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Isolation and Identification of Endophytic Fungi of Licorice and Their Effect on Human Pathogenic Fungi

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ABSTRACT

Background: Endophytes are microorganisms residing within plants, which have recently gained attention for producing bioactive compounds with pharmacological properties like antioxidant, anti-inflammatory, anticancer, and antibiotic effects. Licorice plants (*Glycyrrhiza glabra*) may serve as a source of these bioactive metabolites, particularly those with antifungal potential. This study aimed to evaluate the antifungal properties of endophytic fungi residing in licorice plants against human pathogenic fungi.
Materials & Methods: Licorice plant samples were collected from three locations in Hamadan in October 2022. Endophytic fungi were collected from different parts of licorice plants, identified morphologically using microscopy, and confirmed through molecular methods by DNA extraction and amplification, with sequencing results compared to public databases. The isolated fungi were cultured on PDA (potato dextrose agar) medium, and crude extracts were obtained using ethyl acetate. The antifungal properties of crude extracts were investigated against four fungal pathogens including *Aspergillus niger*, *Candida glabrata*, *Cryptococcus neoformans*, and *Pseudallescheria boydii* using disc diffusion method and ketoconazole as a positive control.
Findings: A total of 21 endophytic fungi were identified, with *Fusarium* as the dominant genus. *F. oxysporum* and *F. redolens* were prevalent in root tissues, while *A. niger* dominated in stems and leaves. *A. niger* exhibited strong antifungal effects against *A. niger*, *C. neoformans*, and *P. boydii*.
Conclusion: Licorice plant roots hosted the highest number of endophytes, likely due to reduced exposure to pollution. *A. niger* and *F. oxysporum* demonstrated significant potential as biocontrol agents against pathogenic fungi, making them promising candidates for sustainable disease management in agriculture and human health.

Keywords: Endophyte, Licorice, Pathogenic fungi

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Introduction

Human reliance on natural sources to discover novel pharmaceutical agents to combat diseases is well-documented [1]. Efforts to find safer, chemical-free, and less toxic therapeutic alternatives have driven the exploration of innovative approaches to address health challenges. As a result, numerous medications such as antifungal, antibiotic, antiulcer, anti-inflammatory, antidiabetic, and antioxidant agents have been derived from a variety of sources like medicinal and edible mushrooms (higher fungi), medicinal plants, and microbial reservoirs such as endophytes, bacteria, and fungi [2-5].

In recent years, bioactive metabolites of both known and novel endophytes have revealed diverse pharmacological properties, including antioxidant, anti-inflammatory, anticancer, antiviral, and antibiotic effects [6]. Endophytes are microorganisms that live within plant tissues without causing disease, which have recently emerged as promising reservoirs of new bioactive compounds [7]. Endophytes dwelling within the internal tissues of plants without causing symptomatic infections have been observed to confer protective benefits to their host plants through [8, 9]. the production of bioactive substances Remarkably, a single plant could host multiple endophytes, contributing to the rich microbial diversity of plant ecosystems [10, 11]. Licorice (*Glycyrrhiza glabra*) is a widely studied medicinal plant in Iran [12]. The medicinal and flavoring properties of licorice have led to its extensive use in the pharmaceutical and food industries [1 3]. Licorice roots contain various secondary metabolites, including chalcones, coumarins, saponins, and flavonoids, which have antioxidant properties and offer numerous health benefits [14, 15]. Studies have demonstrated potent antimicrobial effects of licorice against fungi and bacteria, such as *Candida albicans*, *Aspergillus niger*, and *A. flavus* [13]. Therefore, endophytes in licorice plants may harbor a wealth of biologically active metabolites. This study aimed to evaluate the antifungal properties of endophytic fungi residing in licorice plants against human pathogenic fungi.

Objectives: The primary objective of the present study was to investigate the antifungal activity of endophytic fungal isolates collected from different parts of licorice plants (root, stem, and leaves) against human pathogenic fungi.

Materials and Methods

Collection of plant materials and isolation of endophytes: Samples of licorice plants (*Glycyrrhiza glabra*) were gathered from three locations (Moradbeg, Imamza-

Figure 1) Variations of licorice plants isolated from three regions: (A) Moradbeg, (B) Imamzadeh, and (C) Mavshan

deh, and Mavshan) in Hamadan during October 2022. Licorice plants were identified by a taxonomist in Hamadan, Iran (Figure 1). A testimonial sample (DACB-47653) of each plant was preserved in the herbarium for future reference.

The bark and leaves of healthy fully-grown plants were randomly selected for use in this research. Plant samples were collected in sterile bags and transported to the laboratory, where they were processed within a few hours of collection. Plant parts were placed on a clean glass plate and cut with a sterilized blade. Endophytic fungi were collected from healthy, fresh plant samples according to a modified protocol based on previous studies [16, 17].

Morphological identification of endophytic fungi: To identify the collected fungal isolates, they were placed on culture slides, stained with a lactophenol cotton blue reagent, and evaluated under phase-contrast and bright-field microscopes. Morphological identification of endophytic fungi was performed by considering characteristics mentioned in standard identification manuals, including conidia size and coloration, sporulation and acervulus production, aerial mycelium, margin characteristics, surface texture, colony and medium color, hyphal structure, and growth pattern [18].

Molecular identification of endophytic fungi: DNA amplification and internal transcribed spacer (ITS) sequencing were used for molecular identification of endophytic fungi following a molecular biological protocol [19, 20]. A small segment of fungal hyphae (0.5–1.0 cm^2) was isolated from the Petri dish used for fungal cultivation and lyophilized in a 2 mL Eppendorf tube (Eppendorf, Germany). Lyophilized fungal mycelia were powdered, and DNA was extracted. Fungal DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, USA) based on the manufacturer's protocol. The procedures included cell lysis, RNA digestion, removal of precipitates and debris, and DNA shearing, precipitation, and purification. The isolated DNA samples were amplified using PCR (polymerase chain reaction) and the HotStarTaq Master Mix Kit (Qiagen, USA). The primers ITS1 and ITS4 (Invitrogen, USA) were mixed with the Hot Star Taq Master Mix Kit and DNA template in a total volume of 50 µL. Table 1 shows the primers used in this research.

The initial step was performed for 5 min at 95 °C. Subsequently, 35 cycles of PCR were conducted, which included the following steps: initial denaturation for 30 s at 95 °C, primer annealing for one min at 55.5 °C, and DNA amplification by polymerase for one min at 72 °C. Finally, a terminal extension step was carried out for 10 min at 72 °C.

After the reaction, PCR products were electrophoresed on a 1% agarose gel at 90 volts and analyzed using a gel documentation system. Amplified fungal DNA samples (PCR products) were submitted for sequencing, and the resulting sequences were compared to publicly accessible databases, including GenBank, by employing the BLAST algorithm.

Endophytic fungal cultivation and extraction: Cultivation of isolated fungal strains was performed on Petri dishes using approximately 1 L of PDA (potato dextrose agar) medium for each isolate. The fungal strain was incubated at 28 °C for 21 days, and the culture medium was extracted twice with ethyl acetate to obtain the crude extract

Table 1) Primers used in the present study

Primer Name	Nucleotide Sequence 3′ -5′	Annealing Temperature	Reference
ITS1 (F)	TCCGTAGGTGAACCTGCGG	55.5	[32]
ITS4 (R)	TCCTCCGCTTATTGATATGC	55.5	[33]

[19]. The fungal extract was condensed into a solid residue by solvent evaporation using a rotary vacuum evaporator under reduced pressure, yielding ethyl acetate crude extracts [20].

Antifungal activity test: The antifungal properties of isolated fungi and licorice plant parts were investigated against four fungal pathogens using the disc diffusion technique with slight modifications, including *A. niger*, *C. glabrata*, *Cryptococcus neoformans*, and *Pseudallescheria boydii* [20].

A ketoconazole disc (30 µg/disc) served as a positive control. Solvents acted as negative controls. The inhibition zones around the discs were measured following incubation at 37 °C for 18 to 24 hours for bacteria and at 28 °C for 48 to 96 hours for fungi. The sensitivity of pathogenic fungi was determined by measuring the inhibition zone diameters in millimeters.

Statistical analysis: The experimental data obtained from the antifungal activity tests were recorded as the inhibition zone diameters (in mm).

All tests were performed in triplicate, and the findings were expressed as mean ± SD (standard deviation). ANOVA (one-way analysis of variance) test was used to analyze differences between groups using GraphPad Prism software Version 9.0 (GraphPad Software, San Diego, CA, USA). Post hoc comparisons were conducted by employing Tukey's test to identify substantial differences between groups. The significance level was set at $p < .05$.

Findings

Morphological identification of fungal isolates: Totally, 21 endophytic fungal strains were collected from the leaves and bark of licorice plants, each of which was assigned an internal strain code: MS1, MS2, MS3, MS4, KS4, KS5, KR1, KR2, KR3, KR4, KR5, DR1, DR2, DR3, DR4, DR5, MR1, MR2, MR3, MR4, and MR5.

Microscopic examination revealed that strains DR1, DR2, and DR3 exhibited columnar conidial heads and flask-shaped vesicles with phialides covering half to three-quarters of the vesicle. The conidia of these strains were globose with a finely uneven surface, which displayed a plain green color, indicating their classification within *Aspergillus* species (Figure 2).

Strains MR1, MR2, MR3, MR4, MS3, KR1, and KR3 showed thinly-scattered to plentiful cottony mycelia with pigmentation ranging from colorless to pale violet and white to violet.

These strains contained hyaline conidia or phialospores and exhibited two spore types: unicellular oblong microconidia and multicellular macroconidia with slightly twisted or curved pointed ends. The colonies of these strains were fluffy with cylindrical spores that were either septate or aseptate. Accordingly, strains MR1, MR2, MR3, MR4, MS3, KR1, and KR3 were found to belong to *Fusarium* species (Figure 2). Other fungi were differentiated based on their unique microscopic characteristics.

Molecular identification of fungal

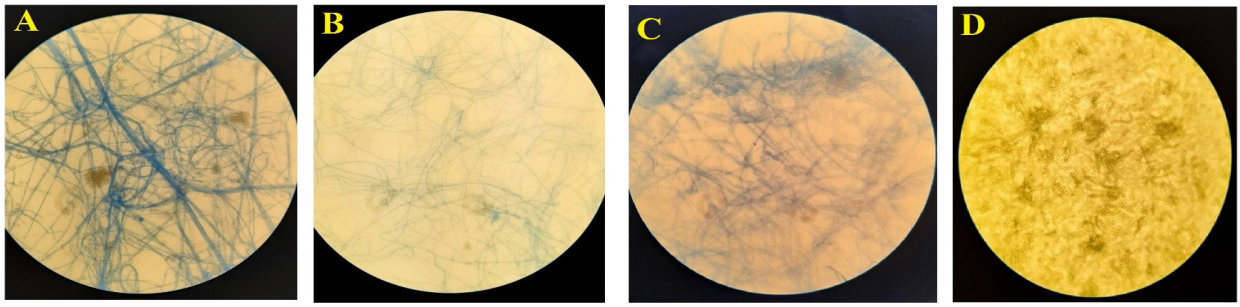

Figure 2) Microscopic structures of endophytes: (A) *Aspergillus*, (B) *Fusarium,* (C) *Aspergillus*, (D) *Fusarium*

isolates: To confirm the identity of the isolated strains, DNA sequence analysis was performed using Mega BLAST software in the U.S. National Center for Biotechnology Information (NCBI) databases. Table 2 provides information on endophytic fungi collected from licorice plants.

Antifungal screening of endophytic fungi isolated from licorice: To assess antifungal activity, extracts obtained from endophytic fungi were screened at a concentration of 100 µg per disc. *A. niger* extract was effective against all tested pathogenic fungi. Analysis of variance and evaluation of the average effect of *A. niger* endophytic fungus revealed that this endophyte had similar effects on *neoformans.*pathogenic fungi *A. niger* and *C* as the control. The other two pathogenic fungi, *P. boydii* and *C. glabrata*, showed less sensitivity to this endophyte than the

Table 2) NCBI BLAST results of fungal isolates recovered from licorice

Isolate	Species	Isolate	Species
MR1	*Fusarium f.sp*	DR4	*Fusarium f.sp*
MR2	*Fusarium oxysporum*	DR5	*Fusarium f.sp*
MR3	*Fusarium f.sp*	MS2	*Aspergillus niger*
MR4	*Fusarium f.sp*	KR1	*Fusarium redolens*
MR5	*Fusarium oxysporum*	KR2	*Fusarium redolens*
MS1	*Aspergillus niger*	KR3	*Fusarium redolens*
MS3	*Aspergillus niger*	KR4	*Fusarium redolens*
MS4	*Aspergillus niger*	KR5	*Fusarium redolens*
DR1	*Fusarium f.sp*	KS4	*Fusarium f.sp*
DR2	*Fusarium f.sp*	KS5	*Fusarium f.sp*
DR3	*Fusarium oxysporum*		

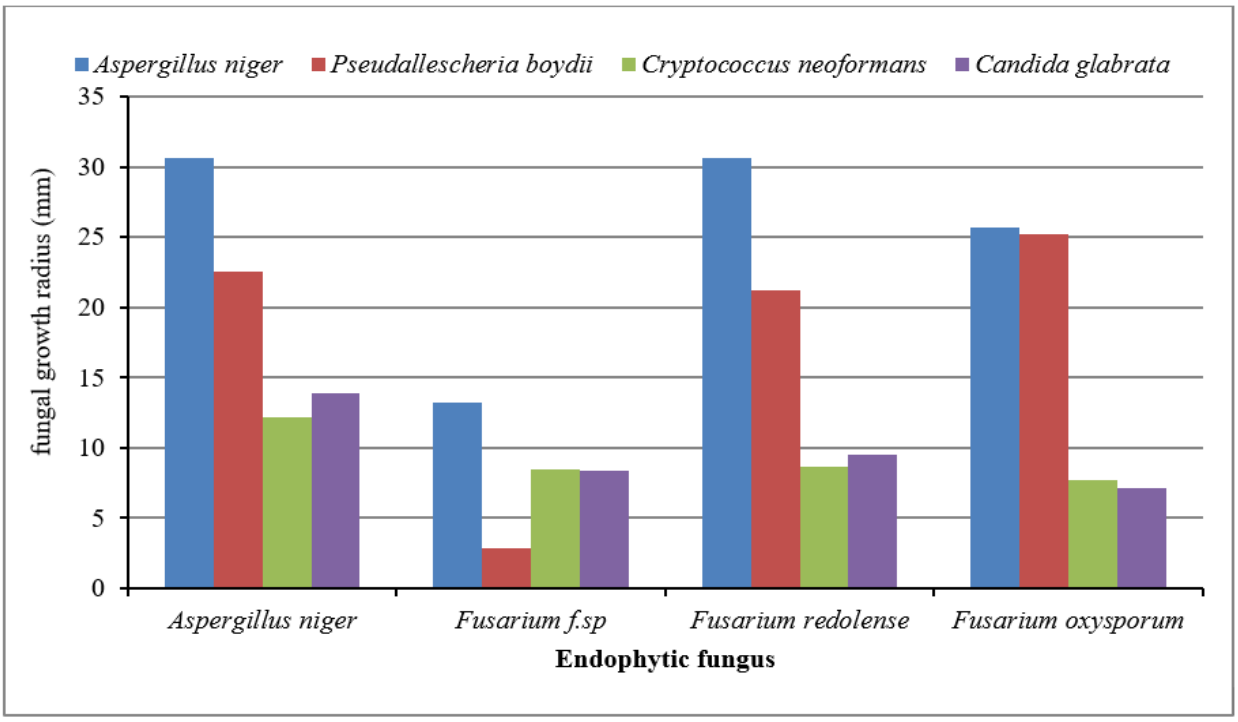

Figure 3) Comparison of the average effect of endophytic fungi isolated from licorice plant on the studied human pathogenic fungi

Table 3) Variance analysis of the effect of fungi isolated from licorice on the studied human pathogenic fungi

Pathogenic Fungus	Endophytic Fungi			
	Fusarium oxysporum	***Fusarium redolens***	***Fusarium f.sp***	***Aspergillus niger***
Aspergillus niger	45.1	39.5	73.5	100.0
Pseudallescheria boydii	27.2	27.6	9.3	43.1
Cryptococcus neoformans	31.0	28.1	69.1	100.0
Candida glabrata	23.4	24.9	82.2	83.9
Control	-	-	-	-

control, with the least sensitivity observed in the pathogenic fungus *P. boydi*. *F. redolens* extract was also found to be effective against pathogenic fungi, the highest and lowest inhibitory effects of this fungus were against pathogenic fungi *A. niger* and *C. glabrata*, respectively. Also, *F. oxysporum f. sp* showed .similar effects on pathogenic fungi *C neoformans*, *A. niger*, and *C. glabrata,* with the greatest effect observed against *C. glabrata* pathogenic fungus. *F. oxysporum* extract showed the highest and lowest inhibitory activities against pathogenic fungi *A. niger* and *C. glabrata*, respectively. The antifungal screening results could be observed in Table 3 and Figure 3.

Discussion

The demand for discovering novel chemical entities to combat various diseases is constantly increasing due to the emergence of new threats such as coronavirus disease 2019 (COVID-19), acquired immunodeficiency syndrome (AIDS), cancer, etc. Moreover, the global concern over antibiotic resistance has further amplified this need. Since ancient times, plants have been used as a source of potential biologically active compounds to combat various diseases. Researchers have recently turned their attention towards plant-associated endophytes as they offer metabolites with greater remedial potential than the plants themselves [21]. Endophytic fungal strains are remarkable microorganisms that reside within plant tissues and are recognized as excellent reservoirs of biologically active compounds [22]. Therefore, investigating the biological activities of natural products, whether medicinal plants or fungi, in order to find potential candidate molecules is highly recommended.

In this study, endophytes such as *F. oxysporum*, *F. redolens*, *F. oxysporum f. sp.*, and *A. niger* were identified within licorice plants. In contrast, a similar study conducted in China on licorice plant endophytes identified *F. oxysporum*, *F.* sp. sporium, and *F. graminearum*, highlighting the diversity of endophytic species depending on geographical locations and environmental conditions [23]. The diversity of endophytes in licorice is in line with the findings of another research by Rachmawaty et al. (2024), where *F. sororula*, *Diaporthe* sp., and *Sarocladium zeae* were isolated from corn plants and demonstrated significant antifungal activity against *F. oxysporum*. These findings reinforce the importance of

exploring different plant species and regions to uncover a broad spectrum of endophytic fungi with potential antifungal properties [24].

The antifungal activities observed in this study underscore the significant potential of endophytic fungi as biocontrol agents. *A. niger* endophytes showed the highest inhibitory activity against *A. niger* pathogenic fungi, demonstrating their potent biocontrol capability. Similarly, previous studies have shown that endophytes such as *Trichoderma koningii* and *T. atroviride* isolated from cedar have substantial growth inhibitory effects on *A. niger*, with *Penicillium viridicatum* showing a 51% inhibition rate in culture [25]. These results support the present study findings and suggest that various endophytic fungi possess strong antifungal properties against *A. niger* and other pathogenic fungi. Moreover, in a study by Xiao et al. (2013), endophytes isolated from *Ginkgo biloba*, specifically *Chaetomium globosum*, inhibited pathogenic fungi like *F. graminearum* and *Sclerotinia sclerotiorum*, illustrating the presence and versatility of a wide range of fungi with antifungal capabilities across different plant hosts [26]. Furthermore, this study revealed that *A. niger* endophytes exhibited significant inhibitory effects against *C. neoformans*, aligning with a previous research on Shirin-ebian plant endophytes that also showed inhibitory activity against *C. neoformans*; the two-way cross-culture method used in this previous research was effective in demonstrating the antifungal capabilities of these endophytes, providing a robust approach for screening potential biocontrol agents [27]. These findings align with the findings of another study by Moussa (2024), where endophytic fungi were highlighted as an underexplored resource of novel antifungal compounds effective against multidrug-resistant pathogens such as *C. auris*, *C. albicans*, *C. neoformans*, and *A. fumigatus*. The potential of endophytic fungi to produce unique antifungal metabolites suggests a promising avenue for developing new antifungal agents, particularly against drug-resistant strains [28].

Interestingly, *F. oxysporum* showed high inhibitory activities against *P. boydii* and *C. glabrata*, mirroring the results of studies on fungal endophytes of cypress and other plants like *Nepeta crispa*. The inhibitory effects of endophytes such as *Alternaria radicina* and *Paecilomyces maximus* CAYPB58 against these pathogenic fungi further validate the antifungal potential of endophytic fungi [29-31].

The present research emphasizes the significant role of the plant root as a reservoir of endophytic fungi, likely due to its favorable microenvironment with lower pollution and dust levels. The findings highlight the importance of considering environmental factors influencing endophytic colonization patterns within plants. Identifying endophytes such as *A. niger* and *F. oxysporum f. sp.* as promising biocontrol agents underscores their potential use in sustainable disease management strategies. These endophytes could be harnessed for applications in crop protection and human health, reducing reliance on chemical fungicides and addressing concerns about antibiotic resistance.

Conclusion

This study revealed that the root part of the plant had the highest abundance of endophytes, likely attributable to lower levels of pollution and dust compared to other plant parts. This finding underscores the importance of considering microenvironmental factors influencing endophytic colonization patterns within plants. Moreover, this investigation identified fungal endophytes such as *A. niger* and *F. oxysporum f. sp* as particularly

promising candidates for biological control and management of pathogenic fungi. These findings not only shed light on the ecological dynamics within plant tissues but also highlight the potential of harnessing endophytic fungi for sustainable disease management strategies in agriculture and beyond. Further research in this area is warranted to elucidate the mechanisms underlying the biocontrol properties of these endophytes and to explore their practical applications in crop protection and human health.

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Conflicts of interests: The authors declare that they have no conflicts of interest.

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Authors' contributions: R.H. designed the present study and analyzed the obtained data, and M.E. contributed to experimental studies and sample collections. All authors read and approved the final version of the article.

Consent to participate: Written informed consent for participation in this study was obtained from the participants and/or their legal guardian(s). All techniques used in this study were conducted according to relevant regulations and guidelines. The findings were reported in accordance with STROBE guidelines.

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