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ORIGINAL ARTICLE

Exploring Iraq's Fungal Diversity Isolation and Characterization of *Canariomyces* and *Petreilla* Endophytes

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ABSTRACT

Background: *Endophytic fungi have been considered by their multifaceted roles in inducing systemic resistance of plant hosts against pathogenic microbial infections.* ***Subjective****: The current study was designed to investigate endophytic fungal species in Sidr leaves tree in Iraq and test their antimicrobial activity against microbial pathogens.* ***Methodology:*** *Twenty leave samples of the Sidr plant (Ziziphus spina-christi) were collected from the Al-Midaina District in Basrah, Iraq. For fungal isolation, solid and moist culture methods were used. The recovered fungal growth from the cute edges was carefully transformed into a new plate for purification.* ***Results:*** *Isolated fungal species were identified using morphological features under a microscope at both macro and micro levels. Genomic DNA was extracted and sequenced using PCR products of a fungal barcode from the internal transcribed spacer (ITS) region. Collectively, the result of morphological and molecular sequencing revealed two novel endophytic fungal species, including Canariomyces microsporus and Petriella musispora, belonging to the class Sordariomycetes. These were documented for the first time in Iraqi. To test antimicrobial activity, Potato Dextrose Broth (PDB) was used as fermentation media. After 21 days of incubation, secondary metabolites were extracted. The result of primary screening of endophytic fungal metabolites against pathogenic bacteria indicated significant inhibitory activity of C. microspores (25 mm; 7 mm) and P. musispora (20 mm; 5 mm) against Staphylococcus aureus and less effectivity against Escherichia coli.* ***Conclusion:*** *This study indicates the possibility of isolation of novel endophytic fungi from Sidr leaves for medical applications such as treating bacterial infections.*

INTRODUCTION

Endophytic fungi inhabit healthy plant tissues during the life cycle without causing disease or markable morphological effects [1]. Generally, endophytic fungi invade the plant tissues and colonize either intra or extra-cellular spaces. On the other hand, endophytic fungi sometimes are considered a source of plant infections under plant immune-stress status, such as *Fusarium* and *Aspergillus* species [2-4]. Molecular identification is essential to the discovery of new endophytic fungi due to difficulties in identification based on morphological properties [5]. Two ways of transmission, vertical and horizontal modes, by which endophytic fungi can enter into inside plant's hosts [6].

Nevertheless, endophytic fungi are involved in promoting the host plants' growth by producing secondary metabolites to influence resistance to biotic and abiotic stresses [7]. The attempts to get the benefits of endophytic fungi have multiplied during the last 30 years for discovering novel bioactive products for medicinal applications such as antimicrobials, anti-inflammatory, and anticancer [8]. During these investigations, there is a need to find new genera or even species of endophytic fungi with relatively unexplored secondary metabolites that have microbial activity, particularly for multi-drug resistance microorganisms [9,10]. The safe usage and cost-effectiveness of fungal metabolites in terms of production compared to chemical processes make fungi promising sources for a wide range of applications [11].

The emergence of antimicrobial-resistant (AMR) bacteria is a serious life-threatening to human public health worldwide. Random and excessive broad-spectrum antibiotic use is the main reason for the increased prevalence of AMR bacteria [12]. For this reason, there is an urgent need to explore natural antimicrobial agents from fungi that have antimicrobial activity against infections of resistant bacteria [13]. Endophytic fungi are considered a pivotal source of bioactive compounds with antibacterial activity [14]. This study aimed to isolate endophytic fungi from plant hosts in Iraq and assess the antimicrobial inhibitory effects of their secondary metabolites.

METHODOLOGY

Samples collection for endophytic fungi isolation:

In June 2024, a total of 20 leave samples from Sidr plants were collected from Al-Midaina District in

Basrah province, Iraq, Latitude (N 30°56′27″) and Longitude (E 47°15′52″). To process endophytic fungal isolation, samples were washed under running tap water and then with sterilized H_2O. Samples were cut under a sterile condition by razor blades. Sample surface disinfection was conducted through processing with 70% ethanol, 0.5% sodium hypochlorite, 70% ethanol, sterilized H_2O, and then air-dried. Samples were laid on Petri plates with potato dextrose agar (PDA) containing rifampicin antibiotic and incubated at 25°C with daily track. Recovered fungi were kept in new PDA plates and slants separately [15].

DNA extraction and endophytic fungi identification:

Genomic DNA of two main isolates of endophytic fungi were recovered from Sidr leave segments and was extracted following the manufacturer's instructions using Genomic DNA extraction Kit (Cat. No. GBYB100) provided by Geneaid, Taiwan. The ITS region of gDNA was amplified using the set of primers, including ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) [16]. PCR amplification condition was conducted following the protocol of Al-Maqtoofi and Thornton [2]. The mixture of the final reaction volume of PCR was (25 µL) 1 µL gDNA template, one µL of each ITS primer, 12.5 µl of Green Master Mix (Promega), 9.5 µL of nuclease-free water. The PCR cycling parameters included an initial denaturation step for 5 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 1.5 min at 72°C, and final 10 min at 72°C. Gel electrophoresed (1% TAE agarose gel), and SYBR GelRed were used to detect the PCR products. The gDNA bands were purified and sent to a Macrogen company in South Korea for genomic sequencing. The species identity was determined by achieving over 95% sequence similarity in the ITS region of the recovered species using the (BLAST) website tool and comparing results with the GenBank database at the NCBI [17]. Sequence alignments for each dataset were performed using MEGAX [18]. Phylogenetic analysis was done by Maximum Likelihood (ML) methods and the model of Jukes-Cantor nucleotide substitution. The stability of the phylogenetic tree was tested by bootstrap analysis with 1000 replicates. Following genomic analysis, the sequence data were deposited to the GenBank for accession number assignment.

Agar well diffusion:

Evaluation of the antibacterial activities of fungal extracts was conducted using agar well diffusion assay on Mueller-Hinton agar (MHA) plates [19]. Fresh overnight bacterial suspension was adjusted in broth media to get the final concentration (1×10^8 CFU/mL). The bacterial suspension was seeded to MHA, and two wells of 5 mm were created. Crude endophytic fungal extracts were dissolved with DMSO and then added to each well. The plates were then incubated for 18-24 h at 37 °C. The negative controls were represented as DMSO only. The inhibition zone diameter was measured in millimeters using a rural.

Statistical analysis:

Data were evaluated statistically using SPSS software, with Student's t-test used to compare the diameter of the inhibition zone of the groups. A *p*-value below 0.05 was deemed statistically significant, reflecting meaningful differences between the groups.

RESULTS

Endophytic fungi identification

Two main endophytic fungal species were isolated from the host plant leaves *Z. spina-christi* in Al-Midaina District in Basrah, Iraq. Findings from microscopic, macroscopic, and molecular gDNA analyses showed that the two isolated endophytic fungal species belonged to two different families, both under the class Sordariomycetes that related to the Ascomycota group. These fungal species were identified as *Canariomyces microspores* belonging to the family Chaetomiaceae and *Petriella musispora* belonging to the Microascaceae family. These two species were identified for the first time in Iraqi Mycobiota, Basrah province (**Figure 1**). The gDNA sequence data of isolated endophytic fungi were deposited and registered in NCBI (the global Gene Bank of The National Center for Biotechnology Information) with their species accession numbers (**Table 1**).

Table 1: Details of isolated *species* area, vegetable sources and ITS accession numbers.

Isolates code	Species	ITS accession numbers
A5	*Canariomyces microsporus*	OP168753
M2	*Petriella musispora*	OR429429

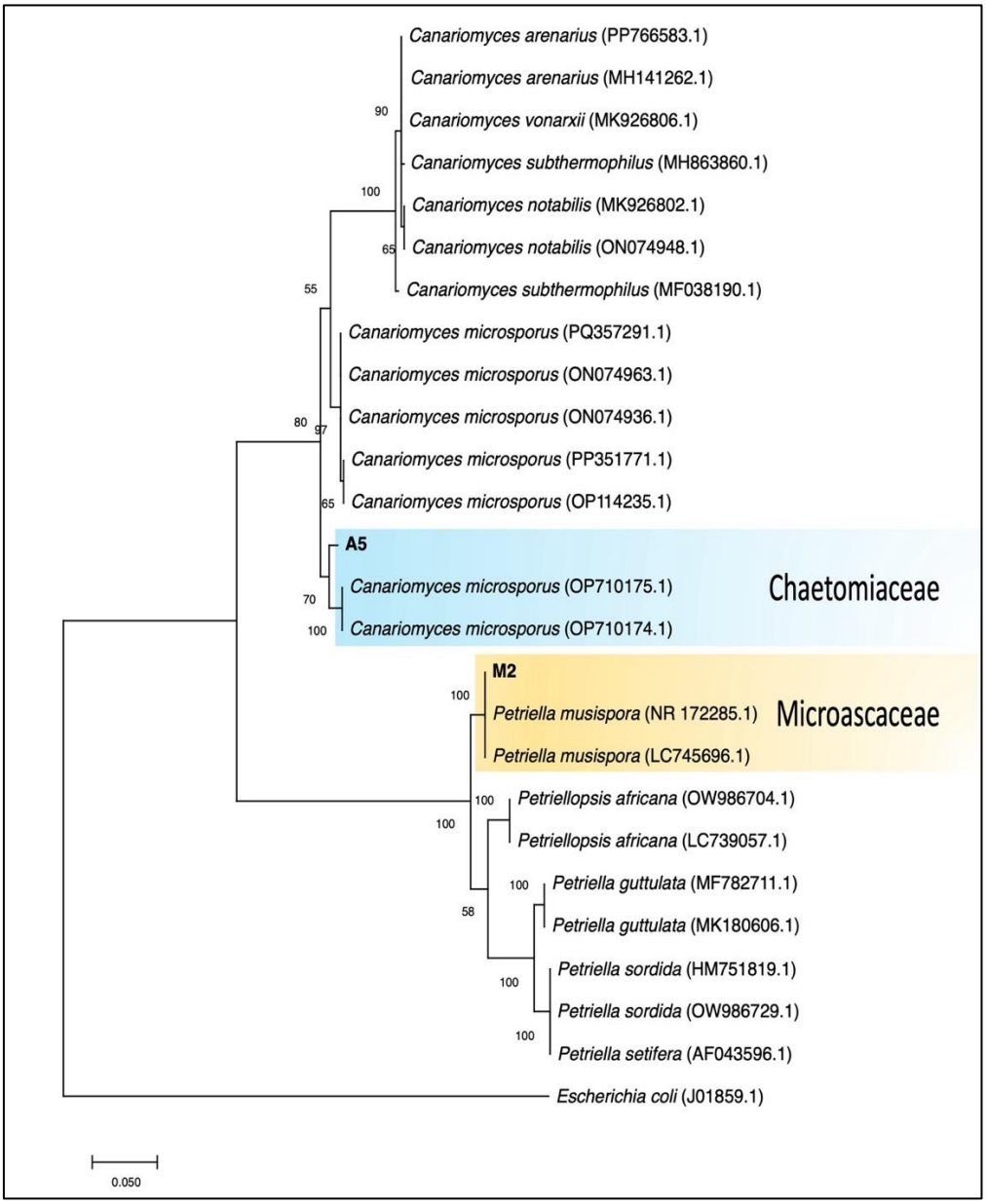

Fig. 1: Phylogenetic tree represents neighbor-joining analysis of ITS domain sequences depicting the relationships of five isolated endophytic fungi (isolate_ No. **A5** and **M2**) with closely related reference sequences of *Canariomyces* species and *Petriella* species retrieved from NCBI. The phylogenetic tree was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. Each numerical value represents the percentage of bootstrap samples, a total of 1000 samples, that support the internal branches with a confidence level of 50% or higher. *Escherichia coli* (J01859.1) represent an outgroup.

Canariomyces microsporus (Mouch.) X. Wei Wang and Houbraken basionym:
Thielavia microspora Mouch., 1973
Culture characteristics: On PDA media, colonies had an entire edge, sparse aerial mycelium, obverse grey, texture floccose, white to smoke grey, the reverse was orange to black when older, about (4-5) cm in diameter at 25°C in seven days. Morphological characteristics: non-ostiolate, *ascomata* superficial, globose or subglobose, (100-150) µm diameter *ascospores* 1-celled, when mature, they appeared as dark brown, solitary to aggregated, ellipsoidal, with attenuated ends, (7-9) × (4-6) µm, smooth, with an apical or subapical germ pore (**Figure 2**).

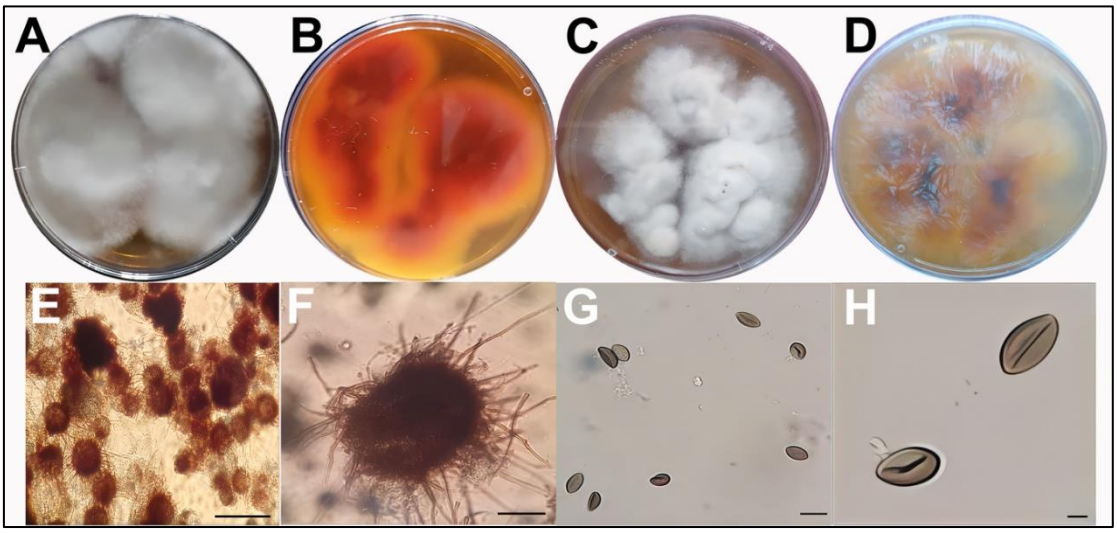

Fig. 2: *Canariomyces microsporus*. **A:** *C. microsporus* on PDA front; **B:** *C. microsporus* on PDA reverse side; **C**, **D**: *C. microsporus* on PCA front and reverse side; **E**, **F**: Ascoma and Ascomatal hairs. **G**, **H**: Ascospore.

Material examined: The fungus was isolated from *Z. spina-christi* leaves that were collected from Al-Midaina District in Basrah province. GenBank accession number was awarded for this species at NCBI (OP168753). The morphological characteristics were similar in description to *Canariomyces microsporus* (syn. *Thielavia microspore*) [20].

Petriella musispora Malloch, 1970

Culture characteristics: Colonies on PDA white to grey or lightly green, flat, slow growing, and wavy, reaching about (3-4) cm in diameter at 25 °C in seven days. The reverse appeared green with a white margin. Morphological characteristic: Ascomata were produced on PDA for 14 days. Perithecia was black, globuse to subglobuse at the base, covered with brown septate hairs, and had a short perithecial neck with (100-400 µm in height) and (150-300 µm in diameter). Asci were clavate to ovoid, reddish brown, and (4–5× 9–11) µm in size. Ascospores were olivaceous brown when mature, ellipsoidal-fusiform, (7-10) × (6-9) µm, with a bubble in the center (**Figure 3**).

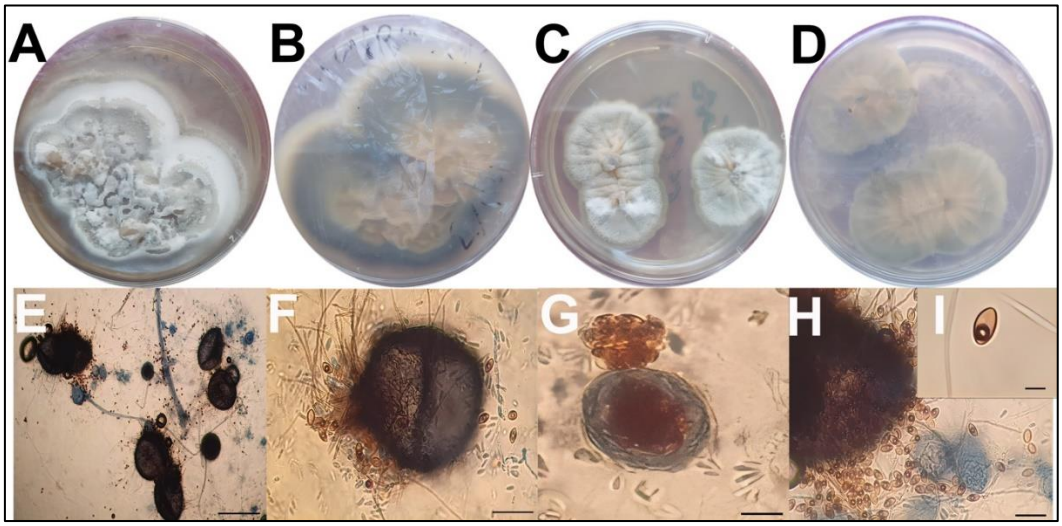

Fig. 3: *Petriella musispora*. **A:** *P. musispora* on PDA front; **B:** *P. musispora* on PDA reverse side; **C**, **D:** *P. musispora* on PCA front and reverse side; **E**, **F:** Fruiting body. **G:** Ascus; **H**, **I:** Ascospore.

Material examined: The fungus was isolated from *Z. spina-christi* leaves that were collected from Al-Midaina District in Basrah province. GenBank accession number was awarded for this species at NCBI (OR429429).

Antibacterial activity

The preliminary antibacterial testing of test extracts against Gram positive and negative bacteria, including *E. coli* and *S. aureus,* was performed using agar well diffusion. The two endophytic fungal cell-free supernatants, crude extract, exhibited antibacterial activity against *S. aureus and E. coli*. The inhibition zone diameter of the cell-free supernatants of endophytic fungi was 20 mm and 15 mm for *C. microspores* and *P. musispora* and 10 mm and 7mm for Gram-negative bacteria, respectively, as represented in **Table 2**. Both secondary metabolites of *C. microspores* and *P. musispora* exhibited significant inhibition activity against Gram positive and less with Gram-negative bacteria.

Table 2: Diameter of zone inhibition of endophytic fungi cell-free supernatant against pathogenic bacterial growth in vitro.

Endophytic fungi extracts	Inhibition zone diameter (mm)			
	E. coli	***Staphylococcus aureus***	***DMSO***	***p-value***
Canariomyces microsporus	7	25	0	< 0.001
Petriella musispora	5	20	0	< 0.001

DISCUSSION

Human activities can introduce antimicrobial-resistant bacteria, antimicrobial-resistant genes, and various anthropogenic chemicals into the environment. These contaminants can promote resistance selection within microbial communities and elevate the frequency of horizontal gene transfer (HGT) [21]. This highlights the urgent need for a continuous discovery of new antimicrobial agents, particularly from endophytic fungi, which live symbiotically within plant tissues and have emerged as promising sources of antimicrobial compounds. Nevertheless, a vast array of novel and potent antimicrobials from these endophytes remains unexplored and undiscovered [11,22,23].

Endophytic fungi are widespread within the tissues of living plants and represent vital elements of plant micro-environments. Endophytic fungi establish a mutualistic relationship with their host plant, resulting in plant growth promotion, microbial pathogen resistance, removing soil pollutants, and enhancing tolerance to abiotic stress [24,25]. Endophytic fungi have been reported to contain antimicrobial secondary metabolites, including flavonoids, peptides, alkaloids, terpenoids, quinones, phenols, acids, steroids, volatile organic compounds, benzopyranones, chinones, saponins, tannins, tetralones, and xanthones, polyketides and wide range of enzymes [26]. For this reason, the current study was designed to investigate the endophytic fungi from the most traditional and widespread plant in Iraq, *Z. spina-christi, and* then assess their secondary metabolites activity again, both Gram-positive and negative pathogenic bacteria. To the best of our acknowledgment, this study is the first study in this field in Basrah, Iraq. *Z. spina-christi* (L.) belongs to the Rhamnaceae family and is an evergreen tree called Sidr and Nabeq in Arabic countries [27].

The result of our study indicated the isolation of two main endophytic fungal species from the Sidr leaves. The morphological and molecular analysis showed that these species of *Canariomyces microspores,* which are related to the Chaetomiaceae family and *Petriella musispora,* belonged to Microascaceae family and both classified under the class of Sordariomycetes that belong to the Ascomycota as phylogenetic tree showed. These species were reported for the first time in Iraqi microbiota from this substrate and globally. Many studies have reported the isolation of many endophytic fungi from Sidr, including species such as *Alternaria*, *Aspergillus*, *Rhizopus*, yeasts, *Cladosporium*, *Drechslera*, *Curvularia*, *Fusarium*, *Ulocladium*, *Penicillium*, *Pestalotiopsis*, *Trichoderma*, *Phomopsis* and *Mucor* [28-31]. Studies showed that the most dominant isolated endophytic fungi species belong to Ascomycota group [5,31,32]. The most common identifying observable characteristic of the species is superficial ascomata with hair extension or stalked ascospores presence [33]. There is a limitation of morphological identification in *Chaetomium* species due to the loss of morphological variation among species, which makes identification based on morphology alone insufficient [34].

Due to the widespread of multidrug-resistant microorganisms that impact human health either directly or indirectly, there has been a growing interest among scientists and researchers to explore alternative sources of safe, eco-friendly bioactive compounds with potent and broad-spectrum antimicrobial activity against pathogenic bacteria [26]. Endophytic fungi are capable of synthesizing a diverse array of secondary metabolites that exhibit broad-spectrum bioactive properties, including antifungal [35], antiparasitic [36], antiviral such as SARS-CoV-2 that causing COVID-19 [37,38], anticancer[39], and immunomodulatory effects [40]. The result of this study showed that both *C. microspores* and *P. musispora* free-cell supernatant (crude extract) of fermentation media possessed notable antimicrobial inhibitory effects against *S. aureus* compared to *E. coli*. Many studies showed that endophytic fungal extracts

exhibited significant inhibitory properties against Gram-positive bacteria compared with less or no activity against Gram-negative bacteria, which might be due to bioactive substances' inability to penetrate the outer membrane barrier of Gram-negative bacteria [41]. This result aligned with other studies that reported endophytic fungal extract showed significant effectiveness against Gram-positive bacteria. Culture broth of *C. microspores* and *P. musispora*, isolated from *Z. spina-christi* (L.), containing bioactive substances such as alkaloids, peptides, steroids, quinones, terpenoids, phenol, phenolic acid, aliphatic, isocoumarins, phenylpropanoids, flavonoids and chinons [8]. More studies are required to identify the specific bioactive compound present in the extracts and inhibit Gram-positive bacteria in addition to the mode of action.

CONCLUSION

Two endophytic fungal species, *C. microspores* and *P. musispora,* that related to the family of Chaetomiaceae and Microascaceae, respectively, all belonged to the class Sordariomycetes in Basrah, Iraq, from Sidr leaves samples. The isolated species exhibited a notable antibacterial inhibitory effect against Gram-positive compared to Gram-negative bacteria. For this reason, they are considered novel natural sources of antimicrobial agents against pathogenic bacteria.

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Informed Consent Statement
This study did not involve human participants; thus, no informed consent was required.
Conflict of interest
The authors declare no conflicts of interest related to this study or its publication.

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