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Endophytic Fungi Associated with Dusty Miller (*Centaurea cineraria* L.) and Judean Wormwood (*Artemisia judaica* L.) and Evaluation of their Antifungal Activities

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ABSTRACT

Endophytic fungi isolated from medicinal plants represent a potent biotechnological source for production of bioactive natural substances. This study aimed to estimate the diversity and anti-dermatophytic activity of the endophytic fungi associated with *Centaurea cineraria* and *Artemisia judaica* cultivated in Botanical Garden of Faculty of Science, Assiut University. Nineteen species belonging to eleven fungal genera were isolated from the studied plants using Potato Dextrose Agar (PDA) and Czapek's Cellulose Agar (CCA) media. The maximum endophytic fungal count was observed on leaf samples of *A.judaica* accounting 44 and 38 CFU/125 leaf segments on PDA and CCA media, respectively. In contrast, the leaf samples of *C. cineraria* showed the widest fungal spectrum contributing 8 genera and 12 species on PDA medium and 7 genera and 9 species on CCA medium. *Aspergillus*, *Penicillium*, *Chaetomium* and *Cladosporium* were the most prevalent fungi. *Ascotricha lusitanica* was firstly recorded in Egypt from *C. cineraria* and was identified morphologically and by gene sequencing. The obtained sequences were deposited in the GenBank under accession number MT256115.The isolated fungi were screened for their abilities to produce antimicrobial secondary metabolites against dermatophytes (*Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes*) using dual culture technique. *A. lusitanica* showed the highest suppressive impact against the tested dermatophytes. In conclusion, this study demonstrated that the endophytic *A. lusitanica* is considered a promising source of antimicrobial natural products involved in the pharmaceutical applications.

INTRODUCTION

Artemisia judaica and *Centaurea cineraria* (Family Asteraceae) are potent sources of natural remedies in all traditional medicine systems since their secondary metabolites exhibit strong antimicrobial, anti-inflammatory and antioxidant activities [1 and 2]. *A. judaica* and *C. cineraria* are widely grown in the Mediterranean region including Egypt [3 and 4]. Endophytic fungi are those that reside within the intercellular and/or intracellular spaces of apparently healthy and asymptomatic host plants [5]. Endophytes occupy a unique ecological status, their relationship with a host plant represents a balance between mutualistic and commensal symbiosis, which is largely controlled via chemicals. That is the reasons why endophytes produce highly specific metabolites [6]. Most of endophytic fungi have a significant association with their hosts protecting them from the pathogens [7]. Moreover, they are key players driving the synthesis of certain biomolecules that enhance plant growth, immunity, and protection against environmental stresses [8]. Endophyte - host relationships can be so close, that microbes can even biosynthesize the same chemical compounds as the plant host [9 and 10]. Interestingly, some fungal endophytes were shown to have biosynthetic capability *in vitro* cultures without a host plant association [11 and 12]. Karuppusamy [13] reported a hypothesis that presented the possible origin of secondary metabolites in plant-endophyte systems, namely (i) parallel coevolution of plants and their microbiota possessing pathways to produce bioactive compounds; (ii) horizontal gene transfer between plants and microbes during their coevolution; (iii) plants or endophytic fungi synthesize and transfer metabolites to each other. Recent studies provided strong indications that fungal endophytes produce invaluable bioactive metabolic compounds beneficial to humans with anticancer, antitumor, anti-inflammatory, antidiabetic and antimicrobial properties [14, 15 and 16]. Some of these bioactive compounds include pestacin, taxol, camptothecin, ergoflavin, podophyllotoxin, benzopyran, isopestacin, phloroglucinol, tetrahydroxy-1-methylxanthone, salidroside, borneol, dibenzofurane, methyl peniphenone, lipopeptide, peniphenone [8].

Dermatophytes including *Microsporum*, *Trichophyton* and *Epidermophyton* are the most significant infectious pathogen, which cause shallow mycosis, and the sores are described by round about attitude, desquamation, alopecia and erythema of the edges [17]. They have the ability to attack keratinized tissue (skin, hair, and nails) of people and different creatures to deliver a disease [18]. Dermatophytosis is known as tinea diseases or ringworm and influence of the all-inclusive community around the world [19]. The incidence of recalcitrant dermatophytoses is increasing due to increasing rates of drug resistance have been recently reported worldwide [20]. Antifungal drug resistance in dermatophytes has today been reported in *Trichophyton* and occasionally in *Microsporum*, but not in *Epidermophyton* species [21 and 22].

Due to the outbreak of drug-resistant dermatophytes, the requirement to search for new compounds with high activity and/or novel mechanisms of action has received a great attention. Our hypothesis addresses the screening for fungal endophytes isolated from medicinal plants as promising sources of novel secondary metabolic byproducts that fulfill the current needs for potent drugs. Therefore, the current study aimed to isolate

endophytic fungi from *Artemisia judaica* and *Centaurea cineraria*. Furthermore screen for their abilities to produce anti-dermatophitic secondary metabolites against *Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes* was also evaluated.

MATERIALS AND METHODS

1- Fungal source and isolation process

The endophytic fungi were isolated from fresh asymptomatic healthy leaves of the medicinal plants *Centaurea cineraria* L (Silver dust) and *Artemisia judaica* L (Wormwood) cultivated in the Botanical Garden, Faculty of Science, Assiut University. Five samples were collected for each plant. The samples were packed in clean polyethylene bags and immediately transferred to the mycological laboratory. Potato Dextrose Agar (PDA) and Czapek's Cellulose Agar (CCA) media supplied with 66.7 mg/L Rose- bengal and 250 mg/L streptomycin were used in the isolation process [23].Fungal isolation was carried out through surface sterilization technique described by Abdel- Hafez *et al*. [24]. Leaves were sterilized by immersing in75% ethanol for 1 min, followed by 1% sodium hypochlorite for 5 min, again in 75% ethanol for 30s. Then, the leaves were washed via sterilized distilled water and dried using a sterilized paper towel under aseptically conditions. Leaves were cut into segments (1 cm^2) and then twenty-five leaf segments were placed on surface of the isolation media in five plates followed by incubation at 25°C for 7 days. The growing colonies were examined and then the total fungal counts were expressed as CFU per 125 leaf segments for each plant species.

2- Fungal purification and identification

The fungal colonies were purified using single spore or hyphal tip techniques suggested by Dhingra *et el.* [25]. Then the purified fungi were identified according to their macroscopic and microscopic characteristics using several keys [26, 27, 28, 29, 30, 31, and 32]

3- Molecular identification of Ascotricha lusitanica

a- DNA extraction

DNA extraction of *Ascotricha lusitanica* was carried out using CTAB method described by Kalyankar [33]. In brief, 50 mg of fungal growth was scraped from PDA, homogenized in liquid nitrogen using a sterile mortar and transferred into 2ml microfuge tubes. Preheated 800 µl CTAB buffer was added and incubated at 65°C for 30 min and then 800 µl of chloroform/isoamyl alcohol mixture (24:1) were gently mixed to the genomic DNA shearing. Samples were centrifuged at 10,000 *g* for 10 min. Then, the aqueous phase was transferred into a new tube; this step was repeated several times to obtain a clear sample. For DNA precipitation, 2/3 volume of a precooled at –20°C isopropanol molecular grade was added and gently mixed. Then, the samples were incubated at 4°C overnight. After centrifugation at 13,000 *g* for 10 min, the supernatant was removed, and the pellet was washed by 200 µl washing buffer (76% ethanol, 10 mM ammonium acetate). The washing buffer was carefully removed, and the pellet was re-suspended in 200 µl TE buffer supplemented with RNAse A (10 µg/ml). After incubation

at 37°C for 30 min, 100 µl of 7.5 M ammonium acetate and 750 µl of ethanol were added and then gently mixed. Furthermore, the samples were centrifuged, and the pellet was re-suspended in a suitable volume of sterile distilled water. DNA concentration and purity were determined by optical density at 260 nm and ratio optical density at 260/280 nm, respectively, using Nanophotometer (Implen GmbH, Germany). DNA samples were stored at −20°C until used.

b- DNA amplification and sequencing

The DNA amplification and sequencing of *Ascotricha lusitanica* were carried out in SolGent Company Limited (Daejeon, South Korea). The ribosomal ITS region was amplified by PCR using a primer pair of ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Oneµl of the fungal DNA (20 ng) was amplified in a 25 µl reaction mixture with Solgent EF-Taq as follows: 10X EF-Taq buffer 2.5 µl, 10 mMdNTP (T) 0.5 µl, primer (Forward-10 picomol) 1.0 µl, primer (Reverse −10 picomol) 1.0 µl, EF-Taq (2.5U) 0.25 µl, Distilled water (to 25 µl). The thermocycling conditions included an initial denaturation for 15 s at 95 °C, followed by 30 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 55 °C, extension at 72 °C for 60 s and a final extension at 72 °C for 10 min. The PCR product was separated by gel electrophoresis on 1% agarose gel run for 75 min in buffer TAE (40 mMTris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2) and detected under a UV illuminator. The PCR product corresponding to ribosomal ITS, according to electrophoretic migration, was eluted from the gel and then purified using purification kit (SolGent, Daejeon, South Korea) depending on to the protocol's instructions [34]. The two strands of amplified ribosomal ITS region were sequenced using primers ITS1 and ITS4. The obtained sequences were analyzed using BLAST search program at the NCBI website: http://blast.ncbi.nlm.nih.gov/Blast.cgi.The alignment was assayed using the multiple sequence alignment program CLUSTALW. Phylogenetic analysis was performed with referential strains from GenBank using MegAlign (ver. 5.01).

c- Screening for antimicrobial secondary metabolites produced by endophytic fungi

Production of bioactive secondary metabolites by endophytic fungi was determined against three human pathogenic dermatophytes (*Microsporum canis*, *Trichophyton rubrum* and *T. mentagrophytes*). These fungal pathogens were obtained from Assuit University Moubasher Mycological Centre (AUMMC). Sabourauds Dextrose Agar medium (SGA) containing (g/L): 40 dextrose, 10 peptone and 15 agar per liter was used for cultivation of dermatophytes.

The ability of endophytic fungi to produce antimicrobial compounds that inhibit the human pathogenic microbes was preliminary determined using dual culture method Omero *et al*. [35]. Petri dishes (9 cm. diam.) containing 12 ml SGA medium were seeded with the human pathogen on one plate edge, then after 48 hours incubation at 28°C± 2 the tested fungal endophyte was seeded on opposite position edge. The endophytic fungi producing bioactive secondary metabolites have the ability to suppress the growth of the human pathogen showing inhibition zone.

RESULTS

Fungi isolated in the present study

Nineteen species belonging to eleven fungal genera in addition to white sterile mycelia were isolated during this investigation. These fungal taxa were recovered from two medicinal plants, belonging to family Asteraceae. These fungi were represented by the following groups: Zygomycota (*Syncephalastrum*), teleomorphic Ascomycota (*Ascotricha* and *Chaetomium*), anamorphic Ascomycota (*Aspergillus* and *Penicillium*) and anamorphs of Hyphomycetes (Table 1). *Aspergillus* and *Penicillium* possessed the highest spectrum of fungal species (6 and 4 species respectively).

The highest endophytic fungal count was noticed on leaf samples of *Artemisia judaica* (44 and 38 CFU/125 leaf segments on PDA and CCA media, respectively). However, leaf samples of *A. judaica* exhibited the lowest spectra of endophytic fungal genera and species either on PDA (6 genera including 10 species) or CCA media (5 genera including 7 species). On the other hand, leaf samples of *Centaurea cineraria* showed the minimum endophytic fungal count of 32 and 24 CFU/125 leaf segments on PDA and CCA media, respectively.The leaf samples of *C. cineraria* produced 8 and 7genera on PDA and CCA media, respectively with number of species up to 12 and 9, respectively (Table 1).

Interestingly, *Aspergillus* was the most prevalent genus recovered from leaf samples of the studied plants comprising 60% of the total collected samples, followed by *Penicillium* which was recovered in moderate frequency of occurrence (40% of all samples).*Aspergillus* was represented by six species and exhibited a percentage of total fungal count ranging between 16.67– 59.09% of total fungi. *Aspergillus flavus, A. niger* and *A. terreus* were recovered with frequency of occurrence ranging from 20 to 40% of total collected samples. Furthermore, *A. sydowii* and *A. versicolor* were exclusively isolated from *A. judaica* on PDA medium with low frequency of occurrence (20% of total collected samples). Also, *A. fumigatus* was recovered only from *C. cineraria* on the PDA medium with low frequency of occurrence (Table 1).

Penicillium was represented by four species which shared with 6.82 – 12.50% of total fungal count on PDA medium and 8.33 – 13.16% of total fungi on CCA media. At the species-level, all *Penicillium* species were isolated with frequency of occurrence contributing 20% of total samples. *Penicillium aurantiogriseum, P. chrysogenum* and *P. waksmanii* were isolated from leaf samples of *C. cineraria*. However, *P. pinophilum* was isolated from leaf samples of *A. judaica* (Table 1).

Chaetomium globosum and *Cladosporium cladosporioides* were moderately isolated (40% of total collected samples) from the leaf samples of the studied plants accounting for 11.36 – 26.32% and 6,82– 9,38% of total fungi obtained on PDA and CCA respectively (Table 1). *Nigrospora sphaerica* was recovered from leaf samples of the studied plants with percentage of total fungal count ranging between 5.26 – 16.67% of total fungi. *Alternaria alternata* was recovered from leaf samples of *A. judaica*. It is worthy to mention that four fungal species namely; *Ascotricha lusitanica,*

Botryotrichum piluliferum, *Cochliobolus spicifer*, *Stemphylium vesicarium* and *Syncephalastrum racemosum* were exclusively isolated from *C. cineraria* (Table 1).

3.1. Ascotricha lusitanica as a new recorded species to Egypt

The endophytic fungus *Ascotricha lusitanica* strain Ca-1 was isolated from healthy asymptomatic leaf samples of *C. cineraria.* To the best of our knowledge, this is the first report of *A. lusitanica* as an endophyte isolated from *C. cinerariain*. The fungus was firstly identified according to colony morphology and microscopic characteristics (Figure 1). *A. lusitanica* strain Ca-1showed slow growth on potato dextrose agar, attaining a diameter of about 35-45 mm in 15 days of incubation at 25 °C. The colonies were initially as yellowish felt covered white aerial mycelium. Then, colonies become greyish black to dark gray sectors appeared on the surface with presence of abundant ascomata.

The cultures were distinguished with yellow mycelia. The texture was floccose with reverse yellow (Figure 1, A-B). Ascomata superficial, black, subglobose to ovoid, 100-150 × 60-120 µm, with a short, distinct neck at apex, ostiolate, extruding ascospores into a mass of dark brown terminal hairs (Figure 1, C).

Terminal and lateral hairs numerous, black, stiff, simple, occasionally branched near the base, producing hyaline ampullae at each joint or on short (one celled) extensions of a joint and also at the apex; hairs 160-258 x 2.8-3.2(4.0) µm slightly narrower toward the apex (Figure 1, D-E). Substrate hyphae producing numerous ampullate hairs like those on the fruiting body, though usually narrower, and together with the dark conidiophores bearing conidia along most of their length and also terminally in groups on short subhyaline branches; ampullate hairs sometimes bearing conidia apically. Conidia were subhyaline to light olive, irregularly ellipsoid to pyriform or tick-shaped, 4.7-6.3 x 3.2 µm, smooth to rough (Figure 1, D-E). Asci extremely delicate, cylindrical to subcylindrical, 8-spored measuring 33.2-47.4 x 6.3 µm containing eight ascospores and arranged in a single row.

Ascospores dark, olive-brown when mature, irregularly oval in face view, ellipsoid, compressed edgewise with a light colored girdle (as an equatorial germ furrow), 7.9-9.5 x 5.6-6.3 µm (Figure 1, G).

Subsequently, *A. lusitanica* was molecularly identified by amplification of the ribosomal internal transcribed spacer (ITS) region. The amplified and sequenced fragment of the 18S ribosomal DNA gene (472 base pair) confirmed that *Ascotricha lusitanica*strain Ca-1 has a 99.34% similarity to *Ascotricha lusitanica* CBS 462.70. The obtained sequences of *Ascotricha lusitanica* strain Ca-1 was deposited in the nucleotide sequence database of GenBank under accession number MT256115. A phylogenetic tree was prepared after alignment with other closely related neighboring sequences of *A. lusitanica* (Figure 2).

Table 1: Endophytic fungal genera and species isolated from leaves of two species from Family Asteraceae (*Centaurea cineraria* and *Artemisia judaica*) using Potato Dextrose Agar (PDA) and Czapek's Cellulose Agar (CCA) media at 25 ± 1 ºC.

Fungi	*Centaurea cineraria*						*Artemisia judaica*					
	PDA			CCA			PDA			CCA		
	TC	%TC	%F	TC	%TC	%F	TC	%TC	%F	TC	%TC	%F
Alternaria alternata	0	0	0	0	0	0	4	9.09	20	0	0	0
Ascotricha lusitanica	2	6.25	20	0	0	0	0	0	0	0	0	0
Aspergillus	15	46.88	60	4	16.67	60	26	59.09	60	19	50	60
A. flavus	3	9.38	40	1	4.17	20	9	20.45	40	10	26.32	40
A. fumigatus	2	6.25	20	0	0	0	0	0	0	0	0	0
A. niger	7	21.88	60	2	8.33	20	4	9.09	20	3	7.89	20
A. sydowii	0	0	0	0	0	0	1	2.27	20	0	0	0
A. terreus	3	9.38	20	1	4.17	20	9	20.45	40	6	15.79	40
A. versicolor	0	0	0	0	0	0	3	6.82	20	0	0	0
Botryotrichum piluliferum	2	6.25	20	0	0	0	0	0	0	0	0	0
Chaetomium globosum	0	0	0	4	16.67	40	5	11.36	40	10	26.32	40
Cladosporium cladosporioides	3	9.38	40	0	0	0	3	6.82	40	0	0	0
Cochliobolus spicifer	0	0	0	3	12.5	20	0	0	0	0	0	0
Nigrospora sphaerica	3	9.38	20	4	16.67	20	0	0	0	2	5.26	20
Penicillium	4	12.5	40	2	8.33	40	3	6.82	40	5	13.16	40
P. aurantiogriseum	2	6.25	20	0	0	0	0	0	0	0	0	0
P. chrysogenum	2	6.25	20	0	0	0	0	0	0	0	0	0
P. pinophilum	0	0	0	0	0	0	3	6.82	40	5	13.16	20
P. waksmanii	0	0	0	2	8.33	20	0	0	0	0	0	0
Stemphylium vesicarium	0	0	0	2	8.33	20	0	0	0	0	0	0
Syncephalastrum recemosum	1	3.13	20	0	0	0	0	0	0	0	0	0
White sterile mycelia	2	6.25	20	5	20.83	20	3	6.82	40	2	5.26	20
TC (CFU / 125 leaf segments)	**32**			**24**			**44**			**38**		
Number of genera (11)	**8**			**7**			**6**			**5**		
Number of species (19)	**12**			**9**			**10**			**7**		

Abbreviations: TC, total fungal count (CFU /125 of leaf segments); %TC, percentage of total fungal counts; %F, percentage of frequency of fungal genera and species per the total collected samples

3.1. Anti-dermatophytic activities of endophytic fungi

In this study, screening on the ability of endophytic fungi to produce antimicrobial secondary metabolites was investigated using dual culture technique. *In vitro*, the suppressive impacts of fifteen fungal strains belonging to thirteen species against three dermatophytic fungi (*Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes*) were tested (Table 2). Among the tested fungal isolates, *A. lusitanica* showed exclusively antifungal activity against *M. canis.* On the other hand, the remaining isolates (fourteen isolates) exhibited no activity against *M. canis* (Table 2).

It is worthy to mention that *A. lusitanica* exhibited high suppressive impact against *T. rubrum* and *T. mentagrophytes* with inhibition zones of 6.33 and 8.33 mm, respectively (Table 2). On the other hand, *Cladosporium cladosporioides* and *Nigrospora sphaerica* had no suppressive impact against *T. rubrum* and *T. mentagrophytes*. Moreover, twelve isolates belonging to four fungal genera showed low inhibitory action against *T. rubrum* and *T. mentagrophytes* producing inhibition zones equal or less than 2 mm. The previous results related to dual culture method revealed that *A. lusitanica* was the most potent strain producing secondary metabolites with antimicrobial impact against the dermatophytes *M. canis*, *T. rubrum* and *T. mentagrophytes* (Figure 3).

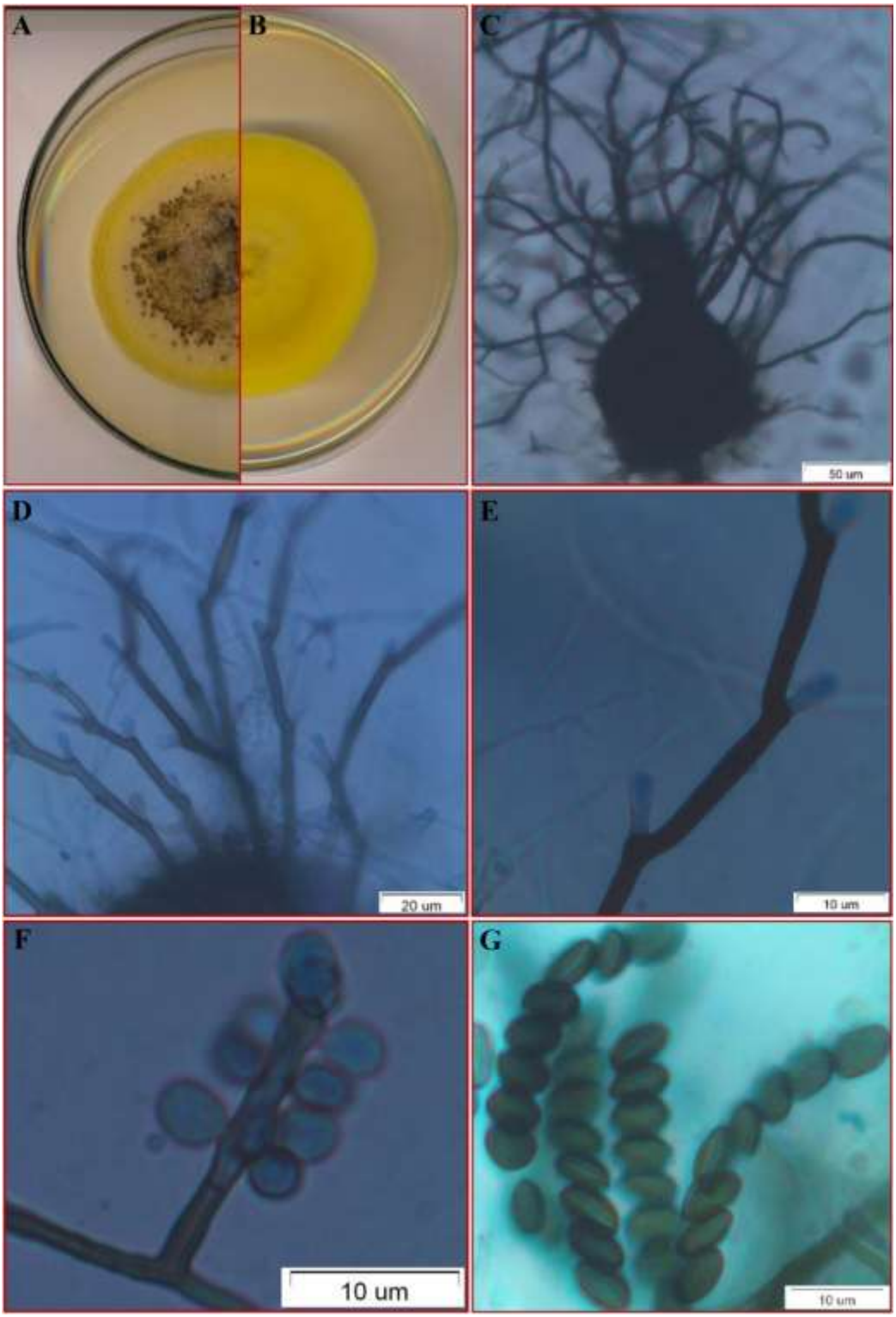

Figure 1: *Ascotricha lusitanica* strain Ca-1: (A-B) colony surface and reverse of 15-days-old culture at 25 °C on PDA medium; (C) Ostiolate ascoma with appendages; (D-E) Ascomatal appendages erect, rigid and geniculate; (F) smooth and ellipsoidal to pyriform conidia; (G) Discoid ascospores.

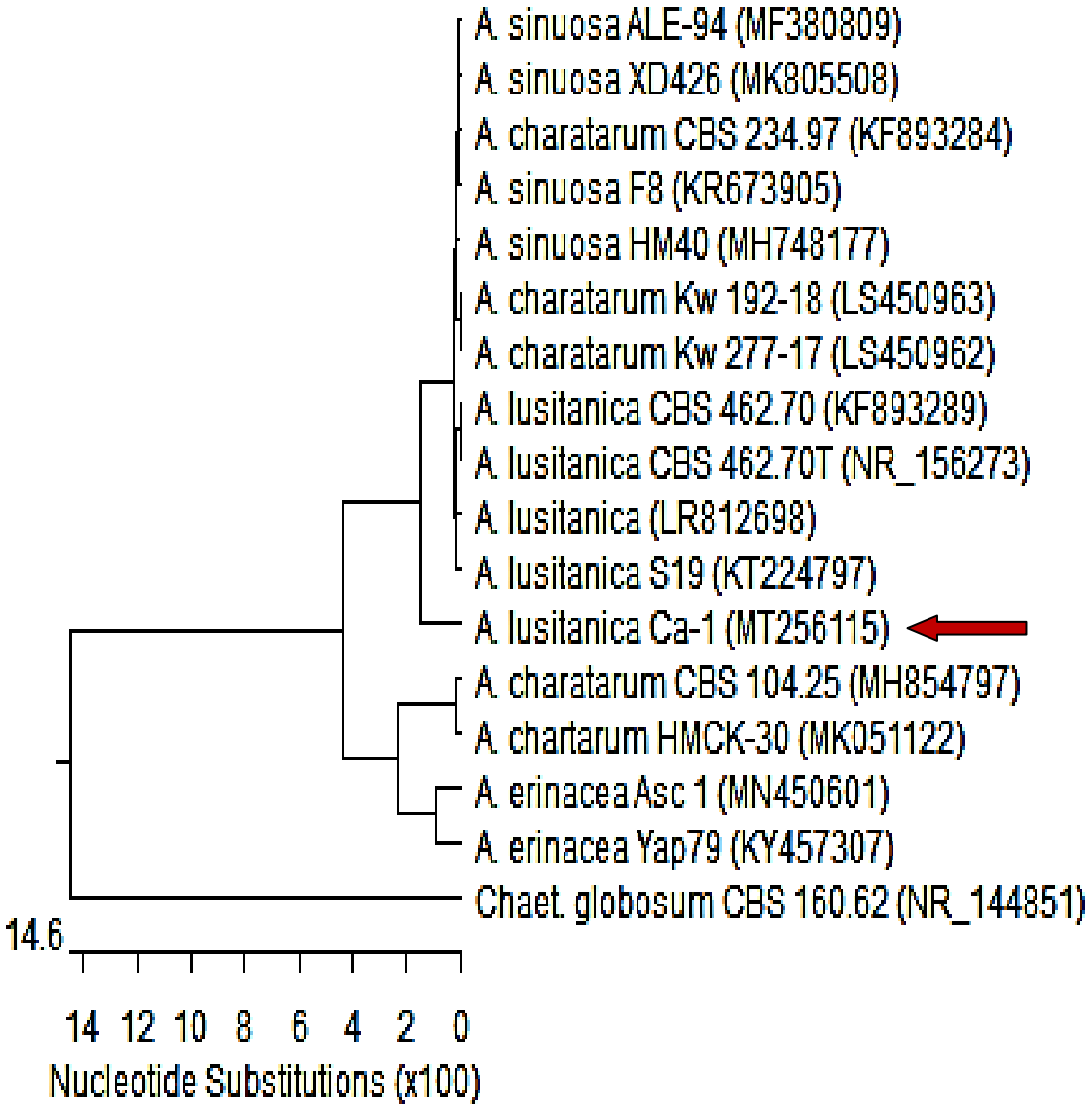

Figure 2: Phylogenetic tree based on ITS sequences of rDNA of the fungal sample isolated in the present study (*Ascotricha lusitanica* strain Ca-1 with GenBank accession No. MT256115, arrowed) aligned with closely related strains accessed from the GenBank. It showed 99.34% identity and 96% coverage with the type strain CBS 462.70T (NR_156273). *Chaetomium globosum* is included in the tree as out-group strain.

Table 2: Screening on secretion of antimicrobial secondary metabolites by endophytic fungi using Dual culture method against three human pathogenic fungi (*Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes*).

Species	Isolate No.	Source	Inhibition zone by mm					
			M. canis		*T. rubrum*		*T. mentagrophytes*	
			Value ± SD	Mark	Value ± SD	Mark	Value ± SD	Mark
Ascotricha lusitanica	Asc-403	*Centaurea cineraria*	0.67 ± 0.58	**L**	6.33 ± 0.58	**H**	8.67 ± 0.58	**H**
Aspergillus flavus	As-16	*Artemisia judaica*	0.00	**N**	1.33 ± 0.60	**L**	1.33 ± 0.58	**L**
Aspergillus flavus	As-15	*Centaurea cineraria*	0.00	**N**	1.33 ± 0.61	**L**	1.33 ± 0.58	**L**
Aspergillus fumigatus	As-11	*Centaurea cineraria*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Aspergillus niger	As-24	*Centaurea cineraria*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Aspergillus sydowii	As-9	*Artemisia judaica*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Aspergillus terreus	As-2	*Artemisia judaica*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Aspergillus terreus	As-25	*Centaurea cineraria*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Aspergillus versicolor	As-22	*Artemisia judaica*	0.00	**N**	1.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Chaetomium globosum	Ch-428	*Artemisia judaica*	0.00	**N**	1.67 ± 0.58	**L**	1.33 ± 0.58	**L**
Cladosporium cladosporioides	Cl-315	*Centaurea cineraria*	0.00	**N**	0.00	**N**	0.00	**N**
Cochliobolus specifer	Coc-316	*Centaurea cineraria*	0.00	**N**	2.00 ± 0.00	**L**	1.00 ± 0.00	**L**
Nigrospora sphaerica	Nig-305	*Centaurea cineraria*	0.00	**N**	0.00	**N**	0.00	**N**
Penicillium chrysogenum	Pen-118	*Centaurea cineraria*	0.00	**N**	2.00 ± 0.00	**L**	2.00 ± 0.00	**L**
Penicillium pinophilum	Pen-119	*Artemisia judaica*	0.00	**N**	2.00 ± 0.00	**L**	2.00 ± 0.00	**L**

H = show high inhibition zone (more than half of the obtained maximum inhibition rate*).
L = show low inhibition zone (less than half of the obtained maximum inhibition rate *).
N = Fungi showing no antagonistic effect (no inhibition zone)

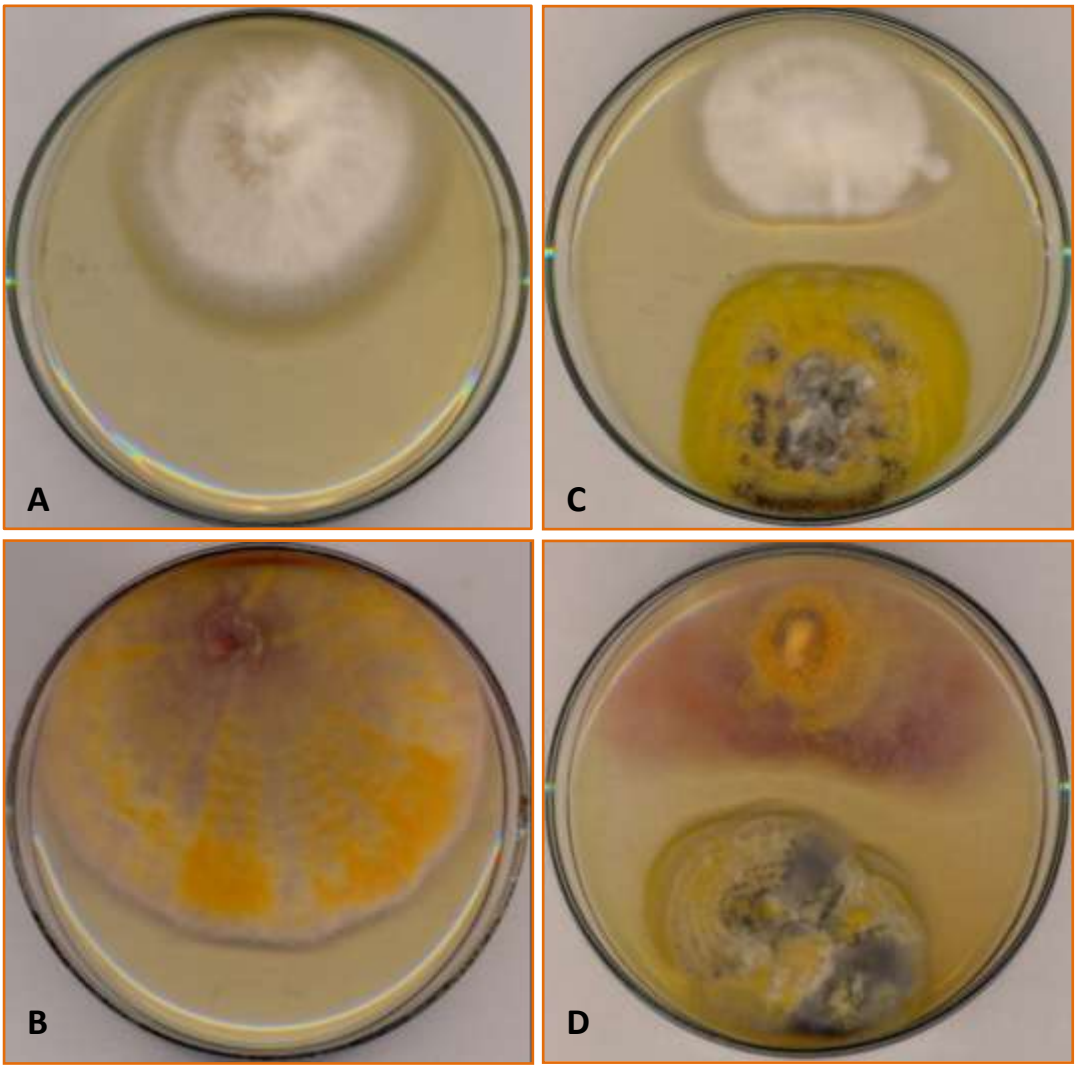

Figure 3: Potentiality of the *Ascotricha lusitanica* strain Ca-1to produce anti-dermatophytic secondary metabolites against **(A)** *Trichophyton mentagrophytes* and **(B)** *T. rubrum* **(C-D)** *A. lusitanica* produced anti-dermatophytic compounds causing considerable inhibition zones.

DISCUSSION

In recent years the increase of microbial infections and the development of microbial resistance to synthetic drugs led to renewed interest to investigate natural sources of bioactive substances for therapy against infectious pathogens [36]. Endophytic fungi isolated from medicinal plants are well known to be valuable sources of a wide range of bioactive metabolites, with antimicrobial, cytotoxic, anticancer, antioxidant, antimalarial, and antiviral activities [37].

Family Asteraceae fulfill the rationale for plant selection to isolate endophytes since most of the species of this family have a long tradition of healing usage, confirmed by modern pharmacognosy [38]. Interestingly, the comparative analysis of the Asteraceae host and/or fungal endophyte therapeutic activity showed similarities [39].

In this study, the diversity of endophytic fungi associated with *Artemisia judaica* and *Centaurea cineraria* as members of Asteraceae was investigated. Nineteen species belonging to eleven fungal genera were isolated and identified. The most common fungal species were affiliated to Ascomycestes, while the remainders were represented by Zygomycota and Hyphomycetes. Reports on endophytic fungi isolated from *A. judaica* are available [40]. Several studies revealed that endophytic fungi associated with medicinal plants were mainly members of Ascomycota or their mitosporic fungi; some are members of Zygomycota, Oomycota, and Basidiomycota [41 and 42]. The widest research program to find endophytes in medicinal Asteraceae has been performed in countries which are localized in the most important biodiversity hotspots, like Brazil, China, the Mediterranean region, Iran, or Thailand [43]. Interestingly, Caruso *et al*. [39] presented a valuable review about endophytic fungi associated with medicinal plants of Asteraceae. They mentioned that endophytic fungi were ubiquitous and isolated from most plant species and environments. Moreover, the most abundant fungi included *Alternaria, Aspergillus, Colletotrichum, Penicillium, Fusarium, Nigrospora, Phoma, Papulaspora, Pestalotiopsis, Chaetomium, Curvularia* and *Phyllosticta.*

Results of the current study revealed that *Aspergillus, Penicillium*, *Alternaria, Chaetomium* and *Cladosporium* were the most predominat fungi recovered as endophytes from *A. judaica* and *C. cineraria.* In accordance with these findings, Zhang *et al*. [44] identified *Aspergillus, Fusarium, Cephalosporium* and *Mucor* as prevailing endophytic genera isolated from *Artemisia annua.* The genus *Mucor* was isolated as an endophyte from the medicinal plant *Centaurea stoebe* [45]. Qian *et al*. [46] reported that *Alternaria alternata, A. tenuissima, Cladosporium cladosporioides, Chaetomium globosum, Fusarium nematophilum* and *Penicillium radicum* were frequently isolated as fungal endophytes from *Artemisia argyi*. Also, Cosoveanu *et al*. [47] found that *Alternaria alternata, Aspergillus flavus* and *Cladosporium* sp. were common endophytes from *Artemisia thuscula*. A recent report on endophytic fungi inhabiting *Artemisia judaica* [40] revealed that *Alternaria, Aspergillus, Chaetomium, Cladosporium,* and *Fusarium* were the most prevalent genera

The endophytic fungus *A. lusitanica* strain Ca-1 was isolated from healthy asymptomatic leaf samples of *C. cineraria.* To our knowledge, *A. lusitanica* is new record to Egypt and this is the first report of *A. lusitanica* as an endophyte isolated from *C. cinerariain*. Moreover, *A.lusitanica* was identified based on the colony morphology, microscopic characteristics and molecular techniques. Indeed, various endophytic fungi isolated from different plant groups have been identified using polyphasic approaches, including phenotypic and genotypic methods [48]. Also, due to the phenotypic similarities of *Ascotricha* with *Chaetomium* and Chaetomium-like fungi, the application of molecular methods is needed for its accurate identification [49].

Generally, *A. lusitanica* appears to be a rare species worldwide. Kenneth [29] reported *A. lusitanica* as a new species of *Ascotricha.* It was isolated from Chickpea (*Cicer arietinum* L.). Fourteen species have been assigned to *Ascotricha*; they mostly occur in soil or on paper, lignum or other cellulosic substrates [50]. *Ascotricha canariensis* was isolated from soil of the Canary Islands, located near the African continent in the Atlantic Ocean [51]. *Ascotricha longipila* and *A. parvispora* were

described as new marine algae-associated fungi [50]. Another report [52] described *Ascotricha amesii* as new species to Japan and it was isolated from garden soil in Osaka. *A. erinacea* and *A. chartarum* were isolated, as new records for the United States, from indoor environments [53]. Furthermore, *Ascotricha hispanica* was described and illustrated as a new species of *Ascotricha* from Spanish soil [54].

In this study, the ability of endophytic fungi were tested for the production of antifungal secondary metabolites against three dermatophytes (*Microsporum canis, Trichophyton rubrum* and *T. mentagrophytes*). It is noteworthy to mention that *Ascotricha lusitanica* showed a significant potentiality to produce secondary metabolites with anti-dermatophitic impact. Development of novel antifungal drugs is an urgent necessity to research new medications and pharmaceuticals, since, in the recent years, because of the rise in pathogenic fungi with different drug-resistant patterns have been observed [36]. Fungi belonging to the genus *Ascotricha* are capable of producing a diverse array of secondary metabolites. In this respect, *Ascotricha amphitricha* produced compound Ascosteroside with valuable anti-Candida activity [55]. A lanostane-type triterpenoid ascosteroside with antifungal property was isolated from *Ascotricha amphitricha* [56]. A cyclonerodiol analogue 3,4-seco lanostane triterpenoid was produced by a marine-derived fungus *Ascotricha* sp. ZJ-M-5 [57]. An antagonist of sphingosine-1-phosphate receptor 1, with anti-angiogenic activity, was isolated from *Ascotricha chartarum* [58].

CONCLUSION

Endophytic fungi isolated from medicinal plants represent a renewable treasure for bioactive metabolites. Due to a rise in the development of drug-resistant fungi, searching a novel antifungal drug is an urgent necessity to new medications. This study concerned with diversity of endophytic fungi associated with *Centaurea cineraria* and *Artemisia judaica.* Interestingly, *Ascotricha lusitanica,* was isolated as a new record to Egypt, and was identified according to phenotypic and genotypic characterization. Screening on the ability of endophytic fungi to produce antimicrobial secondary metabolites against dermatophytes showed the potentiality of *Ascotricha lusitanica* as a source of anti-dermatophitic substances. This species can be considered a promising source of antimicrobial natural products involved in the pharmaceutical applications. Also, more research is required for further elucidation of the active biochemical secondary metabolites produced by *A. lusitanica.*

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