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Evaluation of Antioxidant Potential, DNA Damage Protection and Anticancer Activities of Three Endophytic Fungi Associated with Selected Medicinal Plants

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Abstract

Background: Endophytic fungi refer to such microbes that colonize inside the plant tissues without causing any disease/symptoms. Endophytic fungi associated with medicinal plants often are reported to be an interesting source of bioactive compounds. In this study, we selected three potential endophytic fungi viz. *Daldinia eschscholtzii* (TP2-6), *Daldinia placentiformis* (CP2-11) and *Alternaria alternata* (CPR-9) to evaluate their antioxidant and anticancer potential. **Objective:** The aim of this study was to assess the polyphenol contents, antioxidant capacity of selected endophytic fungi isolated from leaves of *Carica papaya* and *Tridax procumbens* using different assays. Furthermore, DNA damage protection ability and anticancer potential against human cancer cell lines was also investigated. **Method:** Secondary metabolites present in the crude extracts, obtained through ethyl acetate extraction of 20 different endophytic fungi were screened for the presence of phyto-constituents, Total phenolic content (TFC), Total flavonoid content (TFC) and antioxidant potential using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, 2,2'-azinobis(3-ethylbenzothiazoline- 6-sulfonic acid) di-ammonium salt (ABTS) assay, nitric oxide (NO) radical scavenging activity, hydrogen peroxide (H_2O_2) free radical scavenging assay, reducing power assay, DNA damage protection and anticancer potential. **Results:** Phenols, tannins, cardic glycosides, saponins, alkaloids and flavonoids were the main phyto-constituents present in the endophytic fungi. Of the 3 endophytic fungi, *Daldinia placentiformis* (CP2-11) showed the highest TPC and TFC with a value of 118.64±4.61µg GAE (gallic acid equivalent) /mL of crude and 164.87±6.58 µg QE (quercetin) /mL of crude, respectively, while its antioxidant activity for DPPH free radical scavenging assay was 85.02±0.87%. A high positive linear correlation was found between the IC_{50} values of antioxidant activities and phenolic and flavonoid contents r = 0.9158; R^2 = 0.8388 and r = 0.9886; R^2 = 0.9775. Endophytic fungal isolates, *Daldinia placentiformis* (CP2-11) and *Daldinia eschscholtzii* (TP2-6) showed significant DNA damage protection and antioxidant activity in different assays. **Conclusion:** The present study suggests that endophytic fungi associated with medicinal plants could be a potential source of novel natural antioxidant and anticancer compounds.

Keywords
Cell cytotoxicity; DNA damage: Endophytic fungi; Polyphenol content; Secondary metabolites

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INTRODUCTION

Endophytes are mutualistic microbes that dwell in the internal plant tissues without causing any apparent harm to their host (Hirsch and Braun, 1992). These endosymbiotic fungi are ubiquitous, polyphyletic groups of diverse fungi, found to synthesis bioactive secondary metabolites (Schulz et al., 2002; Strobel, 2003) such as phenols, flavonoids, steroids, saponins, tannins and terpenoids which in turn protect the host from various diseases, parasitic infections, insect attack and herbivory (Aly et al. 2010).

Since the symbiosis of endophytes is with the higher plants, which represent a eukaryotic system, the secondary metabolites produced by endophytes may have reduced cell toxicity. Thus, the host itself may act as a selection system that directs microbes to synthesize less toxic metabolites for higher organisms (Aly et al. 2010). Among the endophytic fungi and their hosts, a functional tendency of antioxidant potential exists. Plants possessing medicinal values bestow the beneficial medicinal properties on to endophytic fungi (Strobel et al. 2004). In this study, *Carica papaya* and *Tridax procumbens* were selected as the hosts due to their ethno-botanical importance.

C. papaya leaves are used to treat malaria, dengue, jaundice, etc. They are rich in alkaloids (dehydrocarpaine I & II, carpaine, pseudocarpaine,), flavonoids (myricetin, kaempferol etc.), phenolic compounds (caffeic acid, ferulic acid, chlorogenic acid), the cynogenetic compounds such as benzylglucosinolate etc. Carotenoids namely anthraquinones glycoside, β- carotene, lycopene etc. present in it impart properties such as anti-inflammatory, anti-fertility, abortifacient, hepatoprotective, hypoglycaemic, antihypertensive, wound healing and antitumor activities (Yogiraj et al. 2014). Similarly, *T. procumbens*, has diverse pharmacological properties such as analgesic, anti-oxidant, antidiabetic, antifungal, anti-inflammatory, immunomodulatory, anti-hepatotoxic and antimicrobial activities (Agrawal et al. 2010). Endophytic fungi from mature, green and healthy leaves of *C. papaya* and *T. procumbens* were screened for their antioxidant potential.

Various evidences are increasing and indicating that reactive oxygen species and free radical-meditated reactions lead to oxidative damage to various biomolecules such as DNA, lipids and proteins etc., which may result in atherosclerosis, aging, cancer, heart ailment, diabetes, neurodegenerative disorders, Alzheimer's disease etc. (Halliwell 1994; Finkel and Holbrook 2000). In human body also, free radicals are generated during various biological reactions or due to some exogenous factors. Though human body has many enzyme systems for free radical scavenging, it can be enhanced by exogenous antioxidants.

Antioxidants play a key and effective role in mitigating ROS-mediated tissue damage. This property of free radical scavenging by antioxidants checks cellular damage (Yadav et al. 2014). Antioxidants act by protecting the body against ROS toxicity by precluding ROS, or by scavenging the reactive metabolites or by converting them into less reactive molecules (Sen et al. 1994; Hegde and Joshi 2009). The quest for naturally derived antioxidants is receiving attention in recent years (Schulz et al. 2002; Huang et al. 2007). Recently, endophytic fungi are one of the most explored potential sources that produce novel and important secondary metabolites. Despite that, sparse information is available related to their antioxidant activity (Abirami and Boominathan 2016).

In this study, endophytic fungi associated with medicinal plants were screened for their antioxidant potential and further selected isolates were investigated for their antioxidant potential and anticancer activity. Endophytic fungi were investigated for their phenolic as well as flavonoid compounds along with various phyto-constituents such as phenols, tannins, cardiac glycosides, saponins, alkaloids, etc. Correlation between the IC_{50} values of antioxidant activities and phenolic and flavonoid contents were analyzed. Results in this study show that extracts from endophytes associated with medicinal plants produce alkaloids, phenolic, flavonoids, and saponins etc., which contribute to their antioxidant potential.

MATERIALS AND METHODS

Chemicals and reagents

Gallic acid, 2, 2' - azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium ferricyanide, potassium persulphate, trichloroacetic acid, sodium carbonate, sodium nitrite, aluminium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, Folin-Ciocalteu, ferric chloride, Ascorbic acid, Hydrogen peroxide (30%) - Avra Synthesis Pvt.

Ltd. (India), Dulbecco's Modified Eagle's medium (DMEM) and 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) were supplied by Himedia, Mumbai. Butylated hydroxytoluene (BHT), 2, 2'diphenyl-1-picrylhydrazyl (DPPH) and Quercetin were procured from Sigma Aldrich, India. All the chemicals and reagents used in this study were of analytical grade.

Endophytic fungal isolation

The host plant, viz, *Carica papaya* and *Tridax procumbens* were sampled from the campus of Pondicherry University, India, 12.0219° N, 79.8575° E. Healthy, mature and green leaves from the host plant were collected and surface sterilized (Rashmi et al. 2018). After drying the surface sterilized leaves, they were cut into leaf bits and placed on to petri dishes containing potato dextrose agar (PDA) medium supplemented with antibiotic to restrict bacterial growth. Petri-dishes were incubated at 25±2 °C and were carefully observed for emerging hyphae of endophytic fungi from the margins of leaf bits, starting from second day. To further check the effectiveness of sterilization, sterile leaf imprint was also made on petri dish to check for the trace of epiphytes, if any.

Morphological Identification

The endophytic fungi appearing as emerging hyphae from the leaves were carefully sub-cultured and axenic cultures were obtained by transferring to plates containing appropriate growth media. Pure cultures were further subjected to identification based on axenic culture characteristics and colony morphology with the help of compound microscope and stereo-zoom microscope. Characteristics of the endophytic fungal colonies such as colony appearance (front as well as reverse), texture of colony surface, growth rate, pigmentation produced, conidial formation etc.

Molecular Identification

Apart from morphological observations, the endophytic fungal isolates were also identified on molecular basis. Genomic DNA was isolated from endophytes by using Qiagen, DNeasy Plant Mini Kit. ITS1 (5'-TCC GTA GGTGAA CCT GCG G-3') and ITS 4(5'-TCC TCC GCT TAT TGA TATGC-3') primers were used for amplification of the nuclear ribosomal internal transcribed spacer (ITS) region. The sequencing was outsourced from AgriGenome Labs Pvt. Ltd. The obtained individual ITS sequences were subjected to BLASTN using GenBank database (http://www.ncbi.nlm.nih.gov) (Devadatha et al. 2018) and identification was confirmed.

Crude extract preparation

To obtain endophytic fungal crude extract, ethyl acetate was used as solvent. Axenic endophytic fungal cultures were inoculated in potato dextrose broth (PDB) in Erlenmeyer flask (1000mL). Inoculated flasks were kept under constant shaking condition (100 rpm) at 28±2 °C for two weeks. The broth was filtered by using Whatman no. 1 filter paper and fungal mycelial mass was removed. The fungal broth was extracted twice with double volume of ethyl acetate. The organic phase was separated and vacuum dried to obtain the fungal crude extract. We have used the term "fungal crude extract" throughout the text and it invariably refers to *Daldinia placentiformis* (CP2-11), *Daldinia eschscholtzii* (TP2-6), and *Alternaria alternata* (CPR-9) crude extracts.

Determination of polyphenol content

Total phenolic content (TPC)

Total phenolic content (TPC) of endophytic fungal crude extract was estimated by Folin-Ciocalteu colorimetric assay as reported by Saeed et al. (2012). Briefly, 100µl of Folin-reagent was mixed to 100µl of crude extract (1mg/mL) of endophytic fungi and incubated for 5 min. Further, 1mL of sodium carbonate (7.5%) was added followed by dilution with distilled water. Reaction mixture was incubated for 90 min in dark and finally, absorbance was recorded at 750 nm. Gallic acid was used as a standard phenolic. The TPC was calculated and expressed in terms of µg of Gallic acid equivalents (GAE) per mL of sample quantified on the basis of standard curve of gallic acid.

Total flavonoid content (TFC)

Total flavonoid content (TFC) of the crude extract was performed according to (Kushveer et al., 2018). In the reaction mixture, 300 µL of crude extract, 3.4 mL of 30% methanol, 150 µL of sodium nitrite (0.5M) and 150 µL of aluminum chloride hexahydrate (300 mM), were added sequentially. Later, 1 mL of sodium hydroxide (1M) was added to reaction mixture, after 5 min of incubation, absorbance was recorded at 415 nm. Quercetin (QE) was used as a standard and the results were expressed as µg of quercetin equivalents (QE) per mL of crude sample quantified on the basis of standard curve of quercetin.

Analysis of secondary metabolite constituent

The endophytic fungal crude extracts were analyzed for the presence and/or absence of various secondary metabolite constituents such as alkaloids, anthraquinones, cardiac glycosides, flavonoids, phlobatannins, tannins, terpenoids and steroids. The concentration of endophytic fungal extracts was

analyzed at the concentration of 1mg/mL (Gul et al. 2017).

Antioxidant activities

DPPH radical scavenging activity

The stock solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH), to generate the radicals, was prepared by dissolving 50 µg/mL of DPPH in methanol (Gul et al. 2017). The working solution of methanolic DPPH radical was adjusted to < 1.00 at wavelength 517 nm. To an aliquot of 0.4 mL of crude extract of varying concentrations range (10-200 µg/mL), 0.8 mL of DPPH solution was added with and kept for incubation for 30 min at 37 °C in dark conditions. The absorbance was measured at 517 nm and butylated hydroxyl toluene (BHT) was used as standard. All tests were performed in three independent replicates. The percentage radical scavenging or antioxidant activity was calculated using the following formula:

% Antioxidant activity = $[(A_c - A_s)/A_c] \times 100$

Where, Ac is Absorbance of control at 517nm, As - Absorbance of sample at 517nm.

ABTS Assay

2,2'-Azinobis(3-ethylbenzothiazoline- 6-sulfonic acid) di-ammonium salt (ABTS) assay was performed as reported by Tian and Schaich (2013), with few modifications. A stock solution of ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared in Milli-Q water. ABTS and potassium persulfate solutions were mixed together and left for 16 hours to stand in dark at room temperature. The solution develops a dark blue-green color after incubation. This ABTS solution was further diluted to an absorbance of ~1.0 at 734 nm just before performing the experiment. An aliquot of 100µl of different dilutions of crude extracts (5-200 µg/mL) was added to 1000 µL of ABTS solution. After 5 min of incubation at room temperature, the decrease in absorbance was measured at 734 nm. BHT was taken as a standard.

The following formula was used to calculate the percentage ABTS radical scavenging

% Antioxidant activity = $[(A_c - A_s)/A_c] \times 100$

Where, Ac is Absorbance of control at 734 nm and A_s is Absorbance of sample at 734 nm.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of endophytic fungal crude extract was estimated following Boora et al. (2014) with few modifications. An aliquot of 0.250 mL of 10 mM sodium nitroprusside (SNP) in phosphate buffered saline (PBS) was mixed to 0.500 mL of varying concentration range of crude samples (5-200 µg/mL) and incubated at room temperature for 3 hrs. Finally, an equal volume of freshly prepared Griess reagent (1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid) was added to above mixture. Absorbance was recorded at 546 nm. BHT was used as a standard and % inhibition was calculated as mentioned above.

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was performed according to Prabha and Vasantha (2011). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer of pH 7.4. Endophytic fungal crude extracts (20-500 µg/mL) in methanol were added to a H_2O_2 solution (600 µL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Phosphate buffer without H_2O_2 served as blank. The percentage of H_2O_2 scavenging was calculated as:

H_2O_2 scavenging effect (%) = $(A_c - A_s / A_c) \times 100$

Where A_c is the absorbance of the control, and A_s is the absorbance in the presence of the sample or standards.

Reducing Power Assay

Reducing power ability of the endophytic fungal crude extract was evaluated by the method described by Ferreira et al. (2007) with minor modifications. To an aliquot of 0.2 mL of fungal crude extract of varying concentrations (10-300 µg/mL), 0.5 mL of 1% potassium ferricyanide (C_6N_6FeK) and 500 µL of 0.2 mol/L sodium phosphate buffer was mixed. The reaction mixture was incubated at 50°C for 30 min and 0.5 mL of 10% TCA (trichloro acetic acid) was added to terminate the reaction. From upper layer, 500 µL of reaction mixture was taken and mixed with 500 µL of deionized water and consecutively, 100 µL of 0.1% ferric chloride ($FeCl_3$) was added. The absorbance was measured at 700nm against blank.

Protection of DNA damage assay

Potential of endophytic fungal crude extract to protect pUC19 plasmid DNA (E. coli DH5α) from damaging effect of Fenton's reagent (50 mM Ascorbic acid, 30 mM H_2O_2 and 80 mM $FeCl_3$) was estimated by performing protection of DNA damage assay as described by Golla and Bhimathati (2014). After the optimization of the assay, 50 µg of crude was added to the reaction mixture of Fenton's reagent and 200 ng plasmid. Total volume of reaction mixture was made up to 20 µL using milli-Q water. The reaction mixture was subjected to incubation for 30 min at 37 ˚C. Agarose gel (0.8%) was prepared in TBE buffer and the reaction mixture was subjected to gel electrophoresis.

Anticancer activity

Culturing of cell line

Human Cancer cell line MCF7 and HeLa K were cultured in DMEM containing 10% Fetal bovine serum (FBS) and L-Glutamine-Penicillin-Streptomycin solution at 37 °C in 5% CO_2 condition.

MTT cell viability assay

To perform MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay, in a microtiter 96-well tissue culture plate, 10^3 number of cells were seeded. After 16 hours of seeding, the cells were examined for successful attachment to the culture plate. Then, the cells were given the treatment with the filter sterile endophytic fungal crude extract dissolved in 50% DMSO at varying concentrations (5-500 µg/mL). DMSO (50%) was used as a control. About 72 hours of post crude extract treatment, the MTT assay was performed as described in (Singh et al. 2016). All the experiments were performed in three independent replicates.

Statistical analyses

All experiments were performed in three independent replicates, and the values were represented as mean ± standard deviation. Data were analyzed using the Microsoft excel and graph pad prism version 5.04. Further, statistical analyses were performed with one-way ANOVA, where, p values < 0.05 were considered as significant. The Pearson's correlation coefficient (r) and coefficient of determination (R^2) between TPC-TFC and antioxidant activities were estimated to determine the relationships among them.

RESULTS AND DISCUSSION

In symbiotic communications, antioxidants and reactive oxygen species play a crucial role as they both are universally as well as evolutionarily conserved (White Jr and Torres 2010). Hence, asymptomatic fungi produce antioxidants that help mediate the response of the host plant to tackle the biotic and abiotic stresses by interrupting the oxidative damage (Hamilton et al. 2012). Out of 16 endophytes isolated from the host, *C. papaya* and *T. procumbens* (data not shown here), we selected three isolates, i.e., *Daldinia placentiformis* (CP2-11), *Daldinia eschscholtzii* (TP2-6) and *Alternaria alternata* (CPR-9) for the detailed study of antioxidant and anticancer property.

Morpho-molecular identification of endophytes

The three selected endophytes were identified on the basis of molecular sequence analysis. *Daldinia eschscholtzii* (TP2-6), was isolated from leaves of *T. procumbens*, whereas *Daldinia placentiformis* (CP2-11) and *Alternaria alternata* (CPR-9) were isolated from the leaves of *C. papaya*. The colony morphology of the isolates is shown in Figure 1. With the help of molecular data and morphological characteristics, isolates were identified, and their sequence was submitted in GenBank and accession numbers were obtained. Following are the accession numbers: *Daldinia eschscholtzii* KX987249, *Alternaria alternata* KX987250, *Daldinia placentiformis* MG711819.

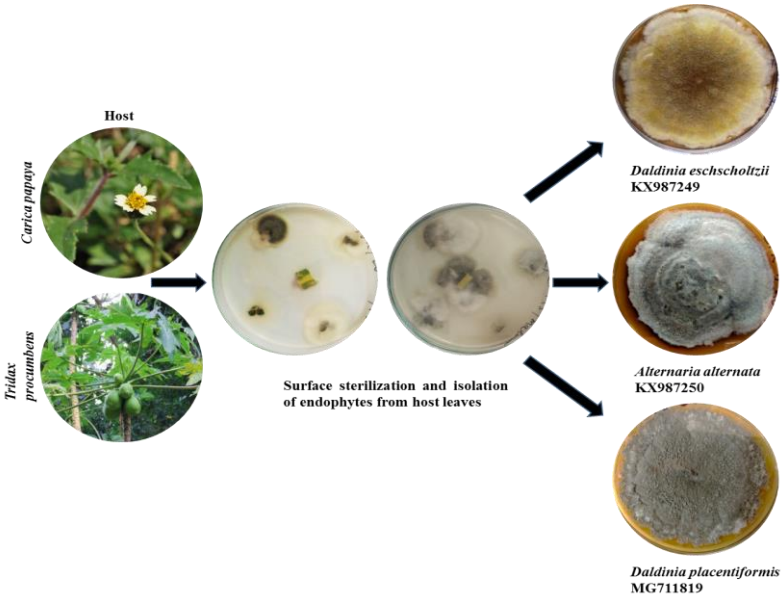

Figure 1. Isolation of endophytic fungi from the two hosts, i.e., *Carica papaya* and *Tridax procumbens*.

TPC and TFC

Studies show that phenolic and flavonoid compounds are dominant antioxidant components that occur naturally in plants (Surveswaran et al. 2007). The prime components of secondary metabolites acting as antioxidants are phenolic compounds. Similarly, flavonoids along with phenolic compounds play a role in lipid stabilization and hence accredited for antioxidant potential (Liu et al. 2007). Hence, the total phenolic content of the endophytic fungal crude extracts were calculated from the gallic acid standard calibration curve (R^2=0.998) and were found to be 118.64±4.61, 87.32±8.96 and 80.28±9.28 µg GAE/mL in CP2-11, TP2-6 and CPR-9, respectively (Table 1, Figure 2). Phenolic content was found to be the highest in CP2-11, while CPR-9 showed least content as is evident from Figure 2. The total flavonoid content of the endophytic fungal crude extracts, was calculated from the quercetin standard calibration curve (R^2=0.994), and was found to be 164.87±6.58, 142.94±5.26 and 126.80±3.17 µg QE/mL in CP2-11, TP2-6 and CPR-9, respectively (Table 1, Figure 2). The TPC of CP2-11 was found to be more when compared to *Chaetomium* sp. isolated from *E. jambolana*, which showed, 60.13±0.41µg GAE (gallic acid equivalent) /mg (Liu et al. 2007).

The endophytic fungal crude analyzed for various constituents of secondary metabolites showed that anthraquinones and phlobatannins were absent in all the endophytic fungal crude extracts. Alkaloids were highest in CP2-11, while, steroids and cardiac glycosides were highest in CPR-9. All the endophytic fungal crude extracts showed positive for the presence of tannins, terpenoids, steroids, flavonoids, cardiac glycosides and alkaloids (Table 2).

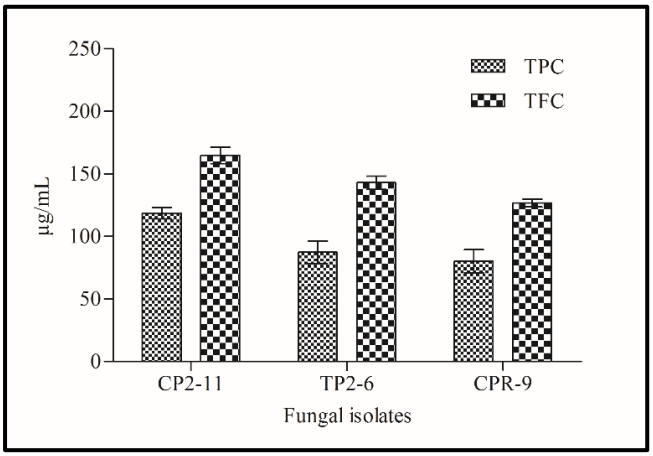

Figure 2. Total phenolic content (TPC) and total flavonoid content (TFC): TPC and TFC of different endophytic fungal crude extracts expressed as µg GAE/mL of crude and µg QE/mL of crude, respectively. Each value represented as a mean ± SD (n = 3). GAE – Gallic acid equivalent, QE – Quercetin equivalent.

Fungal isolates	Total phenolic content (µg GAE/mg of crude)	Total flavonoid content (µg QE/mg of crude)
CPR-11	118.64±4.61	164.87±6.58
TP2-6	87.32±8.96	142.94±5.26
CPR-9	80.28±9.28	126.80±3.17

Table 1. Total phenolic content and flavonoid content: Total phenolic content (TPC) and total flavonoid content (TFC) of the three endophytic fungal crude extracts expressed as µg GAE/mL of crude and µg QE/mL of crude, respectively. Each value represents a mean ± SD (n = 3). GAE – Gallic acid equivalent, QE – Quercetin equivalent.

Endophytic fungal secondary metabolite group	Fungal isolates		
	CP2-11	TP2-6	CPR-9
Tannins	+	+	+
Terpenoids	+	+	+
Steroids	+	++	+++
Flavonoids	++	+	+
Cardiac glycosides	+	++	+++
Alkaloids	+++	+	+

Table 2. Analysis of different groups of secondary metabolite constituents produced by selected endophytic fungi. Where, + = weakly positive, ++ = moderately positive, +++ = strongly positive extracts.

DPPH radical scavenging activity

The DPPH free radical scavenging activity of the endophytic fungal crude extracts was carried out and the results were expressed in terms of IC_{50} values wherein a lower IC_{50} value indicates stronger ability of the extracts that takes place with a color change from purple to faded yellow. The results show that CP2-11 showed the highest scavenging percentage of 85.02±0.87, which was almost similar to *Alternaria* sp. (85.20%) isolated from *M. luteola* (Gunasekaran et al. 2017). This was followed by TP2-6 with 79.67±0.97 and CPR-9 with 74.17±0.99 respectively, whereas BHT served as standard with 88.46±0.97 % scavenging (Figure 3A). BHT was found to have an IC_{50} value of 34.51±0.92 µg/mL when compared to CP2-11, TP2-6 and CPR-9 extracts that had values of 82.02±1.95, 88.57±0.97, and 98.36±2.38 µg/mL, respectively (Table 3). CP2-11 was found to be most active among three fungal isolates whereas, CPR-9 showed least DPPH free radical scavenging activity. DPPH free radical scavenging assay is widely accepted and preferred method to analyze the antioxidant potential of compounds and is also not affected by metal or enzyme inhibition (Gunasekaran et al. 2017).

ABTS Assay

ABTS radical is a stable free radical with the absorption at 734 nm and is frequently used to study the radical scavenging. ABTS free radical scavenging of the endophytic isolates CP2-11, TP2-6 and CPR-9 was found to be 85.13±1.13, 81.56±0.82 and 72.09±0.76 %, respectively. Furthermore, for BHT that served as a standard has shown 92.62±0.84 % scavenging (Figure 3B). The IC_{50} values of ABTS radical cation scavenging activity for BHT was 2.46±0.09 µg/mL, whereas the IC50 values of CP2-11, TP2-6 and CPR-9 were found to be 8.12±0.20, 13.95±0.19 and 16.08±0.21 µg/mL, respectively (Table 3). These results demonstrate that CP2-11 isolate is more potent than an earlier report on *Phyllosticta* sp., which had a scavenging of 72.38 % at a maximum concentration of 1600 µg/mL with the IC_{50} value 580.02±0.57 µg/mL (Srinivasan et al. 2010).

Nitric oxide radical scavenging activity

Inside human body, various physiological processes such as muscle relaxation, inhibition of platelet aggregation, neuronal signaling, and regulation of cell mediated toxicity, get inhibited by nitric oxide, a potent pleiotropic inhibitor. This diffusible free radical serves as effector molecule in various biological systems (Hagerman et al. 1998). Nitric oxide free radical scavenging activity was determined and was found to be least in CPR-9 and highest in CP2-11. Ascorbic acid (standard) showed 87.65±0.93 % radical scavenging activity, whereas percentage nitric oxide radical scavenging activity in endophytic fungal crude in increasing order of nitric oxide radical scavenging activity was 61.92±0.95, 73.65±1.03 and 77.80±0.93 % in CPR-9 < TP2-6< CP2-11, respectively (Figure 3C). The IC_{50} values of the three endophytic fungal crude extracts were found to be 67.37±1.03, 123.26±1.63 and 153.42±1.61 µg/mL in CP2-11, TP2-6 and CPR-9 respectively, whereas it was 35.01±1.20 µg/mL in the case of standard (Table 3). The results obtained are more promising than obtained from *Chaetomium* sp. (62.39 %), followed by *Tricoderma* sp. (59.20 %) and *Colletotrichum* sp. (31.66 %) isolated from *Azadirachta indica* (Kumaresan et al. 2015).

Hydrogen peroxide scavenging activity

The endophytic fungal crude extracts have shown concentration dependent H_2O_2 free radical scavenging activity. The highest H_2O_2 scavenging activity was found to be 85.68±1.00 (CP2-11) and 78.92±0.93 (CPR-9) at a concentration of 300 µg/mL respectively, which was comparable with the scavenging effect of BHT. The results are shown in Table 3 and graphically represented in Figure 3D. The IC_{50} value is inversely proportional to free radical scavenging potential, which was calculated with the help of the linear regression of the percentage antioxidant activity against various concentrations of extract. The extracts of CP2-11, TP2-6 and CPR-9 possess IC_{50} values of 73.35±1.39, 86.32±0.94 and 95.44±1.94 µg/mL, respectively while BHT had a value of 45.89±2.08 µg/mL.

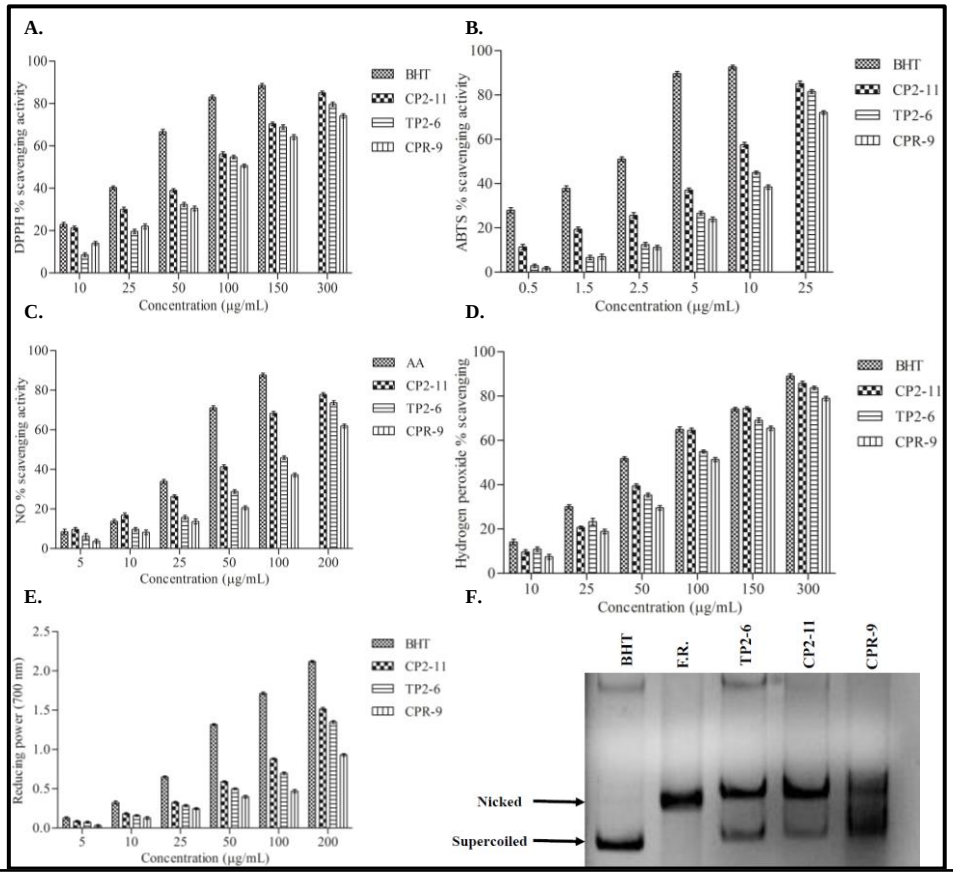

Figure 3. Antioxidant activities of the endophytic fungal crude extracts at various concentrations. Each value represents a mean ± SD (n = 3). A- DPPH radical scavenging activity, B- ABTS radical scavenging activity, C- Nitric oxide (NO) radical scavenging activity, D- Hydrogen peroxide radical scavenging, E- Reducing power ability, and F- Oxidative DNA damage protection activity of endophytic fungal crude extract. BHT = Butylated hydroxytoluene, FR- Fenton's reagent. The concentration of endophytic fungal crude extracts used was 50 µg/mL. *Daldinia placentiformis* (CP2-11), *Daldinia eschscholtzii* (TP2-6), *Alternaria alternata* (CPR-9).

Test samples	DPPH activity IC_{50} Values (µg/ml)	ABTS activity IC_{50} Values (µg/ml)	Nitric oxide activity IC_{50} Values (µg/ml)	Hydrogen peroxide activity IC_{50} Values (µg/ml)
CP2-11	82.02±1.95	8.12±0.20	67.37±1.03	73.35±1.39
TP2-6	88.57±0.97	13.95±0.19	123.26±1.63	86.32±0.94
CPR-9	98.36±2.38	16.08±0.21	153.42±1.61	95.44±1.94
BHT	34.51±0.92	2.46±0.09	-	45.89±2.08
ASB	-	-	35.01±1.20	-

Table 3. IC_{50} values (µg/mL) of radical scavenging activities of selected endophytic fungal crude extracts. Each value represented as mean ± SD (n = 3). ASB = Ascorbic acid, BHT = Butylated hydroxytoluene.

Correlation in terms of Pearson's correlation coefficient (r) & coefficient of determination (R^2)		
Antioxidant activities	**TPC**	**TFC**
IC_{50} of DPPH radical scavenging activity	r 0.9158 R^2 0.8388	r 0.9886 R^2 0.9775
IC_{50} of ABTS radical scavenging activity	r 0.9996 R^2 0.9992	r 0.9580 R^2 0.9179
IC_{50} of NO radical scavenging activity	r 0.9999 R^2 0.9999	r 0.9677 R^2 0.9366
IC_{50} of Hydrogen peroxide activity	r 0.9838 R^2 0.968	r 0.9965 R^2 0.9931

Table 4. Correlations between the IC_{50} values of antioxidant activities and phenolic and flavonoid contents of selected three endophytic fungal crude extracts. r = Pearson's correlation coefficient & R^2 = coefficient of determination. Each value is represented as mean ± SD (n = 3). TPC -Total phenolic content and TFC - total flavonoid content, NO – nitric oxide.

Reducing Power Assay

The reducing power of endophytic fungal crude extracts was found to be in a dose dependent manner which is comparable to the standard BHT. The reducing power was ranked in the order CP2-11> TP2-6> CPR-9. Significantly higher reducing power (1.51±0.01 at 200 µg/mL) was evident in CP2-11 and reducing power for standard BHT was 2.12±0.01 at 200 µg/mL (Figure 3E).

All the IC_{50} values for various assays are shown in Table 3. To evaluate the relationship between antioxidant activity and the TPC/TFC based on DPPH, ABTS, NO, H_2O_2 radical scavenging, Pearson's correlation coefficient was applied. Table 4 represent the values and it further suggests a strong correlation between the IC_{50} values of antioxidant activities and phenolic and flavonoid contents of selected three endophytic fungal crude extracts. Several reports suggest that crude extracts with strong antioxidant activity displays a strong correlation between the phenolic content and antioxidant activity (Wang et al. 2012).

Oxidative DNA damage protection activity

Reactive oxygen species or reactive nitrogen species, cause DNA damage. Free radicals such as hydroxyl radical, hydrogen radical, etc., on reacting with DNA, damages it by adding to bases or removing hydrogen atoms from the sugar moiety. Fenton's reagent damages the plasmid DNA and relaxed open circular form is generated. After optimizing with different concentrations, at 50 µg concentration of endophytic fungal crude extract, DNA damage protection was performed. As evident from the Figure 3F, CP2-11 was most potent in rescuing the plasmid DNA while CPR-9 was least effective in restricting the damage caused by Fenton's reagent.

Anticancer activity

MTT assay was performed to observe the cytotoxic potential of endophytic fungal crude extracts on HeLa and MCF7 breast cancer cells. After treating the cancer cells with endophytic fungal crude extract, cell viability was checked after 72 hours. Cell viability data reflected a significant cytotoxicity in endophytic fungal crude extract treated cells when compared to the DMSO control. The effect of endophytic fungal crude extracts was found to be dose dependent as an increase in concentration of endophytic fungal crude decreased the viability of cancer cells (Figure). CP2-11 was showing highest cell cytotoxicity and least in TP2-6. Approximately, only about 15% cells of HeLa cells were viable when treated with 125 µg of CP2-11 extract, whereas only about 10% viability was seen in MCF-7 breast cancer cell line (Figure 4). Hence, as expected, with increase in concentration of crude extract, a drastic reduction in cell viability was absorbed. Cell viability assay using MTT assay, is a sensitive and colorimetric technique to investigate the effect of endophytic fungal crude extract on cancer cells. Following results further get validation with the fact that sample with high antioxidant activity shows high anticancer potential. Endophytic fungal crude extracts of all the three fungi showed a potential against cancer cells when compared to earlier reports (Almeida et al. 2012). To summarize, the compounds present in crude samples seem to possess relatively a good anticancer potential.

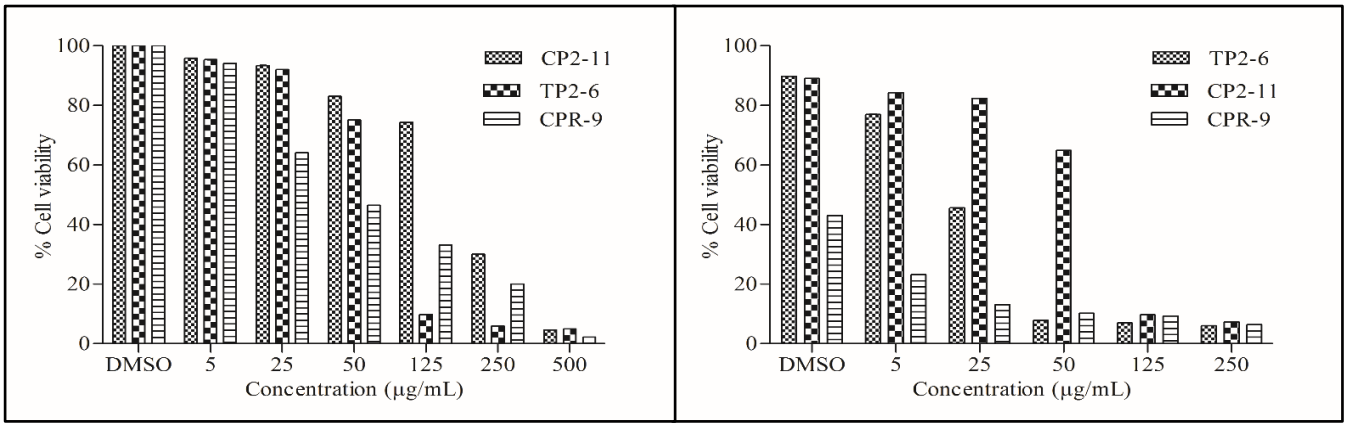

Figure 4. Anticancer activity of endophytic fungal crude extracts on different cancer cell lines. Each value represents a mean ± SD (n = 3): Percentage cell viability of A- HeLa cells and B- MCF7 breast cancer cells, when treated with endophytic fungal crude at different concentrations. DMSO (dimethyl sulfoxide) was used as control.
***Daldinia placentiformis* (CP2-11), *Daldinia eschscholtzii* (TP2-6) and *Alternaria alternata* (CPR-9)**

CONCLUSIONS

The present study focused on antioxidant potential of endophytic fungi isolated from the medicinal plants, viz, *C. papaya* and *T. procumbens*. A positive and strong correlation between TPC, TFC and antioxidant potential of endophytic fungi is established. Antioxidant potential of endophytes was investigated through various assays. Secondary metabolites from endophytes also displayed remarkable anticancer activity against human cancer cell lines. Endophytes associated with medicinal plants taken up in the present study show potential bioactive metabolites having strong antioxidant potential.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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