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ORIGINAL ARTICLE

Antioxidant and Cytotoxic Potential of Endophytic Fungi Isolated from Medicinal Plant *Tragia involucrata* L.

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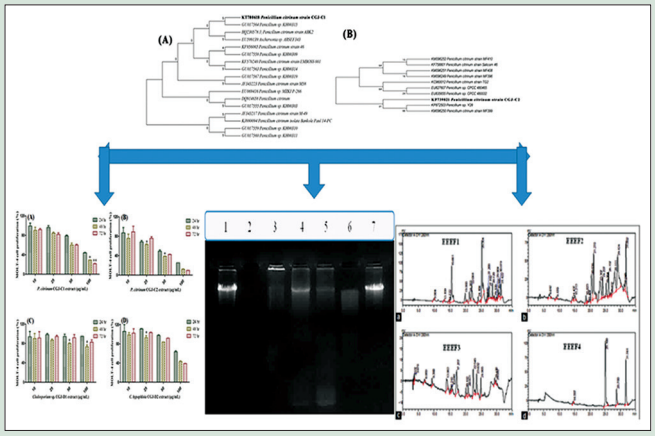
ABSTRACT

Objective: The aim of the present study was to test the antioxidant and cytotoxic and DNA protection activities of ethyl acetate extracts of endophytic fungi isolated from *Tragia involucrata* Linn. (Euphorbiaceae). **Materials and Methods:** The 1, 1-diphenyl-2-picrylhydrazyl scavenging, reducing power, and total antioxidant assay were used to evaluate the antioxidant activity. Cytotoxic activity of endophytic fungal extracts against MCF-7 and MOLT-4 cell lines was carried out using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide method. **Results:** We have successfully isolated four endophytic fungi from *T. involucrata,* namely *Penicillium citrinum* CGJ-C1 (GenBank accession No. KT780618), *P. citrinum* CGJ-C2 (GenBank accession No. KP739821), *Cladosporium* sp. CGJ-D1 (GenBank accession No. KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (GenBank accession No. KT780619). The ethyl acetate extract of *P. citrinum* CGJ-C2 showed the highest antioxidant as well as cytotoxic activity among the four fungal extracts taken for the study. All the extracts showed moderate DNA protection activity. **Conclusions**: Further studies on the isolation and purification of the lead molecule will help in designing the novel therapy for different ailments associated with free radical generation.

Key words: Antioxidants, cytotoxicity, endophytes, *Tragia involucrata*

SUMMARY

Totally four endophytic fungi (*Penicillium citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *Cryptendoxyla hypophloia* CGJ-D2) were isolated from the medicinal plant *T. involucrata*. High amount of phenolic (112.00 ± 4.12 mg/g GAE) and flavonoid content (40.00 ± 1.14 mg/g quercetin equivalent/g) was found in ethyl acetate extract of *P. citrinum* CGJ-C2 and *C. hypophloia* CGJ-D2 respectively. Free radical scavenging ability was measured using DPPH method and a significant anti-radical activity was observed in *C. hypophloia* CGJ-D2 (260.00 ± 1.45 mg/g) extract. The endophytic fungal extracts were subjected to cytotoxic activity against MCF-7 as well as MOLT-4 cell lines using MTT assay. *P. citrinum* CGJ-C2 showed a potential cytotoxicity against cancer cells used in this study. The fungal extracts also exhibited DNA protection capability at higher concentration. We are for the first time reporting on the isolation and biological study of endophytic fungi isolated from *T. involucrata*. Further studies are going on in our laboratory to isolate the novel anticancer lead/s from these fungi.



Abbreviations used: MCF-7: Breast Cancer Cell Line; MOLT-4: Human leukemia cell line; DPPH: 1, 1-diphenyl-2-picrylhydrazyl; MTT:3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; DMSO: Dimethyl sulfoxide; H2O2: Hydrogen peroxide; NCI: National Cancer Institute.

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INTRODUCTION

Endophytic fungi are microscopic organisms that grow within plant tissue without causing immediate symptoms of disease.[1] Natural products from fungal endophytes have vast applications in medicine as they exhibit diverse therapeutic properties.[2,3] Many antitumor agents such as taxol and beauvericin are extracted from the endophytic fungi such as *Pestalotiopsis microspore* and *Fusarium oxysporum* on *Taxus wallachiana* and *Ephedra fasciculate* plants, respectively.[4,5] Even though the endophytes are studied for various applications, still many are unexplored with respect to their therapeutic potential. According to Strobel and Daisy, each plant identified on earth hosts an endophyte.[6] Endophytes acquire the medicinal properties of the host plant in which they exist.[7] Hence, they are the reservoirs for various novel molecules with therapeutic efficacy.

Tragia involucrata belongs to Euphorbiaceae family and widely distributed in Asian subcontinent. This plant has been reported to contain several medicinal properties such as cytotoxic, antitumor, anti-fertility,[8-10] antimicrobial,[11] antidiabetic,[12] wound healing,[11] and

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anti-inflammatory activities.[13] Even though *T. involucrata* has been extensively studied for its medicinal activity, there are no reports on the therapeutic potential of endophytes of *T. involucrata*. To the best of our knowledge, no one has isolated and studied the medicinal properties of endophytes of *T. involucrata.* Hence, the aim of the present study was to isolate endophytic fungi from *T. involucrata* and to study their antioxidant, cytotoxic, and DNA protection potential.

MATERIALS AND METHODS

Collection of plant material

Healthy stems of *T. involucrata* were collected in the month of May 2013 in and around Sullia (T), Dakshina Kannada (D), Karnataka, India. The Global Positioning System locations were 12.5581° N and 75.3892° E. The plant was shifted to laboratory using sterile polyethylene bags at 8°C. The plant was identified by Dr. Jagath Timmaiah, Department of Botany, Karnataka, India. The plant was authenticated by CSIR-National Institute of Science Communication and Information Resources, New Delhi. Herbarium was peevishly deposited by one of the authors to Gandhi Krishi Vignana Kendra, Bengaluru, which has been taken as a reference (Herbarium No. 3687).

Isolation and identification of endophytic fungi from *Tragia involucrata*

T. involucrata was washed with running tap water to remove excess debris followed by washing with distilled water. The samples were cut into small pieces (0.6–0.8 cm) and rinsed with 70% alcohol. The alcohol-washed pieces were again rinsed with 0.5% sodium hypochlorite followed by washing thrice with sterile distilled water. The sterilized samples were transferred aseptically to potato dextrose agar media containing chloramphenicol (150 mg/L) and incubated at (28.00°C ± 1°C) for 7 days.[14]

The genomic DNA was isolated and purified using fungal genomic DNA mini kit using the manufacturer's protocol (xcelgenCat No: XG2416-01 from Xceleries genomics Pvt. Ltd, India), and molecular identification was achieved by internal transcribed spacer (ITS) region and 18S rDNA analysis. The sequences were used as query sequences to search for similar sequences from GenBank using the basic local alignment search tool program. The most similar reference sequences with query sequences were obtained and used for subsequent phylogenetic analyses [Figure 1]. These sequences were aligned using the CLUSTALX program.[15]

Preparation of ethyl acetate extracts of endophytic fungi

Extraction of secondary metabolites was carried according to the procedure explained by Higginbotham *et al*. with slight modification. Each liquid culture with evident growth (incubation for 18 days in static condition at 25°C–28°C) on potato dextrose broth was mixed with an equal volume of ethyl acetate (100%). The mixture was blended using pestle and mortar. The resulting homogenate was filtered with Whatman® No. 1 filter paper and extracted twice with an equal volume of ethyl acetate. The aqueous and organic layers were dried and stored at −20°C until use.[14]

Total phenolic content

The total phenolic content of the fungal extract was estimated using Folin–Ciocalteu (FC) reagent. Different aliquots (0–1 mL) of gallic acid (Sigma-Aldrich, USA) and extracts were taken in tubes and the volume was made up to 1 mL with distilled water. One mL of (1:1 diluted) FC reagent and 1.5 mL of 20% sodium carbonate were added to each tube. After the addition of 6 mL of distilled water, the reaction mixture was incubated at room temperature for 30 min. The absorbance of the sample and tests was measured at 760 nm against blank in ultraviolet-visible (UV-Vis) spectrophotometer (Optima Tokyo, Japan). The total phenolics in fungal extract was calculated using standard curve and expressed as gallic acid equivalents.[16]

Total flavonoid content

Total flavonoid content of the fungal extract was estimated by the protocol published by Zhishen *et al.*[17] Different aliquots of endophytic extracts and quercetin (100 µg/ml) were taken in different tubes, and the volume in each tube was made up to 1 mL with distilled water. 1.5 mL of ethanol, 0.1 mL of 10% aluminum chloride, and 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water were added to each tube and the absorbance of the reaction mixture was measured at 450 nm in UV-Vis spectrophotometer (Optima Tokyo, Japan).

Total antioxidant assay

The total antioxidant assay was carried out using molybdenum reagent. Phosphomolybdate (3 mL) was added into tubes containing either ascorbic acid or extract and incubated at 95°C in boiling water bath for 90 min after shaking vigorously for 15 min at room temperature. The optical density was measured at 695 nm using UV-Vis spectrophotometer (Optima Tokyo, Japan). Total antioxidant potential of endophytic fungal extracts was expressed as ascorbic acid equivalents.[18]

1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical scavenging activity of the fungal extracts was studied by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method based on the procedure of Brand-Williams *et al.*[19] with modifications. Standard ascorbic acid and extracts (100 µg/mL) were taken in different aliquots (0–1 mL) and volume was made up to 1 mL using methanol. Three mL of 0.1 M DPPH (Sigma, USA) was added and tubes were incubated in dark for 30 min. The absorbance was measured at 517 nm using UV-Vis spectrophotometer (Optima Tokyo, Japan). DPPH scavenging activity was calculated by using the following equation,

$$\text{DPPH scavenging (\%)} = (A_{Control}\text{-}A_{Extract}/A_{Control}) \times 100$$

where A denotes absorbance.

Cytotoxic activity

Assessment of cytotoxicity by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide assay

Cytotoxic effect of the endophytic fungal extracts was determined by the procedure explained by Kuriakose *et al.*[20] Cells were grown on 96-well flat-bottomed microtiter plates at a final volume of 100 µL culture medium per well and incubated at 37°C and 5% CO_2 for 24 h, 48 h, and 72 h. Supernatant was removed after incubation and the cells were washed. Fungal extracts (25–200 µg/mL for MCF-7 and 10–100 µg/mL for MOLT-4) were added into the respective labeled wells. After 24 h, 48 h, and 72 h incubation, 10 µL (5 mg/mL) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) solution in phosphate-buffered saline was added to each well and incubated for 2 h in dark. Thereafter, medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Plates were read at 570 nm using a microplate reader (Bio-Rad, USA) and results were expressed as viability percentage against the untreated control cells (100% of viability).

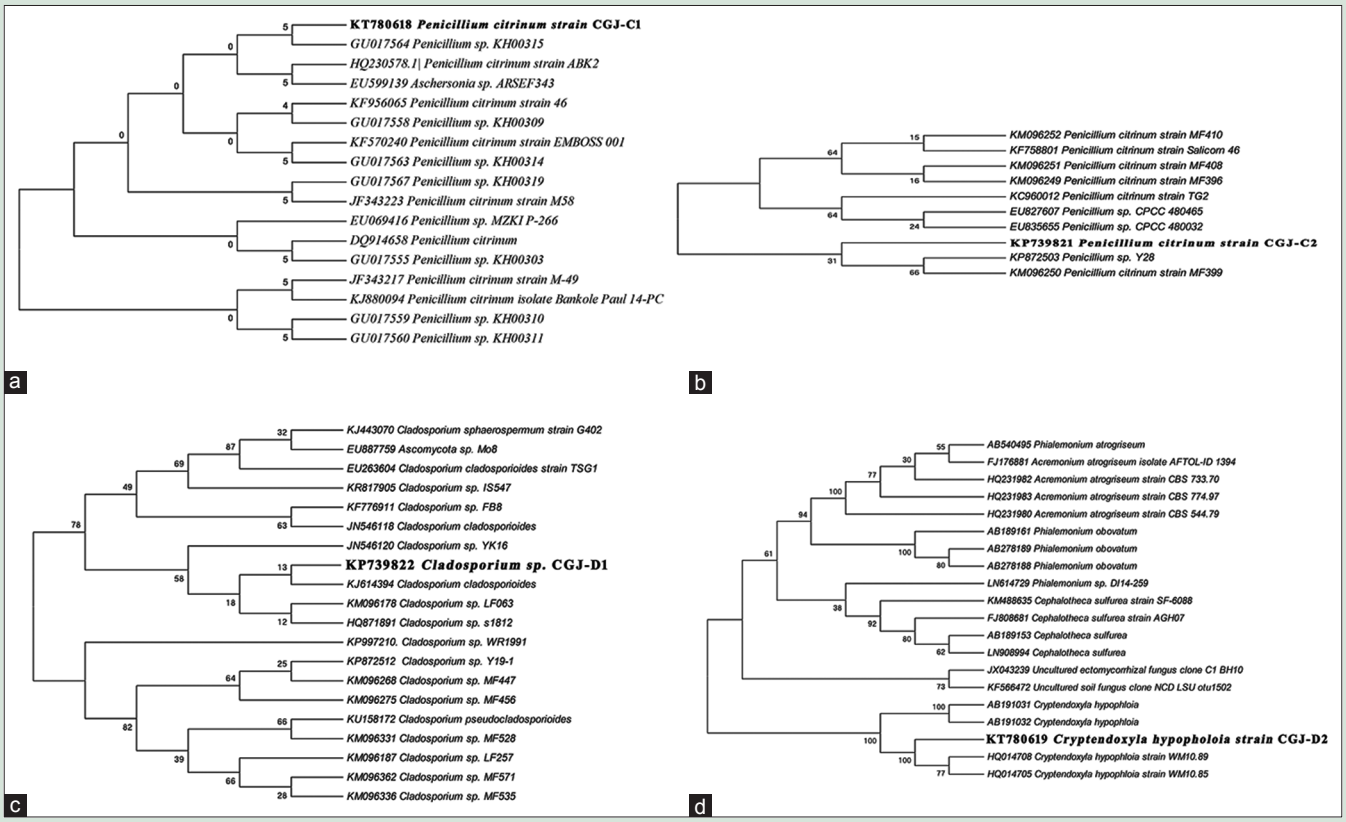

Figure 1: Neighbor-joining phylogenetic tree analysis of fungal endophyte sp. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are shown at the branch nodes. Bootstrap values from neighbor-joining method were determined

DNA protection ability

Oxidative λ-DNA damage was prevented by endophytic fungal extract and it was assayed.[21] λ-DNA (0.5 μg) with and without endophytic fungal extract (200 μg) was incubated with Fenton's reagent (1 mM FeSO4, 25 mM H_2O_2 in Tris buffer 10 mM, pH 7.4) at a final reaction volume of 30 μL for 1 h at 37°C. Relative difference between oxidized and native DNA was analyzed on 1% agarose gel prepared in tris-acetate-ethylenediaminetetraacetic acid buffer (pH 8.5) at 50 V for 3 h at room temperature. The gel was documented (Uvitec Company, software platinum 1D, UK) and the band intensity was determined.

High-pressure liquid chromatography analysis of endophytic fungal extracts

Extract samples were filtered with 0.45 μ membrane filter prior to high-pressure liquid chromatography (HPLC) analysis. The system was equipped with an auto-injector and photodiode array detector (Waters Milford USA). Analysis was performed on a Grace Smart reverse-phase chromatography – 18 column (5 μm, 250 mm × 4.6 mm), at a flow rate of 1 mL/min, using an injection volume of 20 μL, detection at 280 nm. The mobile phases employed were A (methanol 100%) and B (0.5% acetic acid). The gradient program followed was 0–28.6 min, 90%–40% B and 28.6–30 min, 40%–90% B, equilibrated till 35 min.

Statistical analysis

All the experiments were performed in triplicates and the data were compared with one-way analysis of variance using Graphpad prism software version 5.0 (GraphPad Software 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA). A significant difference ($P < 0.05$) among the equation was evaluated by Turkey's test.

RESULTS

Isolation and identification of endophytic fungi from *Tragia involucrata*

Four endophytic fungi were isolated from *T. involucrata*. The identity of the endophytes was established by comparing the 18S rDNA sequence. These endophytic fungi were identified as *Penicillium citrinum* CGJ-C1 (GenBank accession number KT780618), *P. citrinum* CGJ-C2 (GenBank accession number KP739821), *Cladosporium* sp. CGJ-D1 (GenBank accession number KP739822), and *Cryptendoxyla hypophloia* CGJ-D2 (GenBank accession number KT780619) as shown in Figure 1.

Determination of total phenolics

Total phenolic content in the fungal extracts was measured by Folin–Ciocalteu's method. The total polyphenol and flavonoid content of each extract is shown in Figure 2a and b. *P. citrinum* CGJ-C2 extracts showed the highest amount of phenolic content (112.00 ± 4.12 mg/g GAE) followed by *C. hypophloia* CGJ-D2, *P. citrinum* CGJ-C1, and *Cladosporium* sp. CGJ-D1 extracts [Figure 3a].

Flavonoid content

The total flavonoid content of four endophytic extracts was depicted in Figure 3b. Flavonoid content varied in different extracts. Among the four extracts analyzed, the highest amount of flavonoids was found in

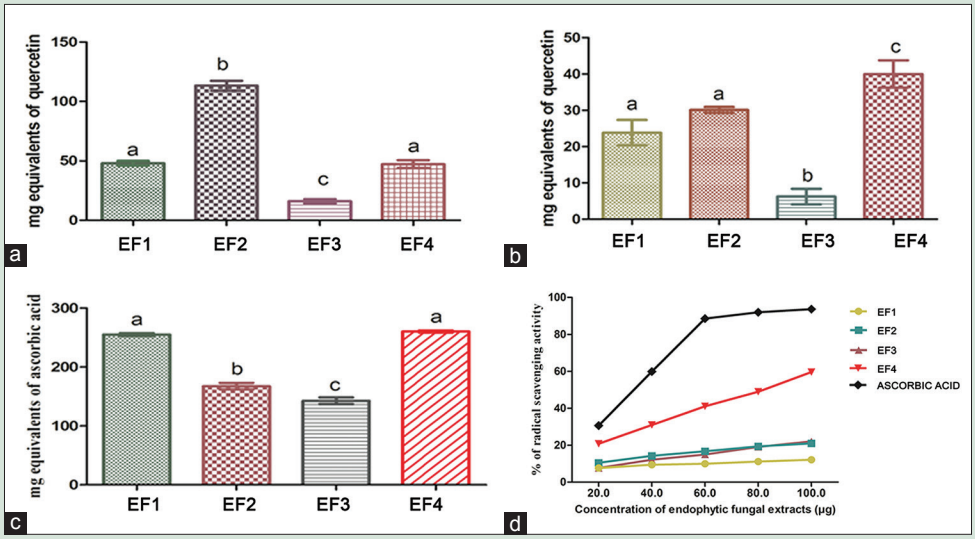

Figure 2: Cytotoxic activity of fungal extracts on MCF-7 cell line. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2 in 48 h and 72 h. (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

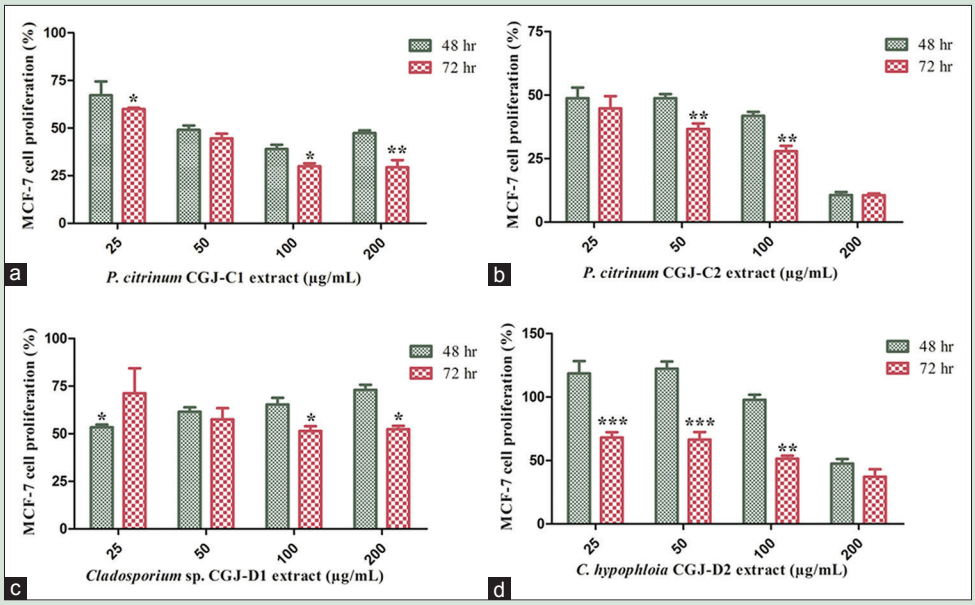

Figure 3: (a) Total phenolic content, (b) Total flavonoid content, (c) Total antioxidant of endophytic fungal extract, (d) 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity of the fungal extracts. All values are reported as means ± standard deviation ($n = 3$). * EF1 (*Penicillium citrinum* CGJ-C1 extract), EF2 (*Penicillium citrinum* CGJ-C2), EF3 (*Cladosporium* sp. CGJ-D1), and EF4 (*Cryptendoxyla hypophloia* CGJ-D2). All the experiments were performed in triplicates and the data were compared with one way analysis of variance using GraphPad prism software version 5.0. Different alphabets above the bars have mean values that are significantly different ($P < 0.05$).

C. hypophloia CGJ-D2 extract (40.00 ± 1.14 mg/g quercetin equivalent/g) compared to the other three extracts.

Total antioxidant activity

The total antioxidant activity of the endophytic extracts was measured spectrophotometrically by phospho-molybdenum method. The principle involved in this method is the reduction of molybdenum (Mo) (IV) to Mo (V) by the extracts, and the final green-colored Mo (V) end product is measured as absorption at 695 nm.[22] In our study, significant antioxidant activity was observed in *C. hypophloia* CGJ-D2 (260.00 ± 1.45 mg/g) extract. The three other extracts also showed considerable total antioxidant activity [Figure 3c].

1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity

The free radical scavenging ability of ethyl acetate extracts of endophytic fungi was evaluated using DPPH substrate. DPPH is a stable free radical with characteristic absorption at 517 nm, and antioxidants react with DPPH and convert it to 2,2-diphenyl-1-picrylhydrazine.

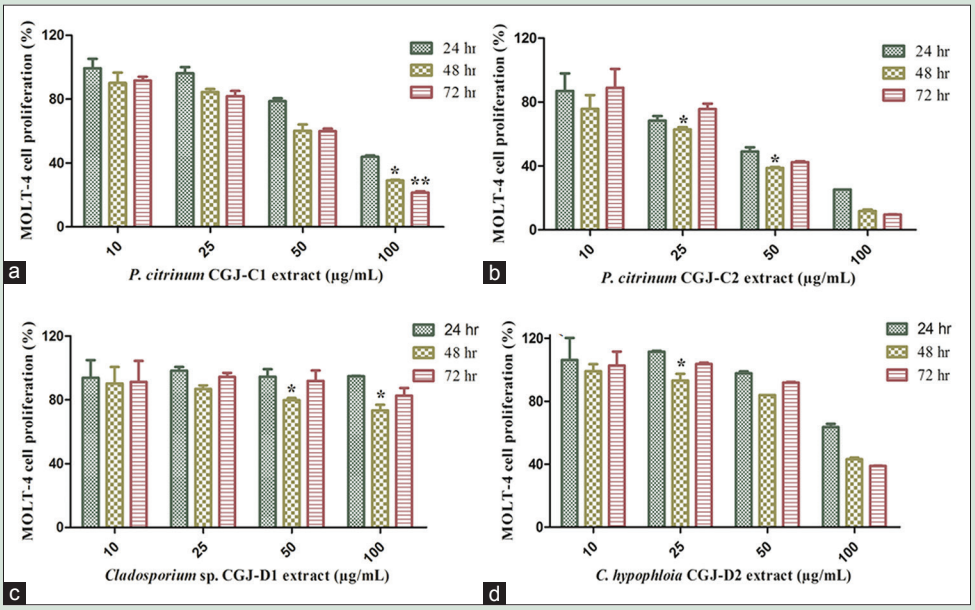

Figure 4: Cytotoxic activity of fungal extracts on MOLT-4 cell line. (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, and (d) *Cryptendoxyla hypophloia* CGJ-D2 in 24 h, 48 h, and 72 h.(*$P \leq 0.05$; **$P \leq 0.01$)

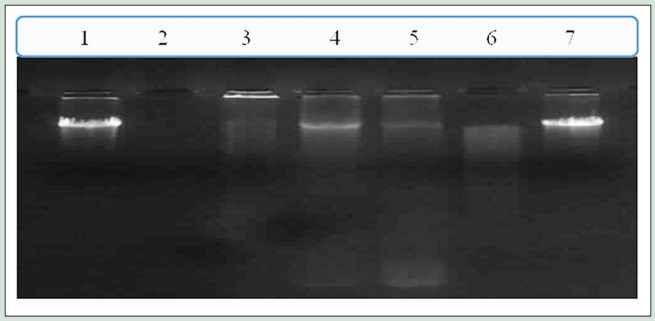

Figure 5: DNA protection by four isolated endophytic fungal extracts. Lane 1: λ DNA (0.5 µg), Lane 2: λ DNA (0.5 µg) + Fenton's reagent (10 µl), Lane 3: λ DNA (0.5 µg) + EF1 (200 µg) + Fenton's reagent (10 µl), Lane 4: λ DNA (0.5 µg) + EF2 (200 µg) + Fenton's reagent (10 µl), Lane 5: λ DNA (0.5 µg) + EF3 (200 µg) + Fenton's reagent (10 µl), Lane 6: λ DNA (0.5 µg) + EF4 (200 µg) + Fenton's reagent (10 µl), Lane 7: λ DNA (0.5 µg) + dimethyl sulfoxide. Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$). * EF1 (*Penicillium citrinum* CGJ-C1 extract), EF2 (*Penicillium citrinum* CGJ-C2), EF3 (*Cladosporium* sp. CGJ-D1), EF4 (*Cryptendoxyla hypophloia* CGJ-D2)

DPPH-scavenging activities of all the extracts were comparable to standard ascorbic acid. *C. hypophloia* CGJ-D2 extract showed 50% radical scavenging activity at 4.01 µg/mL [Figure 3d and Supplementary Table 1].

Cytotoxic activity

Cytotoxic effect of the endophytic extract on MCF-7 and MOLT-4 cell line was assessed using MTT assay. The cytotoxic activity of the endophytic extracts is depicted in Figures 2 and 4. *P. citrinum* CGJ-C2 extract exhibited a significant cytotoxic effect on MCF-7 cell line in 48 h and 72 h compared to other extracts. The cytotoxic activities of all the four extracts were found to be dose dependent. In our study, *P. citrinum* CGJ-C2 extract showed significant cytotoxicity against MCF-7 as well as MOLT-4 cell lines. The IC_{50} value of the *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2 on MCF-7 cells was 1.50, 1.09, 3.87, and 2.90 µg/mL, respectively, at 72 h of incubation after the treatment of extract. The IC_{50} value of the *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2 on MOLT-4 cells was 2.97, 2.38, 8866, and 5.41 µg/mL, respectively [Supplementary Table 2].

DNA protection assay

Incubation of radicals with λ-DNA for 1 h resulted in the total disappearance of DNA bands on 1% agarose gel compared to DNA control and DMSO vehicle (Lanes 1, 2, and 7). However, the addition of 200 µg of endophytic extracts to the mixture of λ-DNA and Fenton's reagent prevented the radical-induced DNA damages (Lanes 3–6) as shown in Figure 5.

High-pressure liquid chromatography analysis

The polyphenols present in the fungal extracts were identified by using reverse-phase C-18 column in HPLC. Different peaks with respect to retention time confirmed the presence of polyphenols in endophytic fungal extracts [Figure 6]. *P. citrinum* CGJ-C1 showed the presence of vanillin, caffeic acid, ferulic acid, quercetin, and coumaric acid. Each extract was quantitated for the presence of phytochemicals, and the quantity of individual chemical was varying with the species. Caffeic acid was found to be a major constituent in *P. citrinum* CGJ-C1 (15.22 ± 0.12 µg/mg), while quercetin (6.904 ± 0.44 µg/mg) and vanillin (1.70 ± 0.84 µg/mg) were present in abundant quantity in *P. citrinum* CGJ-C2 and *Cladosporium* sp. CGJ-D1 extracts, respectively, when compared to other extracts. The chemicals present in the *C. hypophloia* CGJ-D2 did not match any standards that were used for this study [Table 1].

DISCUSSION

The endophytes present in the plant produce similar secondary metabolites as that of the host plant. These secondary metabolites even exhibit the medicinal properties of the plant in which they exist.

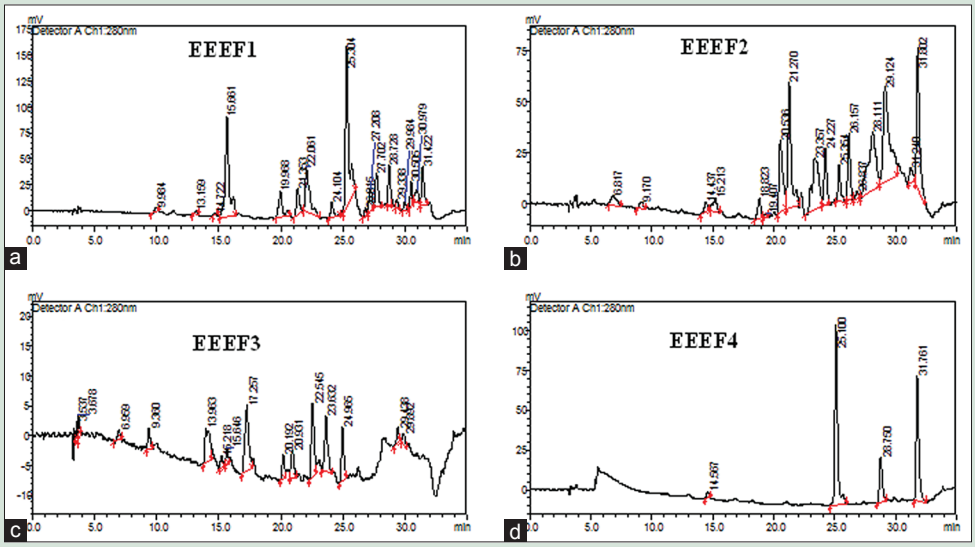

Figure 6: High-pressure liquid chromatography chromatograms of ethyl acetate extract of (a) *Penicillium citrinum* CGJ-C1, (b) *Penicillium citrinum* CGJ-C2, (c) *Cladosporium* sp. CGJ-D1, (d) *Cryptendoxyla hypophloia* CGJ-D2, detected at 280 nm

Table 1: Polyphenolic profiles of endophytic fungal extracts

Polyphenols	Amount of polyphenol (µg/mg)			
	***P. citrinum* CGJ-C1**	***P. citrinum* CGJ-C2**	***Cladosporium* spp CGJ-D1**	***C. hypophloia* CGJ-D2**
Vanillin	0.365±0.08	-	1.70±0.84	-
Caffeic acid	15.22±0.12	2.255±0.095	0.192±0.14	-
Ferulic acid	2.644±0.64	0.148±0.025	0.475±0.18	-
Quercetin	1.239±0.24	6.904±0.44	-	-
Coumaric acid	-	0.554±0.21	-	-

Different extracts were subjected to HPLC analysis along with phenolic acid standard and the peak area, concentrations of phenolic acids were calculated and expressed as mg/g. Values provided are mean of three replicates±SD. HPLC: High-pressure liquid chromatography; SD: Standard deviation; *P. citrinum*: *Penicillium citrinum*; *C. hypophloia*: *Cryptendoxyla hypophloia*

Endophytes isolated from medicinal plants have many advantages over the plant as they can be easily grown and harvested in laboratories. Even the process involved in the isolation of secondary metabolite is less complicated than medicinal plants.[7] This information prompted us to isolate the endophytes from *T. involucrate* and to test the antioxidant and cytotoxic activities as well as DNA protection potential

We have successfully isolated identified four endophytic fungi. Out of the four endophytic fungi, two were different strains of the same species *P. citrinum* CGJ-C1 (KT780618) and *P. citrinum* CGJ-C2 (KP739821). Organisms with 99% or more sequence similarity to be considered from the same species. If the sequence similarity between the organisms ranges from 93% and 98%; the organisms to be considered from the same genus. However, the organism is considered to be previously unidentified if the ITS Sequence similarity is below 93%.[23] The endophyte had 99% similarity to *P. citrinum* CGJ-C1, *P. citrinum* CGJ-C2, *Cladosporium* sp. CGJ-D1, and *C. hypophloia* CGJ-D2.

According to Cui *et al.* (2011), medicinal properties of the endophytic fungi vary even though they belong to the same genus and isolated from the single host species.[24] Our study supported the observation of Cui *et al.* (2011) regarding the different bioactivity in different strains of the same species of endophytes (*P. citrinum* CGJ-C1 [KT780618] and *P. citrinum* CGJ-C2 [KP739821] showed different bioactivity).[24]

Reactive oxygen species (ROS) such as O_2, H_2O_2, and OH– are generated as a result of metabolic process in living organisms. These ROS are involved in various pathophysiologies such as aging, cancer, and neurodegenerative processes. However, they are essential for certain vital functions in the body such as stress-response signaling and programmed cell death. ROS and antioxidants are the key mediators of symbiotic interactions between the host and microorganisms as they are ubiquitous and evolutionarily conserved.[25,26] The microorganism may produce the antioxidant molecules to protect the host plant from different harmful ROS.[27,28]

DPPH free radical scavenging activity is considered to be one of the basic, accurate, and widely used methods to find out the antioxidant potential of various natural products.[19] Electron donation capacity and free radical scavenging potential of a substance determine its reducing power. These properties of antioxidant compounds help them to prevent lipid peroxidation of the membrane as a consequence of a series of reactions of free radicals.[29,30] Some of the previous studies have reported about the linear relationship between total phenolic content and antioxidant activity.[31] However, some researchers have shown that there is no correlation between the phenolic content and antioxidant activity.[32,33] Our study supports the view of a nonlinear relationship between the phenolic content and antioxidant activity. This trend may be attributed to the presence of other phytochemicals along with the phenolics that could act as radical scavengers. Similar results were reported by Wang *et al.*[32] and Devi *et al.*[33]

Assessment of cytotoxicity of natural products is the first step in the development of anticancer compounds.[33] In our study, *P. citrinum* CGJ-C2 showed significant cytotoxicity against MCF-7 as well as MOLT-4 cell lines. All the endophytic extracts except *Cladosporium* sp. CGJ-D1

IC_{50} value much lesser than the National Cancer Institute (NCI) guideline values against *P. citrinum* CGJ-C2 in MCF-7 cell lines. According to the NCI guidelines, any crude extract showing IC_{50} value below 30 μg/mL can be considered as a promising anticancer substance.[34] Interestingly, the earlier work of this group have demonstrated the cytotoxic activity of the methanol extract of *T. involucrata* against KB (subline of the ubiquitous KERATIN-forming HeLa tumor cell line) and MCF-7 cell line, which was well below the NCI guidelines.[8] This finding supports the hypothesis of Zhao *et al*.[7] who proposed that the endophytes share the medicinal properties of a host plant. Our present study will be helpful in finding a new source for anticancer compounds from endophytes. Many studies on endophytes have shown their cytotoxic potential against various cell lines.[5,35] However the IC_{50} value of those endophytic extracts are not within the NCI-guided limits.

Ruma *et al*. reported the PBR-322 plasmid protection by the organic extracts of endophytes (isolated from *Garcinia* sp.).[36] 50 μg/mL was able to protect the plasmid DNA damage by free radicals. However, in the present study, much higher amount of the extract [Figure 5] was needed to protect the λ DNA from Fenton-induced OH− radicals. Polyphenolic compounds are known to have free radical scavenging activity.[35,37] Hence, the radical scavenging activity of different fungal extracts in this study may be contributed by the phenolic compounds. Even though all the extracts showed significant cytotoxic activity against MCF-7 and MOLT-4 cell lines, extracts also prevented the λ DNA damages at higher concentrations. This contradictory trend can be attributed to the presence of various phytochemicals (as we have taken the crude extract) present in the endophytic extracts (as confirmed by HPLC analysis). As these extracts contain several phytochemicals that are responsible for radical scavenging activity; the prevention of Fenton radical generation might have prevented the DNA damage in fungal extract-treated group. Our observation is supported by the finding of Siddaraju and Dharmesh.[37]

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Petrini O, Sieber TN, Toti L, Viret O. Ecology, metabolite production, and substrate utilization in endophytic fungi. Nat Toxins 1992;1:185-96.
2. Chandra S. Endophytic fungi: Novel sources of anticancer lead molecules. Appl Microbiol Biotechnol 2012;95:47-59.
3. Dompeipen EJ, Srikandace Y, Suharso WP, Cahyana H, Simanjunta P. Potential endophytic microbes selection for antidiabetic bioactive compounds production. Asian J Biochem 2011;6:465-71.
4. Strobel G, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM, *et al*. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology 1996;142(Pt 2):435-40.
5. Zhan J, Burns AM, Liu MX, Faeth SH, Gunatilaka AA. Search for cell motility and angiogenesis inhibitors with potential anticancer activity: Beauvericin and other constituents of two endophytic strains of *Fusarium oxysporum*. J Nat Prod 2007;70:227-32.
6. Strobel G, Daisy B. Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 2003;67:491-502.
7. Zhao J, Zhou L, Wang J, Shan T, Zhong L, Liu X, *et al*. Endophytic fungi for producing bioactive compounds originally from their host plants. Curr Res Technol Educ Trop Appl Microbiol Microb Biotechnol 2010;1:567-76.
8. Joshi CG, Gopal M, Byregowda SM. Cytotoxic activity of *Tragia involucrata* Linn. extracts. Am Eurasian J Toxicol Sci 2011;3:67-9.
9. Joshi CG, Gopal M, Kumari NS. Antitumor activity of hexane and ethyl acetate extracts of *Tragia involucrata*. Int J Cancer Res 2011;7:267-77.
10. Joshi GC, Gopal M. Antifertility activity of hexane and ethyl acetate extracts of aerial parts of *Tragia involucrata*. linn. J Pharmacol Toxicol 2011;6:548-53.
11. Samy RP, Gopalakrishnakone P, Houghton P, Ignacimuthu S. Purification of antibacterial agents from *Tragia involucrata* – A popular tribal medicine for wound healing. J Ethnopharmacol 2006;107:99-106.
12. Farook SM, Atlee WC. Antidiabetic and hypolipidemic potential of *Tragia involucrata* Linn. in streptozotocin-nicotinamide induced type II diabetic rats. Int J Pharm Pharm Sci 2011;3:103-9.
13. Rao NV, Benoy K, Hemamalini K, Kumar SS, Satyanarayana S. Pharmacological evaluation of root extracts of *Tragia involucrata*. Int J Pharm Sci Drug Res 2007;2:236-44.
14. Higginbotham SJ, Arnold AE, Ibañez A, Spadafora C, Coley PD, Kursar TA, *et al*. Bioactivity of fungal endophytes as a function of endophyte taxonomy and the taxonomy and distribution of their host plants. PLoS One 2013;8:e73192.
15. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82.
16. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 1999;299:152-78.
17. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem 1999;64:555-9.
18. Kanner J, Frankel E, Granit R, German B, Kinsella JE. Natural antioxidants in grapes and wines. J Agric Food Chem 1994;42:64-9.
19. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol 1995;28:25-30.
20. Kuriakose GC, Singh S, Rajvanshi PK, Surin WR, Jayabaskaran C. *In vitro* cytotoxicity and apoptosis induction in human cancer cells by culture extract of an endophytic *Fusarium solani* strain isolated from *Datura metel* L. Pharm Anal Acta 2014;5:293.
21. Ghanta S, Banerjee A, Poddar A, Chattopadhyay S. Oxidative DNA damage preventive activity and antioxidant potential of *Stevia rebaudiana* (Bertoni) bertoni, a natural sweetener. J Agric Food Chem 2007;55:10962-7.
22. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of Vitamin E. Anal Biochem 1999;269:337-41.
23. Bosshard PP, Abels S, Zbinden R, Böttger EC, Altwegg M. Ribosomal DNA sequencing for identification of aerobic gram-positive rods in the clinical laboratory (an 18-month evaluation). J Clin Microbiol 2003;41:4134-40.
24. Cui JL, Guo SX, Xiao PG. Antitumor and antimicrobial activities of endophytic fungi from medicinal parts of *Aquilaria sinensis*. J Zhejiang Univ Sci B 2011;12:385-92.
25. Ravindran C, Naveenan T, Varatharajan GR, Rajasabapathy R, Meena RM. Antioxidants in mangrove plants and endophytic fungal associations. Bot Mar 2012;55:269-79.
26. Rodriguez RJ, White JF Jr., Arnold AE, Redman RS. Fungal endophytes: Diversity and functional roles. New Phytol 2009;182:314-30.
27. White JF Jr., Torres MS. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol Plant 2010;138:440-6.
28. Hamilton CE, Gundel PE, Helander M, Saikkonen K. Endophytic mediation of reactive oxygen species and antioxidant activity in plants: A review. Fungal Divers 2012;54:1-10.
29. Singh N, Rajini PS. Free radical scavenging activity of an aqueous extract of potato peel. Food Chem 2004;85:611-6.
30. Xing R, Yu H, Liu S, Zhang W, Zhang Q, Li Z, *et al*. Antioxidant activity of differently regioselective chitosan sulfates *in vitro*. Bioorg Med Chem 2005;13:1387-92.
31. Yadav M, Yadav A, Yadav JP. *In vitro* antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* lam. Asian Pac J Trop Med 2014;7S1:S256-61.
32. Wang T, Jonsdottir R, Ólafsdóttir G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Food Chem 2009;116:240-8.
33. Devi NN, Prabakaran JJ, Wahab F. Phytochemical analysis and enzyme analysis of endophytic fungi from *Centella asiatica*. Asian Pac J Trop Biomed 2012;2:S1280-4.
34. Itharat A, Houghton PJ, Eno-Amooquaye E, Burke PJ, Sampson JH, Raman A, *et al*. *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. J Ethnopharmacol 2004;90:33-8.
35. Nascimento do AM, Conti R, Turatti IC, Cavalcanti BC, Costa-Lotufo LV, Pessoa C, *et al*. Bioactive extracts and chemical constituents of two endophytic strains of *Fusarium oxysporum*. Rev Bras Farmacogn 2012;22:1276-81.
36. Ruma K, Kumar S, Prakash HS. Antioxidant, anti-inflammatory, antimicrobial and cytotoxic properties of fungal endophytes from *Garcinia* species. Int J Pharm Pharm Sci 2013;5:889-97.
37. Siddaraju MN, Dharmesh SM. Inhibition of gastric H+, K+-ATPase and *helicobacter pylori* growth by phenolic antioxidants of *Zingiber officinale*. Mol Nutr Food Res 2007;51:324-32.

SUPPLEMENTARY TABLES

Supplementary Table 1: 1 IC_{50} value endophytic fungal extracts and ascorbic acid analyzed by DPPH method

Name of the fungal extracts	IC_{50} (µg/mL)
P. citrinum CGJ-C1	40.81
P. citrinum CGJ-C2	15.81
Cladopsporium sp. CGJ-D1	12.73
C. hypophloia CGJ-D2	4.01
Ascorbic acid	1.55

Supplementary Table : 2 Cancer cells treated with different concentrations of endophytic fungal extracts were assayed for cell viability by MTT method 48 h and 72 h and IC_{50} values were calculated using regression equation

Name of the fungal extracts	IC_{50} value µg/mL			
	MCF-7		MOLT-4	
	48 h	72 h	48 h	72 h
P. citrinum CGJ-C1	2.29	1.50	3.26	2.97
P. citrinum CGJ-C2	1.27	1.09	2.07	2.38
Cladopsporium sp. CGJ-D1	1.25	3.87	34.43	8866
C. hypophloia CGJ-D2	6.24	2.903	5.29	5.41