and were purchased from standard chemical companies.

2.2 Collection of plant material

Mature healthy and disease free leaves of medicinal plants *Justicia adhatoda*, *Memecylon edule* and *Passiflora foetida* were collected from the natural habitats of Narthamalai hills at Pudukkottai District, Tamilnadu, India during the months of January and February 2014. The collected plant was identified by Rev. Dr. S. John Britto, Director, Rapinet Herbarium and Centre for Molecular Systematics, St. Joseph's College, Tiruchirappalli, Tamilnadu, India. The leaf samples from each plant were placed separately in sterile polythene bags and stored in an icebox. The stored samples in chilled condition were used for the isolation of endophytic fungi within 48 h of collection.

2.3 Isolation of endophytic fungi

Isolation of endophytic fungi was carried out by the modified method of Hallman et al. [23]. The collected leaf samples were washed with mild detergent and thoroughly with running tap water to remove the soil particles and adhered debris and then finally washed with sterile distilled water. The leaf samples were subjected to surface sterilization with 70% ethanol for one minute. For further surface sterilization and to remove adhering microorganisms, the leaf samples were immersed in 4% sodium hypochlorite for 3 minutes. The leaf samples were then rinsed with 70% ethanol for one minute. Finally the leaf samples were rinsed with deionised water and blot dried on sterile tissue paper. The leaf samples were cut into 5-10 x 5-10 mm in size using a sterile scalpel. The leaf explants were cultured in petridishes containing Potato Dextrose Agar (PDA) medium supplemented with 100µg/mL of streptomycin to suppress bacterial growth. Petridishes were sealed with parafilm and incubated at 27 ± 2 °C for 15 days under dark

condition and monitored every day. Fungi growing out of the plant explants were subcultured on separate PDA plates and maintained at 4 °C. Fungi were identified in their sporulation state by staining with Lactophenol blue. The fungal isolates were identified based on the colony colour, morphology, hyphal structure, spore size and spore bearing structures and compared with standard manuals of endophytic fungi [24, 25, 26, 27].

2.4 Mass culture of endophytic fungi

The fungal endophytes *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* were mass cultured by placing agar blocks of actively growing pure culture (5mm in diameter) in 250ml Erlenmeyer flasks containing 100ml of potato dextrose broth. The flasks were incubated at 27 ± 2 °C for 14 days with periodical shaking at 150 rpm. After incubation period, the cultures were taken out and used for further study.

2.5 Preparation of fungal extracts

The fungal extracts were prepared according to the modified method of Raviraja *et al.* [28]. After mass cultivation of endophytic fungi, the cultures were filtered through four layers of sterile cheese cloth to separate the mycelial mats. Then the culture filtrate was extracted with equal volume of the filtrate and solvents such as acetone, benzene and ethyl acetate were taken individually in separating funnels and shaken vigorously for 15 min. The solutions were then allowed to stand, the cell mass got separated and the organic phase of solvents so obtained, were collected. The solvents acetone, benzene and ethyl acetate were evaporated and the resultant residue was dried in vacuum evaporator to yield the crude extract (Culture filtrate extract). Mycelial mats were also used for the preparation of acetone, benzene and ethyl acetate extracts followed the same procedure by taking mats instead of filtrate (Mycelial mat extract). After evaporation, the dried extracts were stored at 4 °C until the time of further use.

2.6 Phytochemical analysis - Qualitative methods

Phytochemical analysis for major phytoconstituents of the endophytic fungi was undertaken using standard qualitative methods as described by various authors [29, 30, 31, 32]. The extracts of endophytic fungi were screened for the presence of biologically active compounds such as carbohydrates, proteins, steroids, terpenoids, phenolic compounds, flavonoids, alkaloids, tannins, saponins and anthroquinones.

2.6.1 Carbohydrates

Fehling's test

To 2 ml of endophytic fungal extract, 1 ml of a mixture of equal parts of Fehling's solution 'A' and 'B' was added. The contents were boiled for a few minutes. The formation of a red or brick red precipitate indicates the presence of carbohydrates.

Benedict's test

To 0.5 ml of endophytic fungal extract, 5 ml of Benedict's reagent was added and boiled for 5 minutes. The formation of a bluish green colour showed the presence of carbohydrates.

2.6.2 Proteins

Millon's test

To a small quantity of endophytic fungal extract, 5-6 drops of Millon's reagent was added. A white precipitate which turns red on heating indicates the presence of proteins.

2.6.3 Steroids

Two ml of acetic anhydride was added to 0.5 g of endophytic fungal extract with 2 ml of H_2SO_4 . The colour changed from violet to blue or green, which indicates the presence of steroids.

2.6.4 Terpenoids

Salkowski test

Five ml of endophytic fungal extract was mixed with 2 ml of chloroform and 3 ml of concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration in the interface was formed which showed the positive results for the presence of terpenoids.

2.6.5 Phenolic compounds

Ferric chloride test

To 1 ml of endophytic fungal extract, 2 ml of distilled water followed by a few drops of 10% aqueous $FeCl_3$ solution were added. The formation of a blue or green precipitate indicates the presence of phenolic compounds.

Lead acetate test

1 ml of endophytic fungal extract was diluted to 5 ml with distilled water and to this a few drops of 1% aqueous solution of lead acetate was added. A yellow precipitate indicates the presence of phenols.

Liebermann's test

A small amount of endophytic fungal extract was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution.

2.6.6 Flavonoids

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

2.6.7 Alkaloids

Dragendroff's test

To 0.5 ml of endophytic fungal extract was added to 2ml of HCl. To this acidic medium 1 ml of Dragendroff's reagent was added. An orange red precipitate was produced immediately, which indicates the presence of alkaloids.

Wagner's test

10 ml of endophytic fungal extract was acidified by adding 1.5% v/v HCl and a few drops of Wagner's reagent. The formation of a yellowish brown precipitate confirmed the presence of alkaloids.

Meyer's test

To 1 ml of endophytic fungal extract, a few drops of Meyer's reagent were added. The formation of a pale white precipitate showed the presence of alkaloids.

2.6.8 Tannins

Ferric chloride test

To 1-2 ml of endophytic fungal extract, a few drops of 5% aqueous $FeCl_3$ solution were added. A bluish black colour, which disappears on addition of a few ml of dilute H_2SO_4 was followed by the formation of a yellowish brown precipitate.

Lead acetate test

In a test tube containing about 5 ml of endophytic fungal extract, a few drops of 1% solution of lead acetate was added. A yellow or red precipitate was formed, indicating the presence of tannins.

2.6.9 Saponins

In a test tube containing about 5 ml of endophytic fungal extract, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. A honey comb like froth shows the presence of saponins.

2.6.10 Anthraquinones

Five ml of the endophytic fungal extract was hydrolysed with concentrated H_2SO_4 and 1 ml of dilute ammonia was added to it. Formation of rose pink coloration suggests that positive response for anthraquinones.

2.7 Phytochemical analysis - Quantitative methods

2.7.1 Estimation of phenolic compounds

The content of total phenolic compounds in endophytic fungal extracts was determined by Folin–Ciocalteu method [33]. For the preparation of calibration curve 1 ml aliquots of 0.024, 0.075, 0.105 and 0.3 mg/ml ethanolic gallic acid solutions were mixed with 5 ml of Folin–Ciocalteu reagent and 4 ml (75 g/l) of sodium carbonate. The absorption was read after 30 min at 765 nm at 20°C and the calibration curve was drawn. 1 ml of endophytic fungal extract (10 g/l) was mixed with the same reagents as described above, and after 1 h the absorption was measured for the determination of phenolic compounds. All determinations were performed in triplicate. The total content of phenolic compounds in endophytic fungi extracts in gallic acid equivalents (GAE) was calculated by the following formula:

Total phenolic content (mg/g endophytic fungal extract in GAE) =

$$\frac{\text{Concentration of galic acid established from the calibration curve (mg/ml)} \times \text{Volume of extract (ml)}}{\text{Weight of endophytic fungal extract (g)}}$$

2.7.2 Estimation of flavonoids

The content of flavonoids in the extracts of endophytic fungi was determined by spectrophotometrically using quercetin as a reference compound [34]. One ml of endophytic fungal extract in methanol (10 g/l) was mixed with 1 ml of aluminium trichloride in ethanol (20 g/l) and diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min at 20°C. Blank sample was prepared from 1 ml of endophytic fungal extract and 1 drop of acetic acid, and diluted to 25 ml. The absorption of quercetin solutions was measured under the same conditions. Standard quercetin solution was prepared from 0.05 g quercetin (0.05g/l). All determinations were carried out in triplicate. The amount of flavonoids in endophytic fungi extracts in quercetin equivalents (QE) was calculated by the following formula:
Flavonoid content (mg/g endophytic fungal extract in QE) =

$$\frac{\text{Absorption of endophytic fungal extract solution} \times \text{Weight of quercetin in the solution (g)}}{\text{Absorption of standard quercetin solution} \times \text{Weight of endophytic fungi extract (g)}} \times 10$$

2.8 Evaluation of antioxidant activity

2.8.1 DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of the corresponding endophytic fungi extracts were measured from the bleaching of the

purple-coloured methanol solution of 1,1-Diphenyl-2-picrylhydrazyl (DPPH). In this spectrophotometric assay the stable radical DPPH was used as a reagent [35, 36]. Ascorbic acid standard was prepared in deionised water over the range of 11-88 µg/ml (0.063-0.5 mM). Endophytic fungi extracts were prepared in different concentrations (50-200 µg/ml) in 80% methanol. 4 ml of methanolic solution of DPPH (0.004mM) were added to 1 ml of various concentrations of extract/standard solutions. 1 ml of deionised water/methanol serves as control. The tubes were incubated at room temperature in dark for 20 minutes. After incubation period the absorbance was read at 517 nm. Endophytic fungal extract concentration providing 50% inhibition (IC₅₀) was calculated from the plot of inhibition (%) against extract concentration. Tests were carried out in triplicate. Ascorbic acid standard was used for comparison. Radical scavenging activity is expressed as the inhibition percentage (IC₅₀) of endophytic fungal extract in µg concentration. Radical scavenging activity was calculated by the following formula:

% Inhibition of DPPH radical =

$$\frac{\text{Absorption of blank sample (0 minutes)} - \text{Absorption of tested extract solution (15 minutes)}}{\text{Absorption of blank sample (0 minutes)}} \times 100$$

IC₅₀ is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

2.8.2 ABTS radical cation decolourisation assay

The ABTS radical cation decolourisation assay assesses the total radical scavenging capacity based on the ability of a compound to scavenge the stable 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical in 6 min [37]. For the total antioxidant assay, ABTS was dissolved in deionised water to a 7mM concentration. The ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with a 2.45 mM potassium persulfate (final concentration) and incubating the solution in the dark at room temperature for 12-16 h before use. The radical stock solution was diluted with a 5 mM solution of phosphate-buffered saline (PBS; pH 7.4) to obtain a spectrophotometric absorbance value of 0.700 at 734 nm. Ascorbic acid standard was prepared in deionised water over the range of 0.063-0.5 mM (11-88 µg/ml). Endophytic fungi extracts were prepared in different concentrations (75-200 µg/ml) in methanol. To 40 µl of extract/standard solution, 1.96 ml of ABTS⁺ solution was added and the tubes were kept in darkness for 6 min and read at 734

nm. This was compared to a control where 40 μ l of the solvent was added to 1.96 ml of ABTS⁺ solution. Assays were performed in triplicate. Antioxidant activity is expressed based on the percentage of ABTS radical reduction.

% Inhibition of ABTS cation radical =

$$\frac{\text{Absorption of blank sample (0 minutes)} - \text{Absorption of tested extract solution (6 minutes)}}{\text{Absorption of blank sample (0 minutes)}} \times 100$$

2.9 Statistical analysis

The results of this study were subjected to statistical analysis and the results were expressed as mean \pm standard deviation of triplicates.

3. Results and Discussion

The endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* were isolated from the leaves of medicinal plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda*, respectively. The ethyl acetate extracts of culture filtrate and mycelial mat of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* were subjected to qualitative phytochemical analysis such as carbohydrates, proteins, steroids, terpenoids, phenolic compounds, flavonoids, alkaloids, tannins, saponins and anthroquinones. The availability of different approaches for the discovery of therapeutically important natural products still remains one of the best reservoirs of new structural types. The phytochemical tests of ethyl acetate extracts of culture filtrate and mycelial mat of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* indicated that the presence of carbohydrates, proteins, steroids, terpenoids, phenolic compounds, flavonoids, alkaloids, tannins, saponins and anthroquinones (Table 1).

The presence of phytochemicals in endophytic fungi is an indicator that they can be potential source of precursors in the development of synthetic drugs. Yu et al. [38] published a remarkable review of antimicrobial metabolites isolated from endophytes and belong to alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids. The major natural products of secondary metabolites in plants and fungi are phenolic compounds. The presence of phenolic compounds [39] in endophytes was reported. In this study, the preliminary phytochemical screening has indicated the presence of bioactive compounds in endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium*

janthinellum. Endophytic fungi, a potential source of medicinal compounds, and they are reported that special ecoenvironmental microorganisms may produce special activated metabolites [2].

The total content of phenolic compounds and flavonoids were determined through spectrophotometric analysis in ethyl acetate extracts of culture filtrate and mycelial mat of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* (Table 2). The phenolic compounds (mg/g) are expressed in gallic acid equivalents (GAE), varying between 13.82 \pm 0.82 mg/g and 24.14 \pm 0.46 mg/g (GAE). The highest amounts of phenolic compounds were found in ethyl acetate extracts of mycelial mat of *Penicillium janthinellum* 24.14 \pm 0.46 mg/g and mycelial mat of *Chaetomium globosum* 20.87 \pm 0.78 mg/g followed by mycelial mat of *Cladosporium tenuissimum* 17.36 \pm 0.64 mg/g and culture filtrate of *Penicillium janthinellum* 16.27 \pm 0.47 mg/g, *Chaetomium globosum* 15.57 \pm 0.65 mg/g and *Cladosporium tenuissimum* 13.82 \pm 0.82 mg/g in gallic acid equivalents (GAE).

The amount of flavonoids (mg/g) in quercetin equivalents (QE) varied from 4.32 \pm 0.46 to 7.54 \pm 0.98 mg/g (QE) in ethyl acetate extracts of culture filtrate and mycelial mat of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The highest amount of flavonoids were found in mycelial mat of *Penicillium janthinellum* 7.54 \pm 0.98 mg/g, *Chaetomium globosum* 6.95 \pm 0.43 mg/g and *Cladosporium tenuissimum* 6.14 \pm 0.68 mg/g (QE). The amount of flavonoids were also found in the culture filtrate of *Penicillium janthinellum* 5.48 \pm 0.38 mg/g, *Chaetomium globosum* 4.96 \pm 0.32 mg/g and *Cladosporium tenuissimum* 4.32 \pm 0.46 mg/g (QE), which were lower in amount than in the mycelial mat extracts. The present study results showed that phenolic compounds and flavonoids were found in different quantities in *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*.

The *in vitro* antioxidant activity of the ethyl acetate extracts of culture filtrate, mycelial mat and the mixture of culture filtrate and mycelial mat (1:1) of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* was analyzed by the DPPH radical scavenging and ABTS assays. The results of DPPH[•] and ABTS^{•+} inhibition by ethyl acetate extracts of culture filtrate, mycelial mat and the mixture of culture filtrate and mycelial mat (1:1) of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* are summarized in Table 3 and 4. The ethyl acetate extracts of culture filtrate, mycelial

mat and the mixture of culture filtrate and mycelial mat (1:1) of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* are almost completely inhibited DPPH absorption (90%). These percentages can be considered as a full absorption inhibition of DPPH, because after completing the reaction the final solution always possesses some yellowish colour and therefore its absorption inhibition compared to colourless methanol solution cannot reach 100%. Permanent residual absorption result is upto 7% of total absorption inhibition.

The extracts of mixture of culture filtrate and mycelial mat (1:1) of *Penicillium janthinellum* showed the best radical scavenging activity with the IC_{50} values of $238.64 \pm 10.36 \mu\text{g/ml}$. The culture filtrate and mycelial mat of *Penicillium janthinellum* also showed good radical scavenging activity individually with the IC_{50} values of $344.64 \pm 20.68 \mu\text{g/ml}$ and $296.64 \pm 16.34 \mu\text{g/ml}$, respectively. The extracts of mixture of culture filtrate and mycelial mat (1:1) of *Chaetomium globosum* and *Cladosporium tenuissimum* showed the radical scavenging activity with the IC_{50} values of $276.54 \pm 18.62 \mu\text{g/ml}$ and $296.64 \pm 10.78 \mu\text{g/ml}$, respectively. The culture filtrate and mycelial mat of *Chaetomium globosum* showed good radical scavenging activity individually with the IC_{50} values of $368.88 \pm 28.56 \mu\text{g/ml}$ and $324.36 \pm 18.54 \mu\text{g/ml}$, respectively. The culture filtrate and mycelial mat of *Cladosporium tenuissimum* showed good radical scavenging activity individually with the IC_{50} values of $396.84 \pm 22.42 \mu\text{g/ml}$ and $346.88 \pm 26.48 \mu\text{g/ml}$, respectively. The *Chaetomium globosum* and *Cladosporium tenuissimum* had considerably less radical scavenging activity when compared to *Penicillium janthinellum* extracts, which may be due to the presence of lower quantity of radical scavenging phenolic compounds and flavonoids.

The extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* exhibited antioxidant activity with IC_{50} values were compared to the IC_{50} value of ascorbic acid. The ethyl acetate extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed increased level of antioxidant activity with increasing the concentration of the extracts. Even though the DPPH scavenging aptitude of the extracts was found to be lower than that of the standard antioxidant ascorbic acid. Thus this study suggests that the extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* have antioxidant properties.

Antioxidants are compounds that inhibit or delay the oxidation process by preventing the initiation or propagation of oxidizing chain reactions. A number of methods are available for the determination of free radical scavenging activity but the assay using stable 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the utmost attention owing to the ease of use and its convenience [40]. Ascorbic acid was chosen as the standard antioxidant for the antioxidant study. The DPPH radical contains an odd electron, which is accountable for the absorbance and also for a visible deep purple color. DPPH is decolorized when it accepts an electron donated by an antioxidant compound, which can be quantitatively measured from the changes in absorbance.

The observed colour change from purple to yellow may be due to radical was scavenged by antioxidants in the endophytic fungi extracts through donation of hydrogen and stable DPPH. H was produced. The antioxidant activity of phenolic compounds is mainly due to their reduction and oxidation properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [41]. The DPPH radical scavenging method is a standard procedure applied to evaluate the anti-radical activity [42]. DPPH is a stable free radical having a maximum absorption at 517 nm. In the presence of phenolic compounds in the extracts capable of donating an H atom or an electron, its free radical nature gets neutralized as seen by decrease in absorption at 517nm. The decrease of DPPH absorption in varying concentrations (100-1000 $\mu\text{g/ml}$) of the extracts have been monitored and found that the absorbance due to DPPH decreases continuously upto 800 $\mu\text{g/ml}$ and further increases in the concentration did not change the absorbance. The concentration at which it scavenges 50% of DPPH radical is given as IC_{50} value of the extract.

ABTS radical cation decolourisation assay of the ethyl acetate extracts of culture filtrate, mycelial mat and the mixture of culture filtrate and mycelial mat (1:1) of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed more or less similar results compared to those obtained in the DPPH radical scavenging assay (Table 4). Three different concentrations like 250, 500 and 1000 $\mu\text{g/ml}$ of ethyl acetate extracts of culture filtrate, mycelial mat and the mixture of culture filtrate and mycelial mat (1:1) of endophytic fungi were used in the ABTS absorption inhibition (%) study. The ethyl acetate extracts of culture filtrate of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by $38.98 \pm 2.64\%$,

37.56 ± 2.38% and 43.68 ± 3.24%, respectively at the concentration of 250µg/ml. The ethyl acetate extracts of mycelial mat of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by 45.79 ± 2.48%, 43.96 ± 2.36% and 48.65 ± 3.58%, respectively at the concentration of 250µg/ml. The selected endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* at 250µg/ml ethyl acetate extracts of mixture of culture filtrate and mycelial mat (1:1) had absorption inhibition (%) activity 47.24 ± 3.18%, 45.68 ± 2.82% and 51.86 ± 2.42%, respectively.

The ethyl acetate extracts of culture filtrate of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by 74.65 ± 2.66%, 72.54 ± 2.48% and 77.34 ± 3.52%, respectively at the concentration of 500µg/ml. The ethyl acetate extracts of mycelial mat of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by 79.62 ± 3.62%, 74.42 ± 2.26% and 82.98 ± 2.85%, respectively at the concentration of 500µg/ml. The selected endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* at 500µg/ml ethyl acetate extracts of the mixture of culture filtrate and mycelial mat (1:1) had absorption inhibition (%) activity 81.65 ± 3.56%, 78.42 ± 2.88% and 87.64 ± 3.78%, respectively.

The ethyl acetate extracts of culture filtrate of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by 78.62 ± 2.32%, 76.84 ± 2.64% and 82.28 ± 3.84%, respectively at the concentration of 1000µg/ml. The ethyl acetate extracts of mycelial mat of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* showed percentage of ABTS absorption inhibition activity, i.e by 85.46 ± 2.48%, 81.56 ± 3.46% and 88.68 ± 3.36%, respectively at the concentration of 1000µg/ml. The extracts of mixture of culture filtrate and mycelial mat (1:1) of *Penicillium janthinellum* showed maximum percentage of ABTS absorption inhibition activity, i.e by 90.86 ± 3.46% followed by *Chaetomium globosum* and *Cladosporium tenuissimum*, i.e by 87.24 ± 2.28% and 84.94 ± 2.64%, respectively at the concentration of 1000µg/ml. The standard ascorbic acid showed 94.22 ± 2.68% scavenged ABTS^{•+} activity. The 1000µg/ml ethyl acetate extracts of mixture of culture filtrate and mycelial mat (1:1) had more absorption inhibition (%) activity than

the other concentrations like 250 and 500 µg/ml in all three selected endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The extracts of *Penicillium janthinellum* were the most active, and exhibited fully scavenged ABTS^{•+} compared to the other extracts of *Chaetomium globosum* and *Cladosporium tenuissimum*. Similarly, the researchers were reported that the phytochemicals pestacin and isopestacin are displaying potent antioxidant activity and have been obtained from culture of endophytic fungus *Pestalotiopsis microspora* isolated from host plant *Terminalia morobensis* [43].

In the present study, the culture filtrate and mycelial mat of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* have been shown that they can be used as effective antioxidants. They are excellent free radical scavengers, because of the presence of well known antioxidants like phenolic compounds and flavonoids as confirmed by this study. Several phytochemicals like phenolic compounds, flavonoids, alkaloids, tannins and saponins were known to possess potent antioxidant activity [44]. The content of phenolic compounds and flavonoids with the antioxidant properties of some medicinal and aromatic plant extracts were reported [45].

The observed antioxidant activity in the present study may be due to the presence of phenolic compounds, flavonoids, alkaloids, tannins and saponins. So, the phenolic compounds may contribute to the radical scavenging activity of these endophytic fungal extracts. It is suspected that the presence of these potent antioxidants either in free or bound form in the extracts may be responsible for the overall antiradical and antioxidant activities of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. Phenolic compounds and flavonoids possess a diverse range of beneficial biological activities. Flavonoids have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive species. Tannins also exhibit strong antibacterial, antiulcer, anti-inflammatory, antileishmanial, antimutagenic and apoptotic activities. Antioxidants are known to protect the body against free radical-mediated toxicities. Relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of the different plant extracts. A large number of plants have revealed potent antioxidant activities [46].

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and nitric oxide, may cause disruption of membrane fluidity, protein denaturation and lipid peroxidation by creating

oxidative stress, which could lead to cell injury and death. Under normal conditions, naturally occurring antioxidant enzymes in the body can counteract the cellular effects of ROS. For the reduction of the harmful effect of ROS to the human body, sufficient amounts of exogenous antioxidants are required. So, there is an overwhelming trend to search for naturally occurring antioxidants in the past decades. A number of bioactive compounds with antimicrobial, antitumor, antiinflammatory, and antiviral activities have been previously isolated from endophytes [47].

The antioxidants have different functional properties, such as reactive oxygen species scavenging, inhibition of the generation of free radicals and chain breaking activity and metal chelation. Polyphenolic compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity. Phenolic compounds have also inhibitory effects on mutagenesis and carcinogenesis in humans daily ingested from a diet rich in fruits and vegetables [48]. Many flavonoids have shown strong antioxidant properties and quercetin has been established as a strong antioxidant principle and had been used as standard in antioxidant experiments [49].

Plants containing flavonoids have been reported to possess antioxidant properties [50]. It was reported that flavonoids could remove the $O_2^{\cdot-}$ in human bodies, strengthen the natural disease-fighting system, improve blood circulation and lower blood pressure [51]. Flavonoids are one of the most diverse and widespread groups of natural compounds possess a broad spectrum of radical scavenging properties. There are observed different concentration of phenolic compounds and flavonoids in *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The variability of the phenolic compounds and flavonoids concentration led us to conclude that there will be a corresponding variation in the biological activity. Similarly, the antioxidant capacities of the endophytic fungal extracts were correlated with their total phenolic contents and suggested that phenolics were also the major antioxidant constituents of the endophytes [52].

Suryanarayanan et al. [53] discussed many fungal secondary metabolites with various chemical structures and their wide ranging biological activities and it reflects the high synthetic capability of fungi. About 1500 fungal metabolites had been reported to show antitumor and antibiotic activity [54] and some have been approved as drugs. Similarly a number of novel bioactive compounds were isolated from

endophytic fungi [55, 56]. Reports available on endophytic fungi with novel and bioactive compounds are obtained from medicinal plants [3]. Similar results were observed with *Penicillium* sp isolated from *Centella asiatica* [57]. The present study suggests that phenolics and flavonoides could play an important role in antioxidant properties of ethyl acetate extracts of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The antioxidant properties of the three different endophytic fungi extracts varied widely and it may be due to the variations in the phenolic compounds and flavonoids in the ethyl acetate extracts of *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*. The results of the present study are similar to those in previous report and indicate that endophytic fungi may serve as a potential source of antioxidants [58, 43]. This may be the first report on the *in vitro* antioxidant activity of ethyl acetate extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* isolated from the leaves of medicinal plants *Passiflora foetida*, *Memecylon edule* and *Justicia adhatoda*, respectively.

Table 1. Phytochemical analysis of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*

Name of the phytochemicals	<i>Chaetomium globosum</i>		<i>Cladosporium tenuissimum</i>		<i>Penicillium janthinellum</i>	
	Mycelial mat	Culture filtrate	Mycelial mat	Culture filtrate	Mycelial mat	Culture filtrate
Carbohydrates	+	+	+	+	+	+
Proteins	+	+	+	+	+	+
Steroids	+	-	+	-	+	-
Terpenoids	+	-	+	-	+	-
Phenolic compounds	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Anthraquinones	-	-	-	-	-	-

+ Present - Absent

Table 2. The content of total phenolic compounds and flavonoids in endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum*

Name of the endophytic fungi	Name of the extracts	Name of the phytochemicals	
		Phenolic compounds (mg/g fungal extract in gallic acid equivalents)	Flavonoids (mg/g fungal extract in quercetin equivalents)
<i>Chaetomium globosum</i>	Mycelial mat	20.87±0.78	6.95±0.43
	Culture filtrate	15.57±0.65	4.96±0.32
<i>Cladosporium tenuissimum</i>	Mycelial mat	17.36±0.64	6.14±0.68
	Culture filtrate	13.82±0.82	4.32±0.46
<i>Penicillium janthinellum</i>	Mycelial mat	24.14±0.46	7.54±0.98
	Culture filtrate	16.27±0.47	5.48±0.38

Values are expressed as mean± SD of three determinations

Table 3. *In vitro* free radical scavenging effect (IC₅₀) of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* using DPPH radical scavenging assay

Name of the endophytic fungi	Name of the extracts	IC ₅₀ (µg/ml)
<i>Chaetomium globosum</i>	Culture filtrate	368.88 ± 28.56
	Mycelial mat	324.36 ± 18.54
	Culture filtrate + Mycelial mat (1:1)	276.54 ± 18.62
<i>Cladosporium tenuissimum</i>	Culture filtrate	396.84 ± 22.42
	Mycelial mat	346.88 ± 26.48
	Culture filtrate + Mycelial mat (1:1)	296.64 ± 10.78
<i>Penicillium janthinellum</i>	Culture filtrate	344.64 ± 20.68
	Mycelial mat	296.64 ± 16.34
	Culture filtrate + Mycelial mat (1:1)	238.64 ± 10.36
	Vitamin C	38.46 ± 1.36

Each assay was performed in triplicate

Vitamin C was used as a reference compound of this experiment

The IC₅₀ value is defined as the amount of extract necessary to decrease the initial DPPH radical concentration by 50%

Table 4. *In vitro* free radical scavenging activity of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* using ABTS radical cation decolourisation assay

Name of the endophytic fungi	Name of the extracts	ABTS absorption inhibition (%) of endophytic fungi extracts at different concentrations		
		250 µg/ml	500 µg/ml	1000 µg/ml
<i>Chaetomium globosum</i>	Culture filtrate	38.98 ± 2.64	74.65 ± 2.66	78.62 ± 2.32
	Mycelial mat	45.79 ± 2.48	79.62 ± 3.62	85.46 ± 2.48
	Culture filtrate + Mycelial mat (1:1)	47.24 ± 3.18	81.65 ± 3.56	87.24 ± 2.28
<i>Cladosporium tenuissimum</i>	Culture filtrate	37.56 ± 2.38	72.54 ± 2.48	76.84 ± 2.64
	Mycelial mat	43.96 ± 2.36	74.42 ± 2.26	81.56 ± 3.46
	Culture filtrate + Mycelial mat (1:1)	45.68 ± 2.82	78.42 ± 2.88	84.94 ± 2.64
<i>Penicillium janthinellum</i>	Culture filtrate	43.68 ± 3.24	77.34 ± 3.52	82.28 ± 3.84
	Mycelial mat	48.65 ± 3.58	82.98 ± 2.85	88.68 ± 3.36
	Culture filtrate + Mycelial mat (1:1)	51.86 ± 2.42	87.64 ± 3.78	90.86 ± 3.46
	Vitamin C		94.22 ± 2.68	

Each assay was performed in triplicate

Conclusion

The present study concluded that the presence of bioactive compounds in the extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* which may be responsible for antioxidant activity. Furthermore, the study on isolation and identification of active compounds in the extracts of endophytic fungi *Chaetomium globosum*, *Cladosporium tenuissimum* and *Penicillium janthinellum* and the correlation between the compounds of plant and the endophytes are needed and they may provide a

better source for developing new therapeutic agents as potential natural antioxidants. It is also hoped that this study would lead to the establishment of some bioactive compounds from endophytic fungi.

Conflict of Interests

The authors have declared that there is no conflicts of interests exist.

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