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Investigation the biological activities and the metabolite profiles of endophytic fungi isolated from Gundelia tournefortii L.

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Abstract

Endophytic fungi are microorganisms that are considered as a potential source of natural compounds, and can be applied in various industries. The aims of this research was to investigate the molecular identification, extracellular enzymetic activity as well as phenolic and fatty acid profile analysis, and antioxidant activities of endophytic fungi isolated from the *Gundelia tournefortii* stems. Phylogenetic analysis was done based on the Internal Transcribed Spacer (ITS) region, and five isolates were identified as following: *Aspergillus niger*, *Penicillium glabrum*, *Alternaria alternata*, *A. tenuissima*, and *Mucor circinelloides*. Evaluation of the enzymatic properties showed that *P. gabrum* and *A. niger* had more ability for producing most of the studied extracellular enzymes, unlike to *A. alternata* and *M. circinelloides* which only able for degradation of starch and pectin. The antioxidant activity of isolates showed that *A. alternata* extract (IC_{50}= 417 µg/mL) had the highest antioxidant properties, followed by *A. tenuissima* extract (IC_{50}= 512 µg/mL). Also, the extract of *A. alternata* had the greatest amount of total phenols and flavonoids contents (2.8 mg GAL/g and 2.3 mg QE/g, respectively). The quantification analysis of phenolic acid showed that rosmarinic acid, para-coumaric acid, and meta-coumaric acid (42.02, 7.53, 5.41 mg/g, respectively) were the main phenolic acids in the studied fungi. The analysis of fatty acids confirmed that, in all fungi, the main fatty acids were stearic acid (27.9-35.2%), oleic acid (11.3-17.3%), palmitic acid (16.9-23.2%), linoleic acid (5.8-11.6%), and caprylic acid (6.3-10.9%). Our finding showed that endophytic fungi are a source of bioactive compounds, which could be used in various industries. This is the first report of endophytic fungi associated with *G. tournefortii*, which provides knowledge on their future use on biotechnological processes.

Introduction

Medicinal herbs are an important group of hosts for endophytic fungi. These fungi are endosymbionts that live inside plant tissues without causing disease. They protect the plant against biotic and abiotic stresses by different mechanisms. They can independently synthesize secondary metabolites similar to those made by the host plants. The production of active compounds by them is thought to occur through coevolution or genetic transfer of the host plant to endophytes[1].

Nowadays, the isolation and extraction of bioactive compounds from endophytic fungi are important. The studies have revealed the ability of endophytic fungi for producing many secondary metabolites such as phenols, quinones, xanthones, isocoumarins, terpenoids, alkaloids, and steroids that work as anticancer, antiviral, antibacterial, insecticidal, anti-inflammatory, antifungal, antidiabetic, antioxidant, and etc[2–4].

Phenols are one of the bioactive compounds that can be extracted from endophytic fungi. These compounds have many biological properties such as antioxidant and anticancer[5]. In 2014, Huang et al.[6] could identify kaempferol from *Mucor fragilis*, which was isolated from *Sinopodophyllum hexandrum*. Prihantini and Tachibana[7] showed *Pseudocercospora* sp. isolated from *Elaeocarpus sylvestris* had the highest antioxidant properties. They further isolated 6-methyl-salicylic acid, which had higher antioxidant properties. Das et al.[8] studied the antioxidant activity of the endophytic fungi extracts identified from

Zingiber nimmonii. Based on the results, *Fusarium chlamydosporum*, *Nectria haematococca*, *Aspergillus terreus*, *Alternaria tenuissima* and *Bipolaris specifera* had the highest antioxidant activity. The phenolic acids study in the extract of *B. specifera* showed that this fungus has the ability to produce caffeic acid, para-hydroxybenzoic acid, para-coumaric acid and syringic acid. *A. tenuissima* also produced vanillic acid, chlorogenic acid, ferulic acid and protocatechuic acid. In addition, *A. terreus* was able to synthesize caffeic acid, para-hydroxybenzoic acid and para-coumaric acid. In another study, the relationship between antioxidant activity and phenolic compounds was investigated in endophytic fungi isolated from *Fritillaria unibracteata*. They succeeded in identifying forty-nine endophytic. A detailed study on five endophytic fungi with antioxidant properties, namely *Fusarium* sp., *Fusarium tricinctu*, *Fusarium redolens*, *Plectosphaerella cucumerina* and *Bionectria ochroleuca* showed that these fungi were capable of producing some phenolic acids such as caffeic acid, gallic acid, chlorogenic acid, ferulic acid and rosmarinic acid[9]. Mollaei et al.[2] showed that *F. tricinctum*, as an endophytic fungus isolated from the roots of *Lithospermum officinale*, can be presented as a new source of shikonin. In another study, 28 isolates of endophytic fungi were isolated from *Ferula assa-foetida*, and *Alternaria tenuissima* had the highest antioxidant activity. Analysis of phenolic compounds by HPLC indicated that rosmarinic acid was the most abundant phenolic acid[3]. Hernández-Tasco et al.[10] investigated the antioxidant activity of *Cladosporium* sp. (AC-1 strain) isolated from leaves of *Annona cacans*. They succeed to identify compounds with antioxidant (coumaric acid and cinnamic acid) activity.

Fatty acids are other biologically active compounds that present many applications, due to their physical, biological, and alimentary properties. They were applied in the industries such as pharmaceuticals, cosmetics, and food. They adjust a wide range of functions in the human body, including regulation of inflammatory responses, brain maintenance and function, normal development, vision, vasodilation, vasoconstriction, and blood pressure[12–14]. Endophytic fungi isolated from plants are a common source for producing the fatty acids. Ghaffari et al.[4] studied the fatty acid profile of endophytic fungi isolated from *Ziziphora tenuior*. They concluded that *Alternaria infectoria* and *Aspergillus ochraceus* had the highest amount of fatty acids, and oleic acid, stearic acid, palmitic acid, linoleic acid, and γ-linolenic acid were the main fatty acids. Yang et al.[15] showed *Bionectria ochroleuca* isolated from *Torreya grandis* could produce some fatty acids such as siadonic acid, oleic acid, and linoleic acid.

Enzymes are biological catalysts that catalyze many chemical reactions and are applied in different industries such as detergent, pharmaceutical, food, etc. Due to the industrial use of enzymes, high-scale production with the highest level of activity and stability, as well as the lowest production cost, is very important. Based on the studies, endophytic fungi have the ability for producing different enzymes such as protease, cellulase, amylase, etc. Endophytic fungi such as *Fusarium* sp., *Aspergillus* sp., *Alternaria* sp., and *Penicillium* sp. were reported to produce extra cellular enzymes with a significant yield that can replace the prevailing resources[16].

Gundelia tournefortii L. (belong to Asteraceae family) is an edible spiny, thistle-like plant which grows in the semi-desert areas of Asia[17]. It's called tumble thistle in English and Kangar in Persian. The stem of *G. tournefortii* considered as a food and an herbal drug in Iran. In traditional medicine, *G. tournefortii* has been

used as a hepatoprotective and blood purifier[18], and hypolipidemic and antioxidant agent[19], as well as antidiabetes herb[20]. Since endophytic fungi isolated from plants have the ability to produce bioactive compounds, it is expected that *G. tournefortii* also hosts endophytic fungi that have the ability to biosynthesize active compounds. Therefore, the main goal of this study is to isolate and identify endophytic fungi from *G. tournefortii*, investigating their biological characteristics and studying their metabolites.

Material and Methods

Isolation and molecular identification of endophytic fungi

Plant specimens of *G. tournefortii* were collected from Urmia, Iran (E45°04 , N37°48), during May 2021. Voucher specimen after identifying by Dr. Mostafa Ebadi (ASMUH-40214) was deposited at the herbarium of Azarbaijan Shahid Madani University. Surface sterilization of the plant stems was done based on Sunitha et al.[21] method, and inoculated into PDA to obtain pure colonies according to our previous study[4]. For molecular identification, the extraction of fungi DNA was carried out by Zhu et al.[22]. The extracted DNA was served as template for the amplification of ITS1-5.8S-ITS2 rRNA region. PCR amplification was performed by ITS 1 and ITS 4 as primers in a thermocycler (SensoQuest) programmed. The phylogenetic analyses were created by the MEGA5. Lastly, the phylogenetic tree was constructed by the neighbor-joining algorithm (1000 bootstraps)[4].

Enzymatic activities of endophytic fungi

The amylase, cellulase, pectinase, and protease activates were performed by the Sunitha et al.[21] method. For this propose, the endophytic fungi were cultivated on Yeast-Malt (YM) agar media supplemented with specific indicative substrates. The specific indicative substrates of amylase, cellulase, pectinase, and protease were 1% soluble starch, 1% carboxy methyl cellulose, 1% Pectin, and 1% gelatin, respectively. The enzyme production was determined based on the formation of a clear zone that appeared around the colonies of fungus.

Extraction and analysis of fatty acids

To extract fatty acids, 0.5 gram of dried and powdered endophyte fungi were poured into an Erlenmeyer flask, and then n-hexane (50 mL) was added, and the extraction was done at ultrasonic bath for 30 minutes. Then, the sample was centrifuged for 5 minutes (5000 rpm). After collecting the upper phase, its solvent was evaporated by a rotary evaporator. The obtained extracts were weighed and the yield of the extract containing fatty acid was calculated.

The fatty acids were derivatized by alkaline method (REF), and analyzed by an YL 6100 GC gas chromatography device. The capillary column used (60 m × 0.25 mm × 0.2 micrometers) included 14% cyanopropyl phenyl-86% dimethyl polysiloxane, bonded and cross-linked phase. The initial temperature was 80°C and it was raised to 120°C (20°C/min), and then reached 260°C (3°C/min), and was kept at this temperature for 10 minutes. The carrier gas was nitrogen and its speed was set at 1.1 mL/min. The amount

of sample injection was 1 µL with a ratio of 1 to 20. The detector and injection temperatures were set at 280 and 260°C, respectively. Fatty acids were identified using the standard and the percentage of each fatty acid was calculated using relative area percentages obtained by FID[23].

Total phenolic and flavonoid contents assay

To measure the Total Phenolic Content (TPC), the Folin-Ciocalteu method was used. Briefly, 20 µL of the extract (500 ppm) was added to 100 µL of Folin-Cicaltio reagent (10% v/v), and after 5 minutes, it was mixed with 20 µL of Na_2CO_3 (7% w/v). The samples were placed in the dark for 2 hours. Finally, the absorbance of the samples was read by a spectrophotometer (765 nm). The TPC was determined as mg of gallic acid per equivalent gram of dry fungus (mgGAL/g) by the regression equation derived from the gallic acid standard curve[24].

The Total Flavonoid Content (TFC) in the extracts was obtained by $AlCl_3$ colorimetric method. Briefly, 20 µL of the extract (500 ppm) was added to 100 µL of aluminum chloride (3% w/v) and 0.1 mL of ammonium acetate (1 M). The obtained samples were kept at 25º for 10 minutes, and finally, the absorption of each sample was read at 426 nm. The TFC was calculated by the regression equation derived from the Quercetin, and was expressed as mg of quercetin per equivalent gram of dry fungi (mgQE/g)[24].

Extraction and analysis of phenolic acids

2 ml of Merck's methanol solvent containing 0.1% HCl (1M) was added to 0.2 g of defatted fungi samples and placed inside the ultrasonic device at 25ºC. After 30 min, the extract was filtered and its solvent was evaporated. Water (2 mL) was added to the sample, and was mixed with n-hexane (1:1 v/v). The obtained sample was shaken for two min, and the aqueous phase was separated and mixed with diethyl ether and ethyl acetate solvents (1:1 v/v). After shaking for 5 minutes, the organic phase was separated and evaporated. Finally, the extract was placed inside the oven at 60°C to dry completely.

The separation and identification of phenolic acids was done by HPLC[25]. The column used was C18 (250 mm × 0.46 mm, 5 µm). Also, the mobile phase was HPLC grade methanol and water with 0.1% acid with a flow rate of 0.5 mL/min and a gradient washing system. The injection volume was 20 µL and the type of detector used was UV, which was set at a wavelength of 275 nm. The amount of phenolic acids was calculated by the regression equation derived from standards, and was expressed as mg per gram of dry fungi (mg/g).

Antioxidant activity by DPPH method

The antioxidant activity was evaluated by measuring the amount of free radicals DPPH[26]. Briefly, 100 µL of the extract with various concentrations (0.2, 0.4, 0.7, 1.0, and 1.25 mg/mL) was added to DPPH solution (100 µL, 0.1 mM), and mixed for 30 minutes at 25°C. Then, the absorbance of the samples was read at 517 nm. The inhibition percentage of DPPH free radicals was determined, and finally, the IC_{50} value of the extract was calculated.

Results

Fungi isolation

The results revealed that *G. tournefortii* associated with some endophytic fungi. Totally, five isolates (Gt01-05) were obtained from the *G. tournefortii* stems. According to the phylogenetic analysis, the identified species are placed in three family including Aspergillaceae, Pleosporaceae, and Mucoraceae (Fig. 1). The first clade contained the isolates grouped with 100% bootstrapping to *Aspergillus niger* (Gt05) and *Penicillium glabrum* (Gt02) sequences. The second clade was grouped to *Alternaria alternata* and *A. tenuissima* sequences (Gt01 and Gt03) retrieved from NCBI with 100% bootstrapping. The third clade comprised the isolate (Gt04) grouped with 100% bootstrapping to *Mucor circinelloides* sequences obtained from NCBI (Fig. 1).

Enzyme assay

Evaluation of the enzymatic properties of endophytic fungal isolates showed that *P. glabrum* and *A. niger* (Gt02 and Gt05) had the greatest ability for producing all studied extracellular enzymes (Table 1). *A. alternata* and *M. circinelloides* (Gt01 and Gt04) showed extracellular property for amylase and pectinase; while, they indicated negative extracellular property for cellulase and protease. *A. tenuissima* (Gt03) showed extracellular property for amylase, cellulase, and pectinase. Although maximum extracellular amylase property was obtained in *A. alternata* followed by *P. glabrum* isolate, ANOVA analysis indicated that variations between these isolates to CMC substrates did not reach significant level ($P \leq 0.05$).

Table 1
Extracellular enzymatic activities of fungal endophytes using solid media.

Fungi isolate	Diameter of clear zones (mm)			
	Amylase	Cellulase	Pectinase	Protease
A. alternata (Gt 01)	41 ± 1.3 ^{a}	0 ^{a}	10 ± 0.5 ^{a}	0 ^{a}
P. glabrum (Gt02)	39 ± 1.5 ^{ab}	15 ± 1.2 ^{b}	31 ± 1.9 ^{b}	12 ± 0.9 ^{b}
A. tenuissima (Gt03)	32 ± 0.8 ^{c}	4 ± 0.5 ^{c}	17 ± 1.5 ^{c}	0 ^{a}
M. circinelloides (Gt04)	37 ± 2.1 ^{b}	0 ^{a}	24 ± 0.6 ^{d}	0 ^{a}
A. niger (Gt05)	5 ± 0.3 ^{d}	23 ± 1.7 ^{d}	22 ± 1.8 ^{d}	9 ± 1 ^{c}
Control	0 ^{e}	0 ^{a}	0 ^{e}	0 ^{a}
In a column, values are the means ± SE followed by different letters are significantly different ($P < 0.05$) by Tukey test (n = 3).				

Among the studied endophytes, three isolates could produce cellulase enzymes and developed a clear zone on the CMC medium. A significant difference in cellulase activity was detected between tested isolates and maximum activity was recorded in *A. alternata* (Table 1).

The difference between pectinase properties of *M. circinelloides* and *A. niger* did not reach significant level. But, pectinase activity of *P. glabrum* was significantly higher as compared to other tested entophytes (Table 1).

Among the tested endophytes, *P. glabrum* and *A. niger* showed protease activity, whereas *P. glabrum* significantly exhibited high proteolytic property compared to *A. niger* ($P \leq 0.05$) (Table 1).

Investigation of antioxidant property

The results of antioxidant property showed that the inhibition of free radicals depends on the concentration of the extracts, and with the increase of their concentration, the rate of inhibition of free radicals increases. In the concentration range, ascorbic acid (synthetic antioxidant) had a high antioxidant activity compared to all samples, and in this sense, none of the samples had the ability to compete with ascorbic acid.

Figure 2 shows the IC_{50} value of extracts. Based on the results, *Alternaria alternata* extract (IC_{50} = 417 µg/mL) has the highest antioxidant properties, followed by *Alternaria tenuissima* extract (IC_{50} = 512 µg/mL). It should be explained that compared to ascorbic acid (IC_{50} = 82 µg/mL), the extracts do not have good antioxidant properties. *Mucor circinelloides* and *Aspergillus niger* extracts also had no antioxidant properties. According to the obtained results, it can be assumed that the high antioxidant properties of *Alternaria alternata* and *Alternaria tenuissima* extracts compared to other extracts can be related to their phenolic and flavonoids compounds, and their total phenol and flavonoid contents can justify their high antioxidant activity.

Analysis of phenolic compounds

The polyphenolic extracts of fungi were evaluated in terms of total phenol and flavonoid content. As can be seen from Fig. 3, the extracts of *Penicillium glabrum* and *Alternaria alternata* had the highest amount of total phenols (0.8 and 2.8 mgGAL/gDW, respectively). The TFC results indicated that the *Alternaria alternata* extract had the highest amount of total flavonoids with 2.9 mgQE/g. The *Penicillium glabrum* extract also ranked second with 2.3 mgQE/g. Also, the lowest amount of total flavonoid was related to the *Mucor circinelloides* and *Aspergillus niger* extracts.

The phenolic acids profiles of the endophytic fungi extracts were investigated using HPLC. HPLC chromatogram related to the *Penicillium glabrum* extract was shown at Fig. 4. Table 2 showed the phenolic acids compounds in the extract of endophytic fungi. Based on the results, caffeic acid is the only phenolic acid that was detected in all the extracts. The *Alternaria tenuissima* extract had the highest amount of rosmarinic acid with the amount of 42.02 mg/g dry weight of the fungi (mg/g DW), followed by the *Penicillium glabrum* and *Alternaria alternata* extracts. The second major acid present in the extracts was related to para-coumaric acid, and the *Penicillium glabrum* extract had the highest amount of this compound with the amount of 7.53 mg/g DW. meta-Coumaric acid was another compound that had the highest amount in the extracts of *Aspergillus niger* and *Mucor circinelloides*, and its amounts were 5.41 and 4.37 mg/g DW, respectively. Among the studied phenolic acids, vanillic acid, salicylic acid and cinnamic acid compounds were not detected in any of the extracts. Ferulic acid was also present only in the

Aspergillus niger extract (0.27 mg/g DW). Gallic acid was present in all extracts except *Alternaria tenuissima* extract. Procatechuic acid was also not found only in the *Mucor circinelloides* extract.

Table 2
Amount of phenolic acid compounds (mg/kg dried plant) in fungal isolates

Phenolic acids	*Penicillium glabrum*	*Aspergillus niger*	*Alternaria tenuissima*	*Alternaria alternata*	*Mucor circinelloides*
Gallic acid	0.15	0.03	0.14	0.00	0.02
Protocatechuic acid	0.18	0.08	0.16	0.15	0.00
Rosmarinic acid	4.02	0.00	3.23	41.02	0.00
p-Hydroxybenzoic acid	0.01	0.00	0.00	0.07	0.00
Vanillic acid	0.00	0.00	0.00	0.00	0.00
Caffeic acid	0.15	0.05	0.22	0.32	0.04
p-Coumaric acid	7.53	0.23	0.00	0.00	0.32
Ferulic acid	0.00	0.27	0.00	0.00	0.00
m-Coumaric acid	0.43	5.41	0.00	0.51	4.37
Salicylic acid	0.00	0.00	0.00	0.00	0.00
Cinnamic acid	0.00	0.00	0.00	0.00	0.00

Analysis of fatty acids

Figure 5 showed the yield of hexane extract of endophyte fungi. Based on the obtained results, *Alternaria alternata* extract followed by *Mucor circinelloides* had the highest yield with values of 72 and 48 mg/g DW, respectively. *Penicillium glabrum* and *Aspergillus niger* had the lowest yield (11 and 12 mg/g, respectively).

To investigate the profile of fatty acids, the n-hexane extracts were analyzed by a gas chromatography device. Based on the results (Table 3), twenty fatty acids were identified and the fatty acid percentage of the extracts was reported between 96.9–99.0% (Table 1). In all fungi, the main fatty acids were stearic acid (27.9–35.2%), oleic acid (11.3–17.3%), palmitic acid (16.9–23.2%), linoleic acid (5.8–11.6%), and caprylic acid (6.3–10.9%). Also, Fatty acids are grouped according to the availability of saturated, monounsaturated, and polyunsaturated fatty acids. The results showed that saturated fatty acid were the major group.

Table 3
Percentage fatty acid composition of fungal isolates.

Fatty acid	Common Name	*Penicillium glabrum*	*Aspergillus niger*	*Alternaria tenuissima*	*Alternaria alternata*	*Mucor circinelloides*
C8:0	Caprylic acid	6.3	10.6	6.8	6.3	10.9
C10:0	Capric acid	1.3	4.2	2.0	4.3	4.5
C12:0	Lauric acid	0.5	1.0	1.0	1.0	2.8
C14:0	Myristic acid	1.3	1.1	0.9	0.9	2.2
C14:1	Myristoleic acid	1.1	0.9	1.6	1.3	1.7
C15:0	Pentadecylic acid	1.7	1.7	1.8	1.4	1.9
C15:1	Pentadecenoic acid	1.3	1.0	1.5	1.4	1.2
C16:0	Palmitic acid	18.7	19.8	21.7	23.2	16.9
C16:1	Palmitoleic acid	2.3	0.7	2.3	1.1	2.6
C18:0	Stearic acid	27.9	34.5	30.8	35.2	28.1
C18:1(n-9)	Oleic acid	17.3	10.3	15.3	10.9	13.2
C18:2(n-6)	Linoleic acid	11.6	5.8	6.6	6.2	6.3
C18:3 n3	α-Linolenic acid	2.1	1.7	1.7	0.7	1.5
C20:0	Eicosanoic acid	1.1	1.1	1.0	1.1	1.3
C20:2	Eicosadienoic acid	1.2	0.8	1.1	0.9	0.6
C20:3 n3	Eicosatrienoic acid	1.3	1.0	1.1	0.4	1.0
C20:4 n6	Arachidonic acid	0.0	0.5	0.8	0.3	0.6
C20:5 n3	Eicosapentaenoic acid	0.4	0.5	0.2	0.3	0.3
C22:4 n6	Docosatetraenoic acid	0.0	0.6	0.7	0.0	0.3
C22:5 n3	Docosapentaenoic acid	1.6	0.0	0.0	0.0	0.0
SFA		58.8	74	66	73.4	68.6
MUFA		22	12.9	20.7	14.7	18.7
PUFA						

Fatty acid	Common Name	*Penicillium glabrum*	*Aspergillus niger*	*Alternaria tenuissima*	*Alternaria alternata*	*Mucor circinelloides*
C8:0	Caprylic acid	6.3	10.6	6.8	6.3	10.9
C10:0	Capric acid	1.3	4.2	2.0	4.3	4.5
		18.2	11.1	12.2	8.8	10.6
SUM		99.0	97.8	98.9	96.9	97.9

Discussion

Fungi isolation

The use of molecular phylogeny is very necessary and useful for the accurate identification of fungal species. In this research, according to the results of phylogenetic analysis, the studied isolates were placed in separate clades and their taxonomic position was possible based on the ITS region. The isolates Gt01 and Gt02, which belonged to *A. alternata* and *A. tenuissima*, were placed in the same clade and, although the ITS region was able to show the taxonomic position of these isolates, but it was not able to separate the two species. Similar to this finding, Zhao et al.[27] showed that it is not possible to separate between *A. alternata* and *A. tenuissima* based on the ITS region, and the examination of morphological traits is necessary. The color of the dorsal surface of *A. alternata* and *A. tenuissima* species in the PDA is olive green and gray to dark brown, respectively. Also, the conidial chain of *A. alternata* has two to three lateral branches, while the conidial chain of *A. tenuissima* has one lateral branch. According to morphological characteristics, isolate Gt01 belongs to *A. tenuissima* and isolate Gt03 belongs to *A. alternata*.

Enzyme assay

Five fungal endophytes were isolated from *G. tournefortii*, a medicinal native plant species in Iran, that they have various biological and biochemical properties potentially useful. The results showed that studied isolates have ability for producing different enzymes regarding to degrade starch, carboxy methyl cellulose, pectin, and gelatine. *P. gabrum* and *A. niger* had high ability for producing most of the studied extracellular enzymes, unlike to *A. alternata* and *M. circinelloides* which only able to degrade starch and pectin. Amirita et al.[28] and Sunitha et al.[21] showed high property of extracellular enzymes in fungal endophytes associated with plants. Our results supported that enzymatic property of endophytes plays main role for degradation of protein and polysaccharides, which could imply a resistance strategy of the host plant against pathogenic infection[29].

Antioxidant phenolic compounds from endophytic fungi

Free radicals are compounds with single electrons that irreversibly react with biological molecules such as lipoproteins, lipids, carbohydrates, nucleic acids, proteins and free amino acids. It damages and causes many diseases such as cancer, cataracts, cardiovascular diseases, etc. The antioxidant defense system includes enzymatic and non-enzymatic factors. Enzymatic factors such as glutathione peroxidase,

superoxide dismutase and catalase inhibit peroxide, superoxide and hydrogen peroxide radicals, respectively. Non-enzymatic factors such as phenols inhibit many reactive oxygen species and free radicals. These compounds are valuable secondary metabolites that are abundant in some plants and have many biological benefits. They are classified into different groups such as tannins, stilbenes, lignins, lignans, coumarins, quinones, phenolic acids and flavonoids, and endophytic fungi can be an important source for the production of these compounds[30].

In this study, it was observed that *Alternaria* sp. had the highest amount of phenolic compounds. Also, based on the IC_{50} results, these fungi (i.e. *Alternaria alternata* and *Alternaria tenuissima*) had the highest antioxidant properties. It can be assumed that the high antioxidant properties of these extracts can be related to their phenolic and flavonoids compounds. Up to now, some studied have been done on the phenolic compounds and antioxidant properties of *Alternaria* sp. Elbermawi et al.[31] could identify six phenolic compounds in *Alternaria* sp. isolated from fresh leaves of *Lycium schweinfurthii*, *Pancratium maritimum* and *Cynanchum acutum*. These compounds were named alternariol-5-O-methyl ether, alternariol, altenusin, altenuene, alternarienoic acid, and talaroflavone. Examining their biological properties showed that some of these compounds show strong alpha-glucosidase and lipase inhibitory activities and may act as natural anti-diabetic candidates. Elghaffar et al.[32] evaluated the antioxidant property of the *Alternaria alternata* extract which was isolated from the *Ziziphus spina-christi* leaves. The results showed that this extract with an IC_{50} value of 409 µg/mL had an acceptable antioxidant property and this property was attributed to the presence of phenolic compounds. Ibrahim et al.[33] showed that *Alternaria* species isolated from *Dracaena* plant has a potential antioxidant activity and its IC_{50} was 520 µg/mL. Zheng et al.[34] succeeded in isolating eight endophytic fungi that produce flavonoid compounds from the branches of *Loranthus tanakae* and concluded that *Alternaria tenuissima* ZP28 and ZM148 have better antioxidant activity than others. Then, its flavonoid compounds were identified by LC-MS. In one study, 28 isolates of endophytic fungi including *Plectosphaerella*, *Alternaria, Aspergillus*, and *Fusarium* were isolated from *Ferula assa-foetida*, and their ability to biosynthesis of phenolic acids was studed. The highest amount of total flavonoid, total phenol, and antioxidant activity was reported in *Alternaria tenuissima* supernatant. Isolation and identification of 14 phenolic acids using HPLC showed that rosmarinic acid (64.11 mg/g DW) was major phenolic acid. Finally, *Alternaria tenuissima* was introduced as a rich source of rosmarinic acid[3]. *Alternaria alternata* isolated from *Azadirachta indica* has the ability to produce compounds with strong antibacterial and antioxidant properties, and the investigation on these compounds showed that they belong to flavonoids[35]. In another study, the separation of *Alternaria* sp. Samif01 isolated from *Salvia miltiorrhiza* led to the isolation of seven phenolic compounds belonging to dibenzo-α-pyrones. Examining the antibacterial properties of these compounds showed that some of these compounds have inhibitory properties against Gram-negative and Gram-positive bacteria in the range of 86 to 364 micromolar. Moreover, these compounds showed high antioxidant and cytotoxic activities[36]. Khiralla et al.[37] showed that the extract of *Alternaria* sp. isolated from *Calotropis procera* has antioxidant property (IC_{50} = 236 µg/mL). Gunasekaran et al.[38] concluded that the *Alternaria* sp. extract inhibits DPPH free radicals up to 85.20% at a concentration of 300 µg/mL. In 2015, Anyanwu and Sorensen[39] succeeded in isolating two phenolic compounds named Deoxyphomalone and stemphyperylenol from *Alternaria tenuissima*. Fang et

al.[40] identified several phenolic compounds from *Alternaria tenuissima* isolated from *Erythrophleum fordii*. They included α-Acetylorcinol (belonging to phenylpropenes) and tenuissimasatin (belonging to isocoumarins). Further, they showed that these compounds can have cytotoxic properties against human colon cancer cells. Alternariol monomethyl ether and (4S)-α, β-dehydrocurvularin are phenolic compounds isolated from *Alternaria tenuissima* isolated from the leaves of *Acacia mangium*. These two isolated compounds had inhibitory activity against *Magnaporthe grisea* (the agent of rice blast disease)[41]. Therefore, there was almost a significant relationship between the antioxidant properties of the endophytic fungi studied in this research with previous studies.

Considering the phenolic content of *Alternaria* sp. extracts and their antioxidant properties, their phenolic acids were studied using HPLC. According to the results, rosmarinic acid was the main phenolic acid in the *Alternaria tenuissima* and *Alternaria tenuissima*. This compound is an ester of caffeic acid, which is famous as a phenolic compound. This compound was reported for the first time from the rosemary plant, and then was reported from some other natural sources such as plants and endophyte fungi[42]. In a research done by Parvandi et al.[3], 28 isolates of endophytic fungi were isolated from *Ferula assa-foetida*. Then, their ability for producing phenolic acids was studed, and it was found that *Alternaria tenuissima* was a rich source of rosmarinic acid. Therefore, this research along with our results showed that *Alternaria tenuissima* colud be a source of rosmarinic acid. Since this compound has many biological properties such as antiviral, antibacterial, anticancer, antioxidant, antiaging, antidiabetic, heart protection, liver protection, antidepressant, antiallergy and anti-inflammatory activities, and also, since ancient times, it has been used in folk medicine, cosmetics and food supplements[43].

Penicillium glabrum was another endophytic fungi which antioxidant activity. *Penicillium* species have the potential to produce various phenolic compounds with high biological properties[44]. Antrovenetin, as an antioxidant phenolic compound, was extracted from *Penicillium herquei*, and enhanced the activity of tocopherol, and can be applied at food industries[45]. Jakovljević et al.[46] investigated the chemical compounds and biological activities of two fungal species of *Penicillium*. In this study, antioxidant activity was investigated by various methods. The results showed that *Penicillium chrysogenum* had higher TPC, and better antioxidant property, and also had the ability to chelate iron ions and could be a new potential source of natural antioxidants. Ghanati et al.[47] showed that the endophytic fungi isolated from *Corylus avellana* (especially *Alternaria* sp.) have a high ability to produce many cinnamic acid derivatives, including coumaric acid. According to our results, para-coumaric acid was the major phenolic acid in its extract. para-Coumaric acid is a phenolic acid found in fruits, vegetables and fungi. This compound can be converted into other phenolic acids such as sinapic acid, caffeic acid, and ferulic acid, also other metabolite acids such as lignins and lignin precursors. The studies have shown that para-coumaric acid has several biological activities such as anti-inflammatory, antioxidant, anti-platelet aggregation, analgesic, anti-cancer and neuroprotective [48,49]. According to our results, since *Penicillium glabrum* had the ability to produce para-coumaric acid during a short-term growth period and in relatively high amounts, it could be considered as a suitable source for the production of this compound on a commercial scale.

As a result, from our results and previous studies, it could be concluded that the antioxidant properties of *Alternaria* sp. and *Penicillium glabrum* may be due to the presence of phenolic acids compounds such as rosmarinic acid and para-coumaric acid.

Analysis of fatty acids

To investigate the profile of fatty acids, the n-hexane extracts were analyzed by a gas chromatography device. Based on the results, stearic acid, oleic acid, and linoleic acid were the main fatty acids in studied fungi. Based on the previous studies, these compounds have many properties and are used in different industries.

Stearic acid is a chemical compound that exists naturally in some plants and fungi. This compound is known as a strong organic acid and is used in many chemical, pharmaceutical and agricultural industries. This compound was used as an antibacterial and antifungal agent. Also, this compound is used in the production of detergents, paints, plastics and other industries. Other uses of stearic acid include pH control in various industries, production of alkaline batteries, production of organic fertilizers and production of food preservatives[50, 51].

Oleic acid is an unsaturated acid that is found in abundance in nature. It is widely used in the industry to make colored glass and medicines. Also, it is used in cosmetics and health sectors such as making softening lotions, soaps, etc. This compound decreases blood cholesterol and prevents it from increasing again, regulates blood sugar, strengthens the defense and immunity system of the body, and keeps the skin moist and prevents premature aging. Also, it is one of the important substances to destroy cancer cells (breast cancer in women). In diet programs, the use of this substance causes weight loss[52, 53].

Linoleic acid is a nutrient that has two double bonds. It is one of the unsaturated and essential fatty acids that are often found in vegetable oils. This fatty acid is also known as omega-6 fatty acid. This fatty acid is very essential for human nutrition because its synthesis process cannot be done by the body. Linoleic acid greatly reduces the risk of cardiovascular diseases, promotes healthy skin and hair, improves the health of the brain, strengthens the body's immune function, protects bone density, reduces inflammation in joints, digestive system, lungs and transplanted brain, etc[54, 55].

Conclusion

In this study, the molecular identification, extracellular enzymetic activity as well as phenolic and fatty acid profile analysis, and antioxidant properties of endophytic fungi isolated from the stems of *Gundelia tournefortii* were investigated. According to phylogenetic analysis five isolates were identified as following: *Aspergillus niger*, *Penicillium glabrum*, *Alternaria alternata*, *A. tenuissima*, and *Mucor circinelloides*. Evaluation of the enzymatic properties showed that *P. gabrum* and *A. niger* had more ability for producing most of the studied extracellular enzymes. The antioxidant activity showed that *A. alternata* extract had the highest antioxidant properties, total phenols and flavonoids contents. The quantification analysis of metabolites indicated that rosmarinic acid and stearic acid were the main compounds in studied fungi. The

results indicated that endophytic fungi are a source of bioactive compounds, which could be used in various industries.

Declarations

Author Contribution

Saeed Mollaei and Mostafa Ebadi: Supervision, Conceptualization, Writing-Reviewing and Editing. Fatemeh Ahmadi, Hossein Tahmouresi, and Mohammad Pazhang: Investigation, Methodology, Data curation, Writing-Original draft preparation.

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Figures

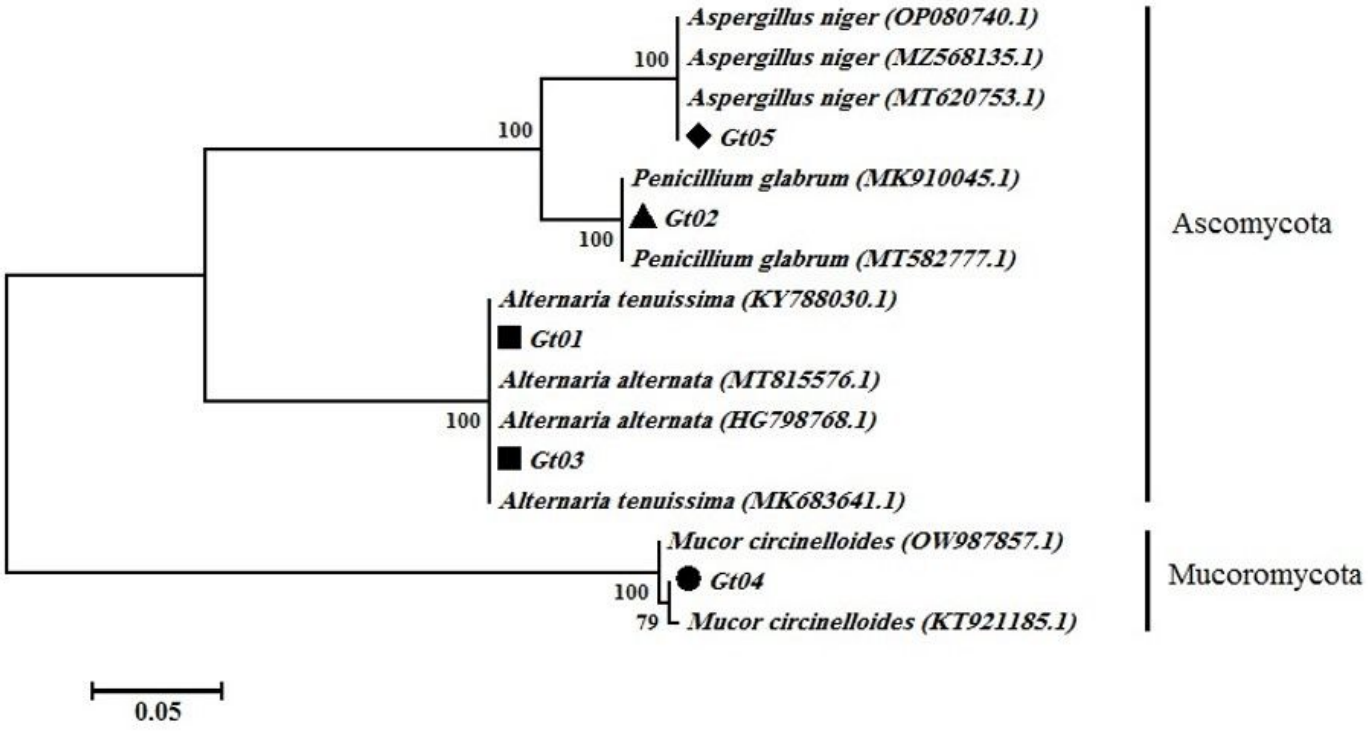


Fig. 1.

Figure 1

Phylogenetic analysis of endophytic fungi isolated from *G.tournefortii* based on the internal transcribed spacer. The phylogenetic tree was constructed using the neighbor-joining method (1000 bootstrap replications).

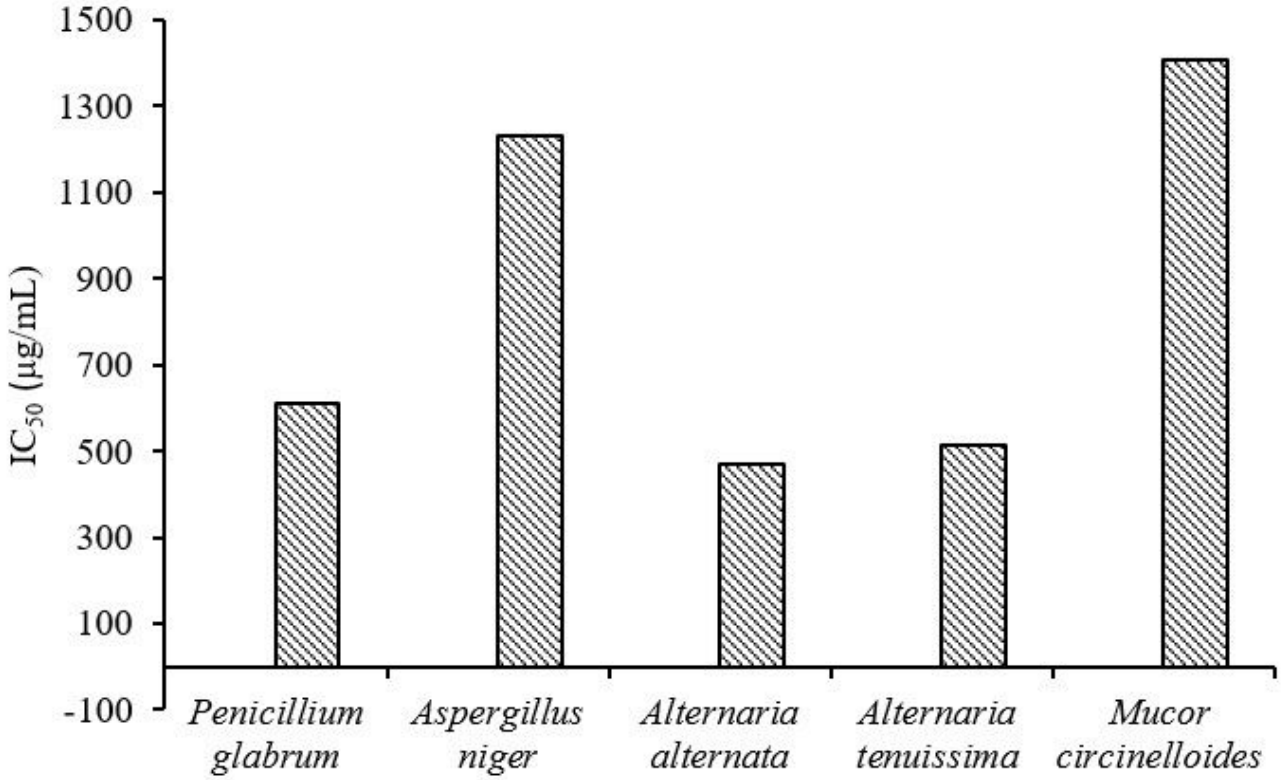


Fig. 2.

Figure 2

Antioxidant activity of the extract of endophytic fungi isolated from *Gundelia tournefortii*

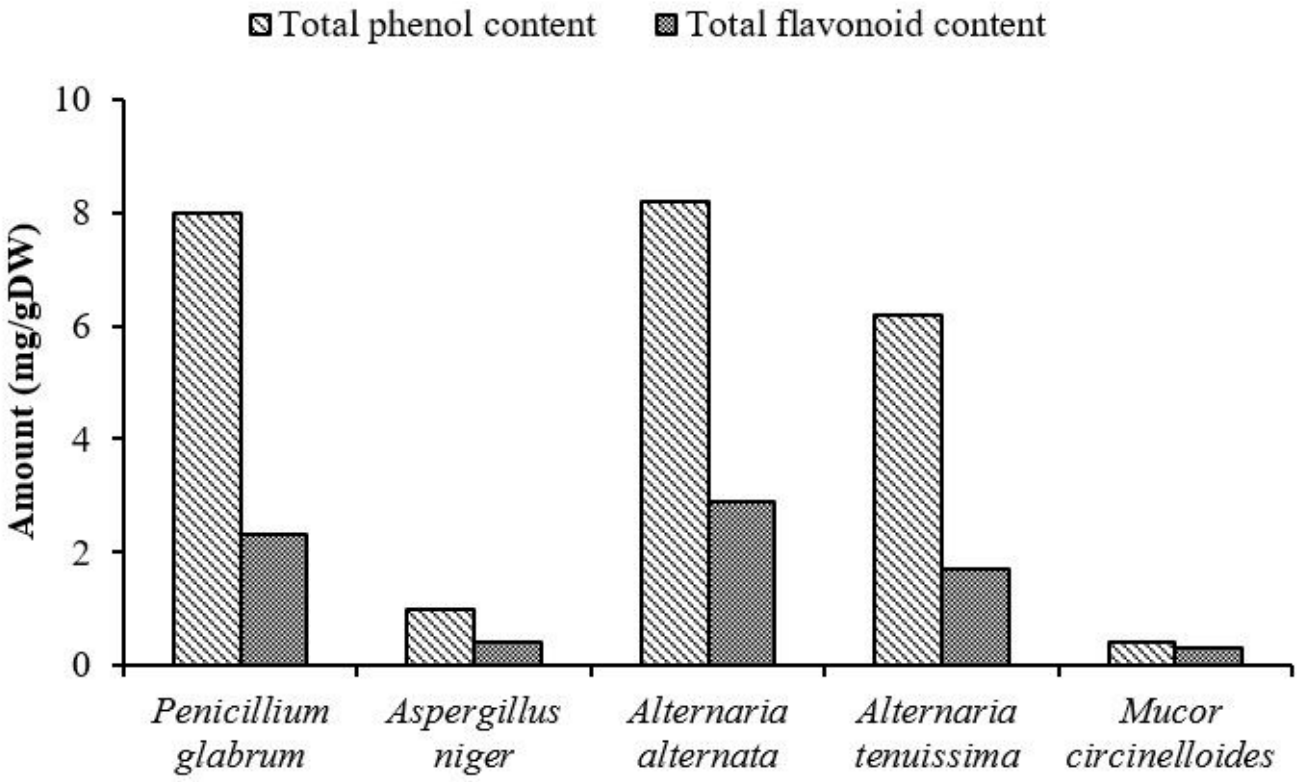


Fig. 3.

Figure 3

Total phenol and flavonoid contents in endophytic fungi isolated from *Gundelia tournefortii*

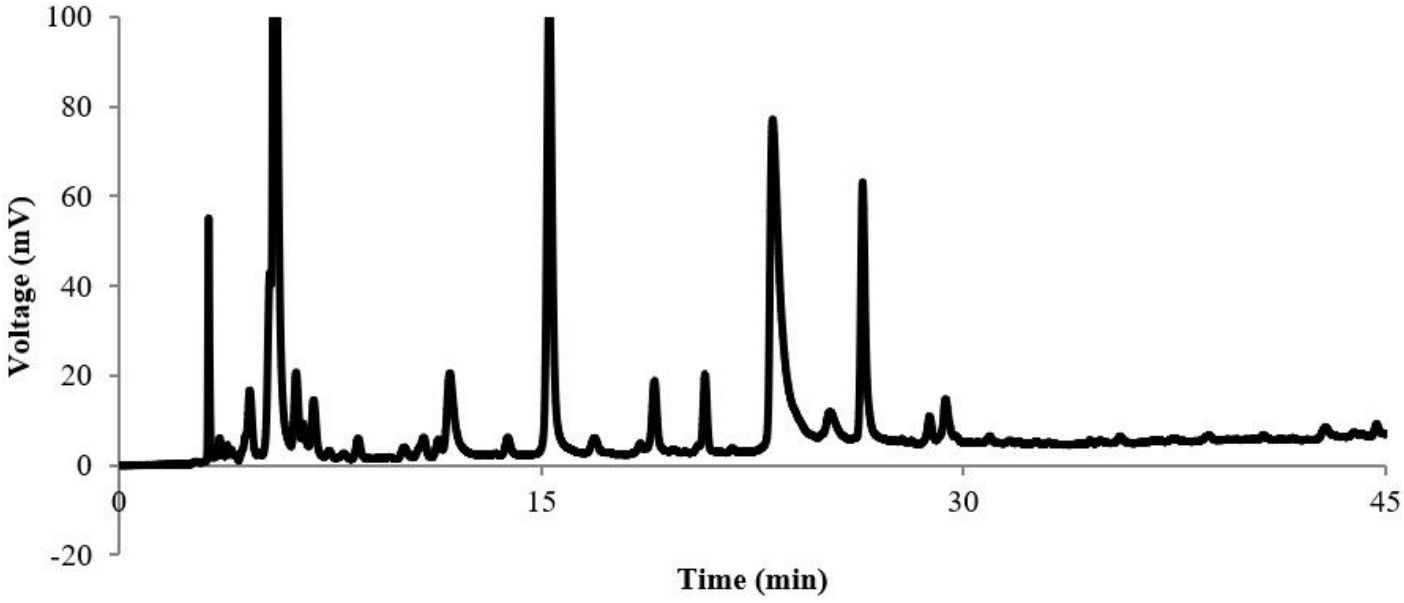


Fig. 4.

Figure 4

HPLC chromatogram related to the phenolic extract of *Penicillium glabrum*

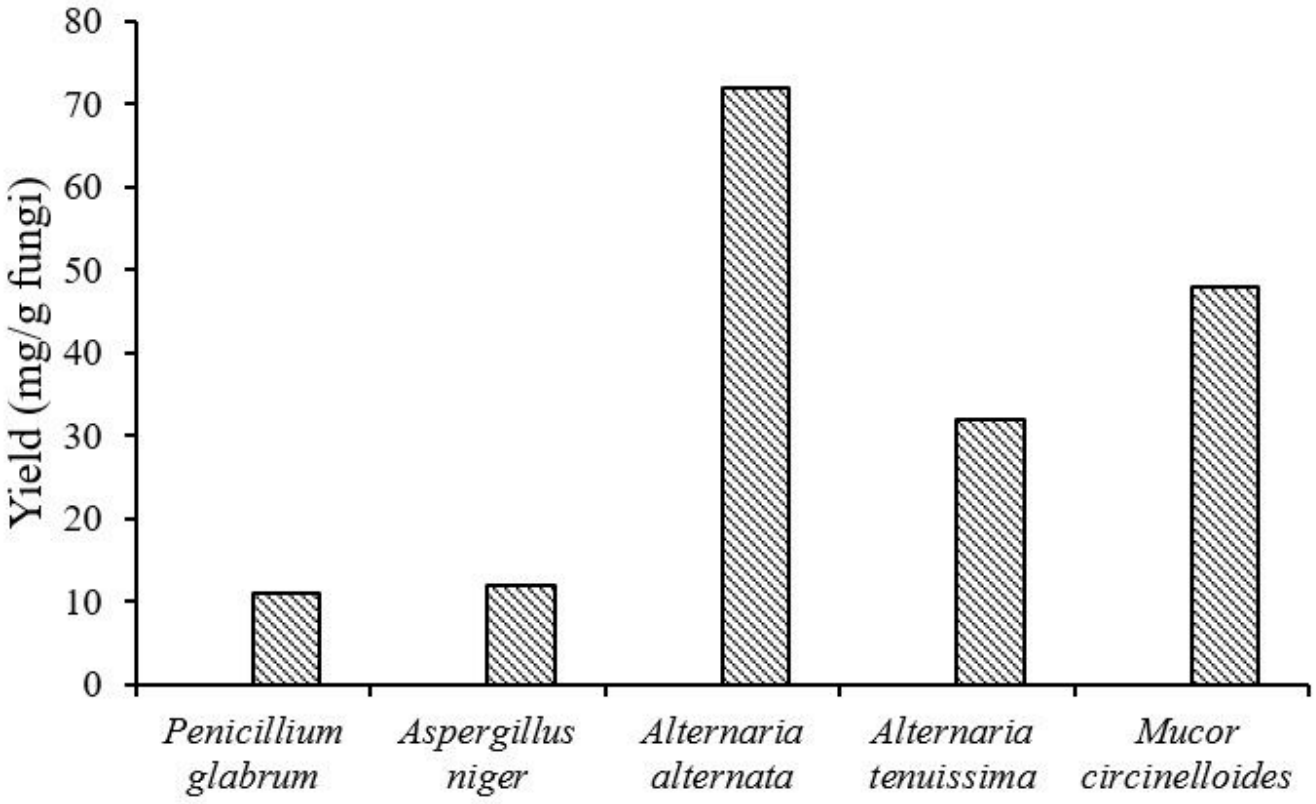


Fig. 5.

Figure 5

Hexane extract yield of endophytic fungi isolated from *Gundelia tournefortii*