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Research Article

Radical scavenging potency, HPLC profiling and phylogenetic analysis of endophytic fungi isolated from selected medicinal plants of Saudi Arabia

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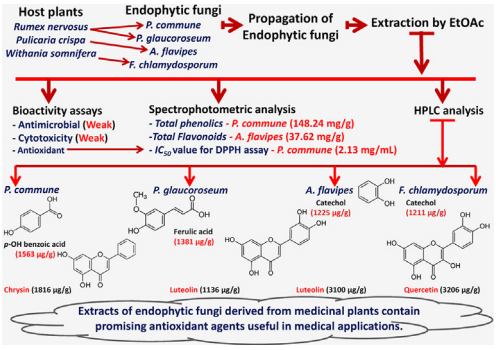
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Endophytic fungi are associated within plant tissues without any harmful effects. They produce a diversity of metabolites and are well known as rich source of bioactive substances. This study aimed to identify fungal isolates from different medicinal plants, evaluate their antioxidant potency and detect components responsible for antioxidant activity.

Results: In the present investigation, 4 endophytic fungal isolates were obtained from different medicinal plants *Rumex nervosus*, *Pulicaria crispa* and *Withania somnifera*. Those isolates were characterized as *Penicilluim commune*, *Penicilluim glaucoroseum*, *Aspergillus flavipes* and *Fusarium chlamydosporum* according to morphological and phylogenetic bases. These strains were propagated on humified rice and their crude ethyl acetate extracts were assayed for their total phenols, flavonoids and antioxidant potency against DPPH free radicals. The results showed that the elevated constituent of phenolics was found in *P. commune* (148.24 mg/g), followed by *F. chlamydosporum* (124.25 mg/g), while the highest total flavonoids were determined in *A. flavipes*, followed by *F. chlamydosporum*, *P. glaucoroseum*, *P. commune*, respectively. Moreover, the maximum hydroxyl radical-scavenging assay was IC_{50} = 2.13 mg/mL exhibited by *P. commune*, followed by *F. chlamydosporum* (2.68 mg/mL). Weak antimicrobial and cytotoxic effects were reported by the studied strains against pathogenic isolates and (HepG2) hepatocellular carcinoma cell

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Penicilluim glaucoroseum
Phenolics

line. The fungal EtOAc extracts were characterized quantitatively by the incidence of different phenols, flavonoids, and phenolic acids, as detected by HPLC.
Conclusions: Endophytic fungi derived from medicinal plants are a promising candidate for the wide industrial production of antioxidant agents with several useful medical and pharmaceutical applications.
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1. Introduction

The maximum phases of reactive oxygen species (ROS), in contrary to the properties of antioxidant, are important as they play an essential role in different incessant age-related illnesses besides maturing. All aerobic microbial cells can create the larger part of their chemical vitality by devouring oxygen through mitochondria. Thus, mitochondria are considered the major intracellular source for oxygen utilization and ROS establishment [1,2]. ROS including hydroxyl anions and superoxide are byproducts of normal metabolism in microorganisms, plants and animals. ROS in high concentrations might cause oxidative destruction to biological molecules as lipids, proteins, and DNA [3]. Living cells produce natural antioxidants to maintain redox status by controlling ROS. The loss of balance free radical and natural endogenous antioxidant production can lead to oxidative stress [4]. Antioxidants from varied species are a wide group of chemical active compounds that found the primary line of protection against various free radical losses; therefore, they are important for keeping up an ideal health and are defensive factors able to stabilize or inactivate free radicals before the free radicals assault cells [5]. The demand level of endogenous natural antioxidants to evade oxidative stress and prevent cell damage can be achieved by exogenous natural antioxidant sources [6]. Antioxidants also exert a significant impact on accumulation of the carcinogenic aflatoxin B_1 by the fungus *Aspergillus flavus* as described earlier [7].

Multiple antioxidants recently are artificially synthesized via industrial sector although they cause health problems such as liver carcinogenesis [8]. Conversely, natural antioxidant derivatives, such as those produced by endophytic fungi, appear to be not harmful. Particularly, as a result of high biodiversity and biochemical advancement [9], endophytic fungi have the potential to utilize various substances, producing a large variety of active metabolites [10]. Antibiotics, anticancer agents, antiviral, antidiabetic, immunosuppressive compounds, insecticidal by-products, in addition to antioxidants well reported as active metabolites from fungal endophytes, and remedial plants also have been considered as a great storage of endophytic fungi producing new substances of biopharmaceutical and industrial values [11].

Medicinal plants supply therapeutic molecules such as terpenoids, phenolics, alkaloids and flavonoids. Among these, flavonoids and polyphenols play a critical function in antioxidant free radical scavenging and several other related bio-pharmaceutical activities [12]. Dietary antioxidants, including polyphenolic compounds are most successful in the avoidance of oxidative stress related infections [13]. Moreover, polyphenolic compounds are remarkable active metabolites existing in both plants and fungi [14]. A vast number of naturally occurring products were established to have antioxidant properties, including phenolics and other flavonoids [15]. Fungal endophytes combined with therapeutic plants are a promising antioxidant resource [16]. Many members diversity, maximum antioxidant potency revealing, bioactive metabolites abundant endophytic fungi relate to divisions of mainly Ascomycota followed by Basidiomycota [17].

Extracts obtained from endophytic fungi were found to possess various biological activities. Endophytic fungus *Alternaria tenuissima* AUMC14342 isolated from *Artemisia judaica* L. possessed a potent antimicrobial efficiency [18], while members belonging to class Ascomycetes endophytic fungal extracts showed significant antibacterial activity against one or more multidrug resistant bacteria [19]. The ethyl acetate extracts of the endophytic *Alternaria alternata* PGL-3, *Cochlibolus lunatus* PML-17, *Nigrospora sphaerica* EPS-38, and *Emerecilla nidulans* RPL-21 from Egyptian medicinal plants exhibited potent inhibition of HCV NS3/4A protease [20]. Metabolites from the mangrove endophytic fungus *Penicillium chermesinum* exhibited cytotoxicity toward certain cancer cell lines [21]. *Chaetomium* sp., *Aspergillus peyronelii* and *A. niger* endophytic fungi isolated from *Eugenia jambolana* showed highest antioxidant activity [22].

The DPPH method is among the most widely applied *in vitro* assays to check the capability of tested compounds acting as antiradicals or hydrogen donors that could be employed easily, performed promptly and is inexpensive [23]. Based on the investigated antioxidant activity of *R. nervosus* [24], *Pulicaria crispa* [25], and *Withania somnifera* [26], this research aimed to: (i) identify fungal isolates in correlation with target medicinal plants, (ii) evaluate the antioxidant potential of promising isolated strains, and (iii) determine the phenolic and flavonoid components responsible for antioxidant activity using HPLC analysis.

2. Materials and methods

2.1. Sample collection

Three medicinal plants, *Rumex nervosus*, *P. crispa* and *W. somnifera* were collected from Al Mandaq, (20.113607°N, 41.2854624°E), Al Bahah, Saudi Arabia. For isolation and purification of fungal endophytes, whole plants including roots and aerial parts were stored in sterile plastic bags in a cooler with ice during collection and refrigerated, then transferred to the laboratory under aseptic conditions. All samples were processed within collection time of 24–48 h. Collected samples were then rinsed with deionized water to avoid soil particles and debris. Plants were identified by staff members, specialists in Botany based on reference books with species descriptions, keys, and illustrations for plant identification, and the voucher specimens were preserved at the department herbarium.

2.2. Isolation procedures

The freshly gathered leaves of selected plants were carefully surface washed and then sterilized for the isolation process as previously reported [27]. Briefly, the isolation procedures of fungi were conducted on potato dextrose agar medium (PDA) contained dextrose (20 g/L), potato infusion (200 g/L), and agar–agar (20 g/L) into 1 L distilled water with a pH of 5.6 ± 0.2. All culture plates were then incubated for 10–14 d at 28°C. The obtained dominant

fungal mycelia protubering from the plant pieces were picked, subcultured and identified according to morphological characteristics and microscopical examination. Then, all isolates were stored on slants containing PDA medium at 4°C for further molecular level identification with the help of the internal transcribed spacer (ITS) region of DNA.

2.3. Molecular identification of isolated fungi

2.3.1. Isolation of genomic DNA

Czapek-Dox broth culture medium was employed to cultivate the pure fungi along 5 d at 25–28°C. The extraction procedures of the total DNA from each isolate were performed using a specific kit "Norgen Plant/Fungi DNA Isolation Kit (Sigma, Thorold, Canada)" according to Hassan et al. [28]. DNA was kept after elution at −20°C for further studies.

2.3.2. PCR amplification and nucleotide sequence analysis

Specific universal primers ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used in the present to amplify the ITS region of selected strains as previously suggested by Mohamed et al. [29]. PCR was achieved as reported by Hassan et al. [28], and the mixture of PCR tubes contained of 25 μL *viz.*, 12.5 μL of PCR master mix, 1 μL from both forward and reverse primers (approx. 20 pmol), genomic DNA 1 μL and 9.5 μL of ddH_2O. The amplification of DNA was carried out in Thermocycler "C1000 Touch™ (Bio-Rad, Germany). The obtained PCR products were resolved on agarose gel 1.5% using TBE buffer, and visualized through a gel documentation system; then, the gel extraction kit (Omega Bio-tek) was applied to purify the PCR product based on the manufacturer's instructions. All PCR products were sequenced with the gene analyzer 3121 sequencing service (Macrogen Co., Seoul, South Korea). The four sequences of selected strains were inspected with BLAST search tool at (NCBI) to detect sequence similarity. Obtained sequences were checked and analyzed using BioEdit software program version No. 7.2.5. Tested isolates were identified by comparison to isolates from the sequencing databases using a BLAST of the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Fermentation process of fungal metabolites

Fermentation process and ethyl acetate (EtOAc) extraction of metabolites of the isolated strains were performed on solid rice medium. The fermentation flasks were inoculated with approximately 1 mL of freshly fungal spore suspension ($10^6 \sim 10^7$ spores/mL), and then incubated in dark for 25–30 d at 30°C [18]. The fermented medium was extracted two times by 500 mL EtOAc as a universal organic solvent used to extract many bioactive polar and non-polar compounds to assay their effectiveness [30], and the extract was carefully filtered over anhydrous sodium sulfate and dried at 40–50°C using a vacuum rotary evaporator.

2.5. Estimation of total phenolic content

Total phenolic content assay was assessed using modified procedures [31]. Briefly, a 0.5 mL of the specimen (10 mg/mL) was added with the same volume of Folin–Ciocalteu's phenol reagent, followed by adding 1 mL of 10% Na_2CO_3 after 3 min to the reaction solution. Incubation of the mixture was proceeded under shaking and dark conditions at 180 rpm for 60 min at 25°C. Measurement of the absorbance was at 750 nm. Phenolic content was declared as gallic acid equivalent (GAE) (mg/g) through the later equation due to the standardization curve: $y = 0.0169x - 0.1172$, $R^2 = 0.9588$. The standard curve of gallic acid was linear between 0.5 and 100 μg/mL.

2.6. Determination of total flavonoid content

A previous method reported by Quettier-Deleu et al. [32], was conducted to measure total flavonoid content. 0.5 mL of each extract (10 mg/mL), was carefully mixed with 1.0 mL of a 2% (v/v) $AlCl_3.6H_2O$ ethanolic solution and the absorbance was measured at 430 nm after 10 min. Total flavonoid content was expressed as quercetin equivalent (QE) (mg/g) by employing the later equation due to the standardization curve: $y = 0.0208x - 0.2381$, $R^2 = 0.9678$. The calibration curve of quercetin was linear between 0.5 and 100 μg/mL.

2.7. Radical scavenging activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay

The antioxidant properties of the test samples were measured as scavenging activity or hydrogen donating form based on the procedure reported before by Brand-Williams et al. [33]. While the DPPH radical is scavenged, the color was changed from purple to yellow with a 517 nm absorbance decreasing. Then, 1.8 mL of 0.1 mM DPPH (Sigma-Aldrich, Germany) (4 mg/100 mL of methanol) mixture solution was added to 0.2 mL of the tested samples in absolute methanol at various concentrations (10, 5, 2.5, 1, 0.5, 0.1 and 0.05 mg/mL) beside the blank. The mixture was left aside at room temperature (shaken vigorously in-between) for 30 min. and absorbance was determined by spectrophotometer (Jenway 7315) at 517 nm. Butylated hydroxytoluene (BHT) was employed as a positive control and all measurements were performed in triplicate. The following formula was carried out to determine the capacity to scavenge the DPPH radical:

$$\% \text{ DPPH radical scavenging} = \frac{A\text{-}B}{A} \times 100$$

where A is the negative control absorbance (methanol and DPPH) and B is the sample absorbance (DPPH, methanol and sample). The IC_{50} was obtained by interpolation from linear regression analysis, where the obtained regression equation was $y = ax + b$, IC_{50} was calculated as $IC_{50} = (0.5 - b)/a$. IC_{50} value denotes the level of the antioxidant capacity of the tested extracts. The IC_{50} value is inversely proportional to the free radical scavenging property of the sample.

2.8. Quantitative analysis of phenolics and flavonoids using high performance liquid chromatography (HPLC)

HPLC (Agilent 1100, U.S.A.) with automatic injection, equipped with a quaternary gradient pump, in-line degasser, and dual wavelength UV/Vis detector was used. The chromatographic separations were fulfilled on a C18 column (25 × 0.4 cm, particle size 5 mm). The temperature of the used column was set to 40°C, the injection volume was adjusted to 10 μL, and the flow rate was set to 1 mL/min. Phenolics were separated using gradient mobile phases, glacial acetic acid/acetonitrile/water (5/20/980, v/v/v, pH 2.68) and acetonitrile/glacial acetic acid (1000/5, v/v) with detection at 325 nm. Flavonoid quantification was performed via a mobile phase composed of 1% formic acid and acetonitrile in distilled water (90:10, v/v) with detection at 280 nm. The resolved compounds were detected, identified and quantified on the basis of chromatographic retention times of pure standards. External flavonoid and phenolic standards were used for the identification and quantification of compounds. Based on peak area computation, the quantification of compounds was analyzed [34].

2.9. Antimicrobial assay

The antimicrobial activities were carried out by well diffusion method as reported by Jahangirian et al. [35] against pathogenic isolates including two Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*), two Gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumonia*) and two yeasts (*Candida albicans* and *C. glabrata*). Well diameter was 8 mm filled with 50 μL of the 100 mg/mL (w/v) test samples. Chloramphenicol (0.5%) was used as positive control for bacteria and clotrimazole (0.5%) for yeasts, while dimethylsulfoxide (DMSO) was used as negative control.

2.10. Cytotoxic activity

The HepG2 were cultured in DMEM (supplemented with 100 mg/mL streptomycin, 10% heat-inactivated fetal bovine serum, and 100 units/mL penicillin), in a humidified, 5% (*v/v*) CO_2 atmosphere at 37°C. The optical density (OD) was calculated, and the determination of cell viability percentage was as follows:

$$\text{Viability of the cells } (\%) = \frac{\text{OD of treated cells}}{\text{OD control}} \times 100\ \%$$

The WST-1 assay was performed to assess cell viability using Abcam® kit ab155902 WST-1 Cell Proliferation Reagent [36] and the fungal EtOAc extracts were assayed at 10 and 100 μg/mL along with camptothecin as a standard.

2.11. Data analysis

All tests and measurements were performed thrice. Using the SPSS, software program (version No. 16), and one-way ANOVA, the values were expressed as the mean ± SE at the 0.05 significance level.

3. Results

3.1. Isolates and sequence data

Based on the culture features shown in Table 1, four dominant endophytic fungal species were derived from the leaf parts of three different tested plants: *Penicilluim commune* and *Penicilluim glaucoroseum* from *R. nervosus*, *Aspergillus flavipes* from *P. crispa* and *Fusarium chlamydosporum* from *W. somnifera*. The multiple nucleotide sequence alignment of the ITS region in various fungal isolates (*P. commune* TU-17, *P. glaucoroseum* TU-13, *A. flavipes* TU-32 and *F. chlamydosporum* TU-36) were derived. The resulting ITS sequences were aligned with different sequences, using the online searching tool BioEdit (http://www.mbio.ncsu.edu/BioEdit/page2.html). The intra- and interspecies variables were detected. Sequence alignments of the four fungal isolates with other sequences on the database showed 97–100% similarity within all strains. *P. glaucoroseum* TU-13 showed similarity ranging from 97 to 99% with *P. commune* MN589647 and *P. commune* KU743963. *P. glaucoroseum* TU-13 showed 100% similarity with *P. glaucoroseum* MH865551 and *P. glaucoroseum* MT529148. *A. flavipes* TU-32 also, showed 100% similarity with *A. flavipes* MT322622 and *A. flavipes* KY964058, respectively. However, *F. chlamydosporum* TU-36 showed similarity ranging from 98 to 99% with *F. chlamydosporum* KC778405 and *F. chlamydosporum* MG748660. Larger differences between the other fungal strains were also observed. Additionally, 100% homology was observed between the two *A. flavipes* TU-32 and *P. glaucoroseum* TU-13 strains with related strains in the database of GenBank. The ITS sequences positions, counting the variations among the tested strains, are listed in Fig. 1. Phylogenetic investigation of a phylogeny with the nucleotide sequences of the four fungal strains of the cloned ITS regions which were constructed using special software MEGA 5.1 was done.

The selected strains were divided into two distinct phylogenetic subclades (Fig. S1). Group 1 contained *P. glaucoroseum* TU13 *P. glaucoroseum* TU-13, *A. flavipes* TU-32 and related strains in the GenBank database. However, group 2 included *F. chlamydosporum* TU-36, *F. chlamydosporum* KC778405 and *F. chlamydosporum* MG748660. A phylogenetic study of all ITS nucleotide sequences showed a significant phylogenetic distance of roughly 100 percent. The similarity index data reflect the similarities within the isolates. As a result, the ITS analysis clearly distinguished the four species.

3.2. Assay of total (phenols and flavonoids) and antioxidant assay of selected strains

The purpose of the present work was to achieve fungal isolates with their antioxidant assay from chosen medicinal plants. Total flavonoids and total phenols, which are major components associated with antioxidant activity, were investigated. Our findings exhibited significant variation among various tested extracts. A maximum yield of total phenols was detected in *P. commune* extracts (148.24 mg/g), followed by *F. chlamydosporum* (124.25 mg/g), *P. glaucoroseum* (113.56 mg/g), and *A. flavipes* (105.16 mg/g), while total flavonoids were within the arrangement of *A. flavipes* > *F. chlamydosporum* > *P. glaucoroseum* > *P. commune* as shown in Table 2. Thus, *P. commune* and *F. chlamydosporum* exhibited the highest phenol content, while *A. flavipes* and *F. chlamydosporum* exhibited the highest flavonoid content, which was responsible for their higher effectiveness in scavenging radicals. IC_{50} values indicated that all four fungal isolates had potentially maximum DPPH radical-scavenging activities. The maximum activity reached up to (IC_{50} = 2.13 mg/mL) in case of *P. commune*, followed by *F. chlamydosporum* (2.68 mg/mL), *A. flavipes* (3.09 mg/mL), and *P. glaucoroseum* (4.03 mg/mL), and the IC_{50} value of the positive control BHT was 4.50 mg/mL.

3.3. HPLC analysis

In the current study, phenolic and flavonoid components of EtOAc extracts from isolated fungi were investigated by HPLC. The analysis of phenolics displayed that the *P. commune* strain produced high amounts of *p*-OH benzoic at 1563 μg/g, while ferulic and protocatechulic acids were detected in *P. glaucoroseum* strain EtOAc extract at (1381 and 1016 μg/g), respectively. Strain *A. flavipes* produced catechol and cinnamic acid at (1225 and 1044 μg/

Table 1
Selected endophytes isolated from different host plants, and their classification.

Fungal strains	GenBank accession nos.	Host Medicinal plants	Fungal Phylum; Class; Order	Plant family
P. commune TU-17	MK804731	*Rumex nervosus* Vahl.	Ascomycota; Eurotiomycetes; Eurotiales	Polygonaceae
P. glaucoroseum TU-13	MK804727			
A. flavipes TU-32	MK804746	*Pulicaria crispa* (Forssk.) Benth. ex Oliv.		Asteraceae
F. chlamydosporum TU-36	MK804750	*Withania somnifera* (L.) Dunal	Ascomycota; Sordariomycetes; Hypocreales	Solanaceae

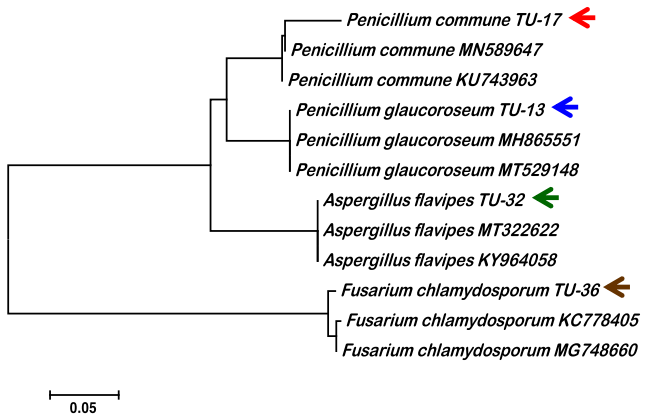

Fig. 1. Neighbor-joining (NJ) phylogenetic tree derived from concatenated sequence alignment of ITS sequences showing genetic diversity of the fungal isolates aligned with other closely related strains accessed from GenBank database.

Table 2
Contents of flavonoids, phenolics, and IC_{50} values from antioxidant assays from tested fungal endophyte strains of medicinal plants.

Fungal strains	Phenols (mg/g)	Flavonoids (mg/g)	IC_{50} value (mg extract/mL DPPH radicals)
P. commune	148.24 ± 0.962[a]	22.68 ± 0.116[d]	2.13 ± 0.004[a]
P. glaucoroseum	113.56 ± 1.657[c]	34.28 ± 0.354[c]	4.03 ± 0.016[d]
A. flavipes	105.16 ± 1.092[d]	37.62 ± 0.032[a]	3.09 ± 0.014[c]
F. chlamydosporum	124.25 ± 0.921[b]	35.12 ± 0.097[b]	2.68 ± 0.010[b]
BHT	-	-	4.50 ± 0.012[e]

(-): Not detected. The data were given as averages of three replicates (Mean ± SE). Values followed by the different letters are significantly different at $p < 0.05$.

g), respectively, while catechol and *p*-OH benzoic acid were determined in the strain *F. chlamydosporum* extract at (1211 and 1016 μg/g), respectively (Table 3 and Fig. 2).

The results of flavonoid analysis using HPLC (Table 4 and Fig. 3) exhibited that the *P. commune* strain produced chrysin, acacetin and epicatechin at (1816, 1433 and 1732 μg/g), respectively, while apigenin and luteolin at 1023 and 1136 μg/g, respectively, was produced by *P. glaucoroseum* strain. The *A. flavipes* strain produced rutin, apigenin, luteolin, acacetin and quercetin at (1030, 3100, 2040 and 1130 μg/g), respectively, while a high amount of quercetin (3206 μg/g) was detected in the extract obtained from *F. chlamydosporum* strain. These results demonstrated that the obtained fungal strains from medicinal plants are prospect origins of naturally antioxidant products. Fig. 4 illustrates structures of detected phenolic and flavonoid compounds in different fungal endophyte extracts.

3.4. Antimicrobial activity

The antimicrobial activity of the tested fungal extracts showed that all tested extracts had weak or no activity against tested pathogenic bacteria and *Candida* in comparison to that of their reference control (Table 5).

3.5. Evaluation of cytotoxicity activity using WST-1

The cytotoxicity results getting from the WST-1 testing were evaluated based on the cell viability and correlated with diluted DMSO as a control. As demonstrated in Table 6, the cytotoxicity assay indicated that the EtOAc extract of chosen strains at concentrations of 100 and 10 μg/mL showed weak cytotoxicity against HepG2.

4. Discussion

Endophytic fungi produce a diversity of metabolites and are well known as rich source of bioactive substances. In the current study, four endophytic fungal strains (*P. commune*, *P. glaucoroseum*, *A. flavipes*, and *F. chlamydosporum*) were obtained from selected medicinal plants. Similar to our study by Atri et al. [37] isolated *Fusarium* sp. from *W. somnifera* leaves parts, while, Das et al. [38] identified *F. chlamydosporum* fungal endophyte from *Zingiber nimmonii*. Sequence analysis was used by Bergamaschi et al. [39] to distinguish between *P. glaucoroseum* and *P. commune*, which is similar to our findings. ITS sequences have been used by many other researchers to identify *F. chlamydosporum* [40]. Various alignments of the ITS sequences of all tested fungi revealed nucleotide sequence discrepancies that allowed these organisms to be distinguished. Both ends of the ITS region displayed nucleotide sequence differences in the multiple alignment. The 5.8S ribosomal DNA gene sequences were established to have perfect homology [41].

In the present study, we investigated the total phenols and flavonoids in the obtained strains based extracts; our findings showed that maximum phenols and flavonoids were detected. On the other hand, the total phenolic content (TPC) of the *F. chlamydosporum* extract was 26.64 mg GAE/g dry weight. Meanwhile, in *F. chlamydosporum,* the TFC was achieved as 22.9 mg catechin equivalent/g dry extract [38]. However, tannins and coumarins were screened as traces in extra- and intrafungal extracts of *F. chlamydosporum* soil isolate [42].

Moreover, the EtOAc extracts' DPPH radical-scavenging activities were detected at maximum levels in the isolated strains. *P.*

Table 3
HPLC analysis of total phenolic components of fungal extracts.

No.	Retention time	Phenolic compounds	Concentration (μg/g)			
			P. commune	*P. glaucoroseum*	*A. flavipes*	*F. chlamydosporum*
1	3.0	Catechol	-	-	1225	1211
2	4.1	Caffeic acid	655	461	98	344
3	4.98	Ferulic acid	424	1381	514	507
4	6.89	Gallic acid	-	507	864	-
5	8.12	Chlorogenic acid	588	677	-	-
6	8.89	*p*-OH benzoic acid	1563	-	-	1016
7	10.0	Cinnamic acid	462	-	1044	-
8	11.4	Salicylic acid	56	-	-	-
9	12.0	Ellagic acid	609	-	765	-
10	13.0	Pyrogallol	-	-	-	714
11	14.2	Protocatechulic acid	-	1016	-	-

(-): Not detected.

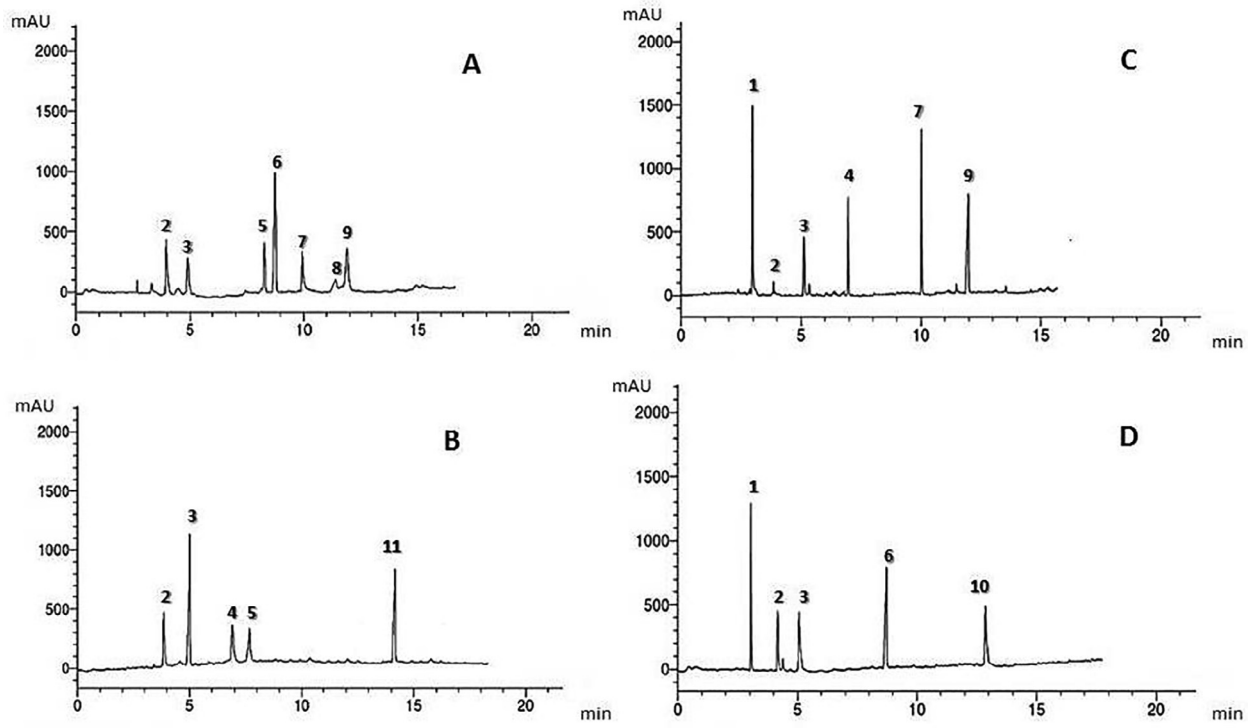

Fig. 2. HPLC chromatogram of phenolic components in fungal endophyte extracts (A) *P. commune*, (B) *P. glaucoroseum*, (C) *A. flavipes*, and (D) *F. chlamydosporum*.

Table 4
HPLC analysis of flavonoid components in fungal endophyte extracts.

No.	Retention time	Flavonoid compounds	Concentration (µg/gm)			
			P. commune	*P. glaucoroseum*	*A. flavipes*	*F. chlamydosporum*
1	4.02	Rutin	420	304	212	-
2	5.0	Apigenin	-	1023	1030	612
3	7.0	Chrysin	1816	69	-	588
4	8.0	Luteolin	189	1136	3100	-
5	9.5	Acacetin	1433	-	2040	-
6	10.2	Naringenin	-	-	822	201
7	11.5	Kaempferol	-	-	412	-
8	12.11	Isovitexin	-	77	-	416
9	13.0	Quercetin	217	869	1130	3206
10	14.0	Epicatechin	1732	-	914	-

(-): Not detected.

commune (marine-derived fungus from mollusks) EtOAc extract exhibited IC_{50} 42.35 µg/mL) antioxidant activity determined by DPPH assay [43]. An earlier study by El-Sabagh et al. [44] proved that a *P. crispa* methanolic extract was compelling in repressing DPPH free radicals with IC_{50} values of 0.51 mg/mL and 172.24 µg/mg total flavonoid content (TFC). Meanwhile, the *F. chlamydosporum* ethyl acetate extract showed (IC_{50} 226.9 µg/mL) DPPH radical scavenging capacity [38]. Most endophytic fungi are famed to have synthetic capacities higher than their host plants, based on their long coevolution integrated with hereditary recombination as reported by Fernandes [9]. In this respect, high concentration levels of phenolics and flavonoids were determined in fruits, roots and leaves of *W. somnifera* ranging from17.80 to 32.58 mg/g (dry mass), and significant DPPH radical scavenging activity ranged between 59.16 and 91.84% [26].

Phenolics including catechol, pyrogallol, caffeic, ferulic, chlorogenic, *p*-OH benzoic, cinnamic, salicylic gallic, ellagic acids and protocatechulic acid, as well as, flavonoids including rutin, apigenin, chrysin, luteolin, naringenin, kaempferol, isovitexin, acacetin, quercetin and epicatechin were detected at different concentrations within the fungal ethyl acetate extracts using the HPLC analysis. Das et al. [38] detected quercetin concentration of 5.82 mg/g by ESI-MS/MS analysis of HPLC fragments of *F. chlamydosporum* ethyl acetate extract. GC–MS/MS analysis compounds from the *A. flavipes* ethyl acetate extract were chemically confirmed as amodiaquine, isovitexin, 7,3,4,5′-tetramethoxyflavanone, and 3-hydroxy-2′,4,4′,6′-tetramethoxychalcone at concentrations of 11.4, 26.4, 9.1, and 35.8%, respectively [45]. In another study by Diblasi et al. [46], they detected lovastatin in fermented products from a *P. commune* culture with a yield of 1.56 g/L. Meanwhile, withanolides produced by *Talaromyces pinophilus fungus* were isolated from leaves of *W. somnifera* in the culture medium [47]. Withanolides have been obtained from the leaves and root parts of *W. somnifera* that dominate minimum levels [48].

Multiple polyphenols (syringic, gallic, vanillic acids, p-coumaric and benzoic, in addition to kaempferol, catechin, and naringenin) were verified by the analysis of HPLC different parts of *W. somnifera*. Moreover, catechin was determined at the maximum concentration ranging between 13.01 and 30.61 mg/g, compared to other polyphenols [26]. Another analysis using (UPLC/HRMS) was

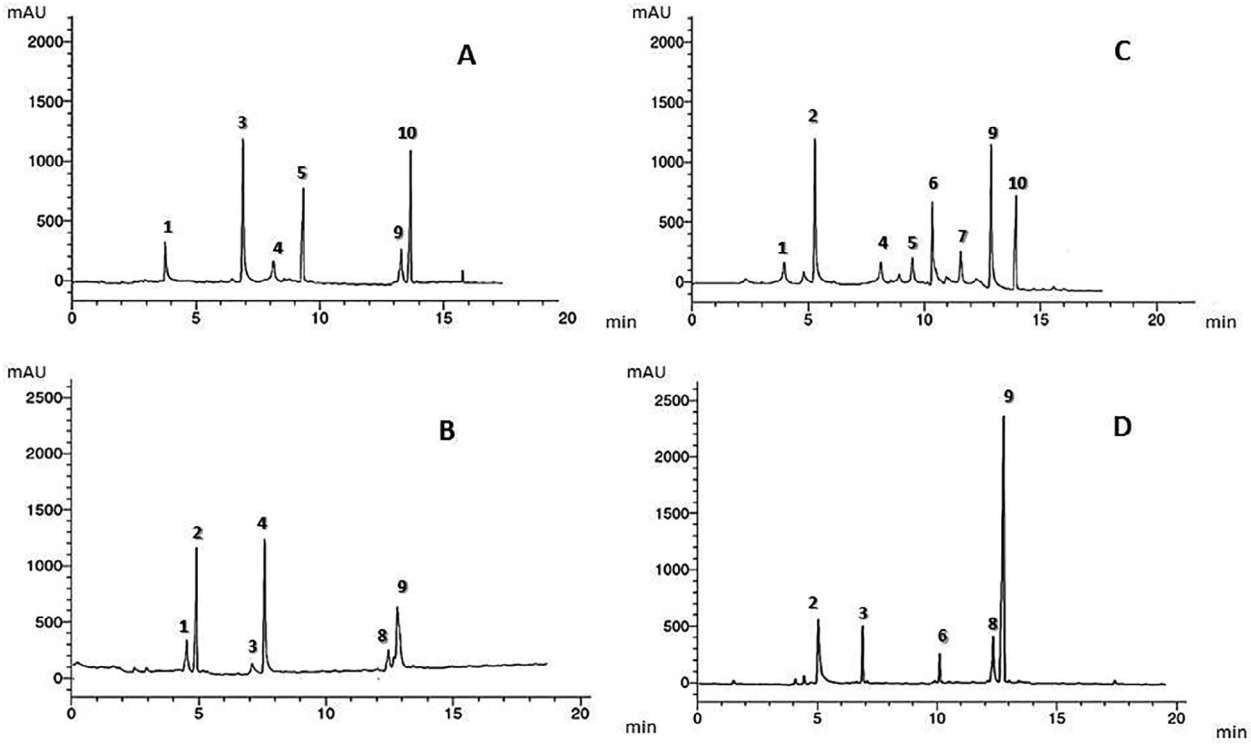

Fig. 3. HPLC chromatogram of components in fungal endophyte extracts (A) *P. commune*, (B) *P. glaucoroseum*, (C) *A. flavipes*, and (D) *F. chlamydosporum*.

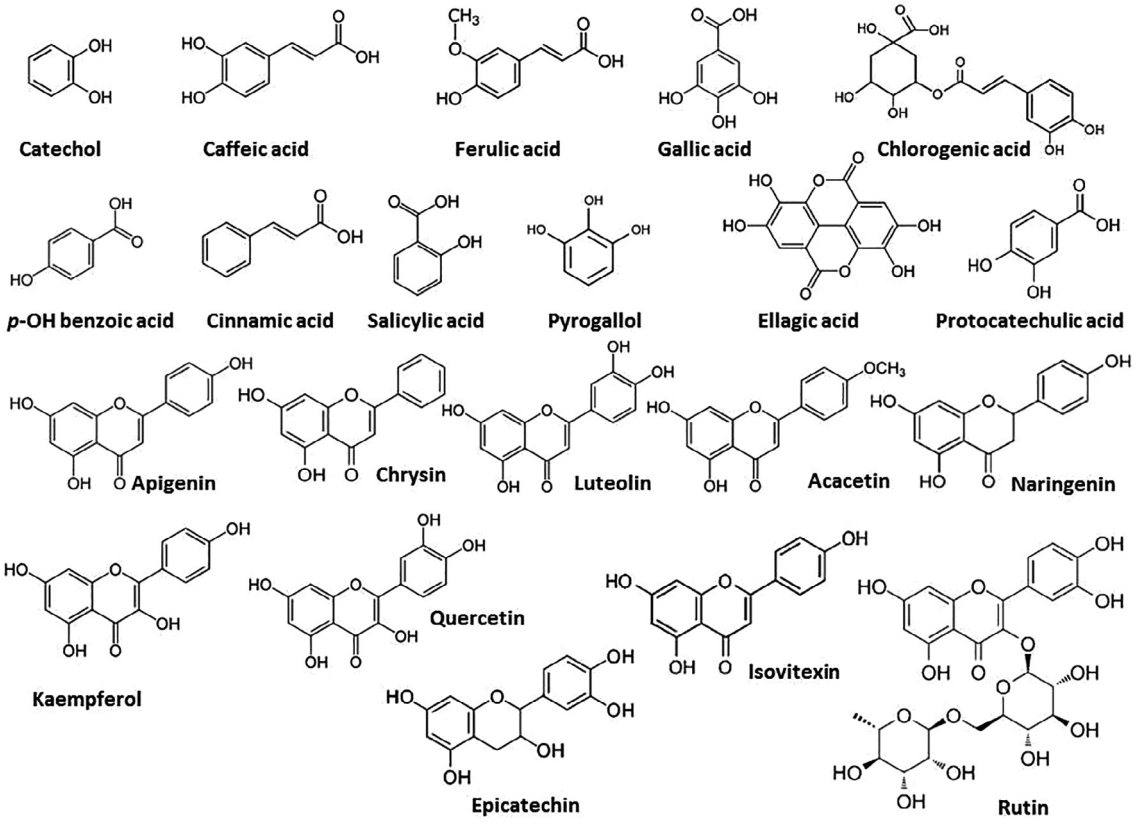

Fig. 4. Structures of detected phenolic and flavonoid compounds in different fungal endophyte extracts.

employed to determine the *P. crispa* metabolic profiles and revealed 15 phenolic acid and phenolic acid derivatives and 22 flavonoids [44]. Besides the antioxidative potency of flavonoids, they exhibited other benefits, for example, rutin being a potent of α-glucosidase inhibitor that is useful for diabetes control [49]. The cytotoxic activity of the *P. commune* EtOAc extract showed an

Table 5
Antimicrobial activity assay of tested fungal endophyte extracts.

Fungal strains extracts	Diameter of inhibition zone (mm)					
	B. subtilis	*S. aureus*	*E. coli*	*K. pneumonia*	*C. albicans*	*C. glabrata*
P. commune	11	10	-	-	9	10
P. glaucoroseum	-	-	-	-	10	-
A. flavipes	10	-	-	-	-	-
F. chlamydosporum	-	-	9	-	-	-
Chloramphenicol	21	19	16	18	-	-
Clotrimazole	-	-	-	-	20	23

(-): Not detected.

Table 6
Cell viability assessed by WST-1 assay of fungal endophyte extracts.

Fungal extracts Conc.	Viability%			
	P. commune	*P. glaucoroseum*	*A. flavipes*	*F. chlamydosporum*
100 µg/mL	89.46 ± 0.55	96.23 ± 0.24	94.32 ± 0.81	96.23 ± 0.74
10 µg/mL	98.30 ± 0.31	98.46 ± 0.09	98.22 ± 0.79	98.23 ± 0.26

inhibitory effect against colon carcinoma (HCT-116) cell line with IC_{50} (182.98 µg/mL), where camptothecin was applied as a positive standard drug [43].

5. Conclusions

In this study, endophytic fungal crude extracts of *P. commune* and *P. glaucoroseum* from *R. nervosus*, *A. flavipes* from *P. crispa* and *F. chlamydosporum* had shown to contain high levels of phenolic and flavonoid contents, in addition to bioactivity against DPPH free radicals. Moreover, the potential antimicrobial and cytotoxic activities were evaluated by the studied strains. This study used HPLC analysis for the EtOAc organic solvent extract of the selected strains, resulting in the determination of several bioactive chemical substances that have antioxidant features. Concerning research on antioxidants and their bioactivity, further work is needed to study the related genes involved in their activity and biochemical pathways. Additionally, the production of phenolics and flavonoids may help in various industrial applications. Therefore, our work concludes that the associated endophytic fungi with selected plants might be proposed to be a potent candidate in industrial high scale production of antioxidants, medical sectors, and in pharmaceutical advancement.

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Conflicts of interest

The authors declare no conflict of interest.

Supplementary material

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