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Original Article

OCCURRENCE AND IDENTIFICATION OF FUNGI ASSOCIATED WITH DRIED MEDICINAL PLANTS IN KURDISTAN REGION-IRAQ

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ABSTRACT

Medicinal plants are widely used in primary healthcare; however, they are frequently affected by fungal infections, posing health risks to users. This study is concerned with the isolation and identification of fungi found on five medicinal plants (Stinging nettle, Germander, Mesquite, Millepertuis scabre, and Hawthorn) bought from herbal shops in the cities of Duhok and Zakho, Iraq. Using moist blotting paper, direct plating methods, and genetic analysis, the current study found 26 different types of fungi from seven groups, including *Penicillium, Aspergillus*, and *Alternaria. Aspergillus, Penicillium*, and *Alternaria* were the predominant genera. The highest frequency was 67% for *Botrytrichum murorum,* 67% for *Alternaria atra,* and the lowest frequency was 4% for *Penicillium, 7% for Alternaria,* demonstrating considerable heterogeneity across plant types and collecting sites. The internal transcribed spacer (ITS) region, a commonly used DNA barcode for fungi, was used to identify the fungus. These findings offer essential insights into trends in fungal contamination and highlight the necessity of rigorous quality control protocols in the herbal medicinal plant supply chain.

KEYWORDS: Medicinal plants, Fungal contamination, Molecular identification, Phylogenetic analysis, ITS sequencing.

1. INTRODUCTION

Historically, medicinal plants have been essential in providing primary healthcare to human communities (Adhikari *et al.*, 2010; Ozioma & Chinwe, 2019; Okaiyeto & Oguntibeju, 2021). These plants are predominantly harvested from the nation's mountainous regions, deserts, and coastal zones, rather than cultivated. Diverse medicinal plants and their components are recognized as vulnerable to various microbial agents. They are frequently conveyed without sufficient packaging and are generally stored in containers such as tins, plastic, jute, cartons, and sacks. Jute and polyethylene bags are primarily utilized for packing during transportation. A considerable fraction of these plants and their constituents are compromised or tainted by microbes, especially fungi, due to insufficient packing. Fungal infections have been noted to significantly affect medicinal plant components, both in the field across various growth stages and in raw medicinal materials stored in warehouses, storage facilities, and herbal shops. Such events might result in both qualitative and quantitative losses of medicinal plants and their components. The quality of these plants is affected by storage conditions (Snowden, 2010; Tiwari *et al*., 2022).

The use of diseased or contaminated herbal components in medicinal preparation is likely to compromise the quality of the resultant medicine, perhaps rendering it dangerous instead of therapeutic. Global usage and demand for medicinal plants are rising since they constitute over 50% of all medications employed worldwide (Weideman, 2005; Ozioma & Chinwe, 2019; Salmerón-Manzano *et al.*, 2020). In the USA, 78.7% of the most often prescribed medications are sourced from natural resources. Data from industrialized nations indicate that medicinal herbs are widely employed owing to scientific proof of their effectiveness (Ahmad *et al*., 2021; Ur Rehman *et al.*, 2021). Additionally, approximately 80% of Asian and African countries, including South Africa, frequently depend on medicinal plants for healthcare (Wyk *et al*.,1997; Mahesh & Satish, 2008; Ojah *et al*., 2021). Even though medicinal plants are widely used and beneficial, their safety can be threatened by germs, including many types of fungi that number over a million (Alvin *et al*., 2014; Caruso *et al*., 2020; Tsipinana *et al.*, 2023). Various factors, including geographical location, climatic conditions, physiology, and the uniqueness of colonized tissues, influence the fungal infection of plants used for medicine (Singh *et al*., 2015; Fang *et al*., 2019).

Improper handling and inadequate storage conditions are significant problems (Ashiq *et al*., 2014; Fontana *et al.*, 2021). A lot of research has shown how easily medicinal plants can get mycotoxins and fungal infections *(*Ałtyn & Twarużek., 2020*;* Areo *et al.*, 2020; Ndoro *et al.*, 2022). People are worried about public health because more people are using medicinal plants for food and health benefits, but they do not know how safe or

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effective they are. Fungi getting into medicinal plants is a big problem because they can kill plants and make them go bad, which can be bad for people's health in a number of ways (Ashiq *et al*., 2014; Othman *et al*., 2021). Food safety and purity must be protected from fungi and their toxins, and it is important to know which fungi make more toxins than others to do this (Abdullah *et al.*, 2009).

Historically, mycologists have depended on morphological traits for the identification of fungal species (Al-Zamel, 2009; Haleem *et al*., 2016). Molecular diagnostics offer much better accuracy, specificity, and speed in distinguishing between different fungal species and subspecies compared to traditional diagnostic methods (Liu *et al*., 2000). In combination with conventional morphological techniques, molecular identification of fungi is a crucial tool for precise taxonomic classification. Because of its high species variation and conserved flanking regions that are appropriate for primer binding, the internal transcribed spacer (ITS) region of ribosomal DNA is widely acknowledged as the universal DNA barcode for fungi among a variety of molecular markers (Schoch *et al.*, 2012). The ITS1–5.8S–ITS2 region, which allows for accurate differentiation among closely related fungal taxa, was amplified in this study using the ITS1 and ITS4 primers. This method has been effectively used for a variety of fungal genera, enabling accurate phylogenetic analysis and identification of the isolates derived from medicinal plants. Research on the fungal colonization of agricultural goods is minimal, and knowledge regarding the mould contamination of medicinal herbs in the country is scarce. The aim of this study was to identify the fungal species colonizing frequently used dried medicinal plants in Zakho and Duhok, Iraq, with particular emphasis on potentially toxigenic fungi that may affect the safety and quality of these herbal products.

2. MATERIALS AND METHODS

Sample Collection:

Between 1st October and 1st December 2024, fifteen samples of five distinct dried medicinal plant species were collected at randomly from three respected herbal stores in the cities Zakho and Duhok (Table 1). The selection of these medicinal plants was predicated on their availability and prevalent use. Sterilized paper bags were utilized to convey the samples to the mycology research laboratory at the University of Zakho. The bags were labelled, securely packed, and stored in a refrigerator at 4°C until required.

Table 1: Medicinal Plants, Their Families, and Parts, used for fungal isolation:

Common name	*Scientific name*	*Family*	Plant part
stinging nettle	*Urtica dioica L.*	Urticaceae	Shoot
Germander	*Teucrium chamaedrys L.*	Lamiaceae	Leaves
Syrian Mesquite	*Prosopis farcta*	Fabaceae	Fruits
Millepertuis scabre	*Hypericum scabrum*	Hypericaceae	seed
Hawthorn (azerole)	*Crataegus azarolus L.*	Rosaceae	leaves

Isolation Methods:

Isolation on Standard Moist Blotter Paper Method:

Sterilize plates using three layers of 90 mm filter paper that have been wet with 10-15 milliliters of sterile distilled water were used to remove the harmful activity of chemical agents from the samples. Five grams of each sample were surface sterilized for 1-2 minutes in a sterile conical flask using a 5% sodium hypochlorite solution (NaOCl). This was followed by three (1.5-2 min.) washing with distilled water. Using sterile forceps, the disinfected samples were moved into a Petri dish with sterilized filter paper. Depending on the size of the particles, the larger samples were sliced into tiny pieces at a pace of 5-10 pieces per plate (Toma & Abdulla, 2013).

After making three duplicates, the plates were incubated at 25°C for 5-7 days. Fungal growth appeared on sterilized malt extract agar (MEA) plates containing 0.5 mg of chloramphenicol per milliliter to inhibit bacterial growth of bacteria. Microscopic and morphological features were used to identify fungal colonies.

Fungal Isolation by Agar Plate Method:

The procedure used for fungal isolation was previously explained by Abdullah *et al.* (2002). Five grams of each sample were surface sterilized for 1-2 minutes in a sterile conical flask using a 5% sodium hypochlorite solution (NaOCl) to remove the harmful effects of the chemical agent from the samples. Three times for 1-2 minutes, washed with distilled water, followed by this step. Larger samples are broken up into smaller pieces by particle size, then transferred using sterile forceps onto a Petri dish containing sterile malt extract agar (MEA) at a density of 5–10 pieces per plate. MEA was supplemented with 0.5 mg of chloramphenicol per milliliter to prevent the growth of bacteria (Abdullah & Nashat, 2014).

Morphological Identification:

Conidial size, shape, and color were among the cultural and morphological traits used to identify fungal isolates from pure cultures on a MEA medium using a light microscope (40x). The fungal isolates were identified by consulting several important taxonomic sources, including Ellis (1971), Simmons (1986), Klich (2002), Pitt and Hocking (2009), Abdullah and Atrosh (2014), and Hussein and Abdullah (2017).

Molecular Identification:

DNA Extraction:

To extract DNA, the fungi isolated from dried medicinal plants were grown on MEA culture medium. The plates were incubated at 25°C for 5 to 7 days. The pure colonies were pulverized using sterile slides, and 40 to 60 mg of fungi were collected and transferred to sterile 1.5 ml tubes, subsequently frozen for one day, pulverized using a small pestle, and stored in the freezer until DNA extraction was performed. The DNA of the isolated fungus has been extracted with the Add Prep Genomic DNA Extraction Kit (AddBio, Korea, Catalog No. 10023)

according to the manufacturer's instructions. The purity of the extracted DNA was quantified and evaluated using the NanoDrop2000c spectrophotometer. The extracted fungal DNA was amplified using primers ITS1 (5′ TCCGTAGGTGAACCTGCGG –3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC –3′)(White *et al*., 1990).

DNA Amplification Using Conventional Polymerase Chain Reaction:

In a total reaction volume of 50 µl, we used 25 µl of Taq PCR master mix, 2.5 µl of forward primers, 2.5 µl of reverse primers (both at 10 pmol/µl), 5 µl of genomic DNA (25-50 ng/µl), and 15 µl of sterile deionized distilled water to amplify the ITS region or ribosomal DNA (ITS1, ITS4). The negative control was a reaction using nuclease-free water rather than template DNA. The subsequent amplification settings were employed during amplifications in an Eppendorf AG thermal cycler. The ITS region was initially denatured for three minutes at 94°C before being amplified by PCR. After that, there were 35 cycles of denaturation at 95°C for 35 seconds, annealing at 52°C for 1.5 minutes, and extension at 72°C for 1.5 minutes. To guarantee full synthesis of all DNA fragments, the reaction was finished with a final extension at 72°C for 10 minutes (White *et al*., 1990). After separation by 1.5% agarose gel electrophoresis, the 500-bp ITS-amplified PCR products were examined under a UV transilluminator. Verkley *et al*. (2014) used a DNA ladder with a molecular weight of 100 bps to measure the band size. The forward and reverse primers from the single-plex PCR were sent to Macrogen Company in South Korea to sequence the positive PCR results. Using SeqTrace (0.9.0), forward and reverse DNA sequencing were performed to generate the consensus sequence for each sample. The sequences were edited and then assembled. The NCBI's BLAST search was used to identify the fungal species from the sequences (Verkley *et al*., 2014).

Table 2: The reaction components for PCR.

Component	Volume
Maseter mix	25 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
DNA Template	2 µl
Distilled water	18µL
Total reaction volume	50 µl

Table 3: PCR conditions for the ITS region of fungal isolates.

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	94	3 min	1
Denaturation	95	35 sec	35
Annealing	52	1.5 min	
Extension	72	1.5 min	
Final extension	72	10 min	1

Phylogenetic Analysis:

The data from the 500 bp ITS region (Internal Transcribed Spacer) were used to perform a phylogenetic analysis using maximum likelihood methods. The MAFFT (Multiple Alignment using Fast Fourier Transform) and substitution models (K2: Kimura 2 parameter) were used to align the phylogenetic tree. Missing data was used to fill in alignment gaps. Based on the total character differences, maximum likelihood trees were built, and bootstrap values were computed from 1,000 replications. The evolutionary analysis was carried out using MEGA 11software (Sevindik *et al.*, 2022; Tamura *et al.*, 2021).

3. RESULTS

Distribution of endophytic fungi:

A total of 15 medicinal plant samples were analyzed for fungal contamination, with their common and scientific names, and the plant parts summarized in Table 1. Fungal genera were isolated using two methods: the Standard Moist Blotter (SMB) and the Agar Plate Method (APM). Both methods were applied to surface-sterilized plant materials. Overall, seven fungal genera comprising 26 species were identified through a combination of conventional morphological and molecular techniques. The genera included *Penicillium* (*P. chrysogenum*, *P. citrinum*, *P. griseofulvum*), *Aspergillus* (*A. niger*, *A. flavus*, *A. quadrilineatus*, *A. terreus*, *A. fumigatus*, *A. versicolor*, *A. oryzae*, *A. chevalieri*, *A. amstelodami*), *Rhizomucor pusillus* (Zygomycota), *Chaetomium elatum*, *Botrytrichum murorum*, *Curvularia specifera*, *Fusarium brachygibbosum*, and several species of *Alternaria* (*A. malorum*, *A. tenuissima*, *A. brassicicola*, *A. destruens*, *A. consortialis*, *A. infectoria*, *A. atra*, *A. ventricosa*).

Endophytic fungi were isolated employing the moist blotter technique, resulting in the identification of 12 fungal species across four genera (*Aspergillus, Alternaria, Fusarium*, and *Curvularia*) in the studied medicinal plants (Table 4). The richness and distribution of these fungi fluctuate according to plant species and sampling site. *Urtica dioica* consistently exhibited *Aspergillus flavus* contamination across all three samples, but *Fusarium brachygibbosum* and *Curvularia specifera* were observed occasionally. The fungal counts for these samples were 3, 2, and 1, respectively. *Aspergillus niger* was predominantly found in all samples of *Prosopis farcta*. *A. flavus* and *A. terreus* were identified in two of the three samples,

although *A. fumigatus* was detected in only one, yielding fungal counts ranging from 2 to 3 per sample. *Teucrium chamaedrys L.* exhibited notable contamination variety, especially in the initial sample, which revealed the presence of *A. niger, A. flavus, A. fumigatus,* and *C. specifera* (total = 4 fungi). *A. niger* and *A. flavus* were prevalent in all samples. *Hypericum scabrum* demonstrated the highest fungal diversity among the examined plants. The second sample comprised six distinct fungi: *A. niger, A. flavus, A. fumigatus, Alternaria malorum*, *A. brassicicola*, and *A. alternata*. The first and third samples similarly exhibited numerous fungi, though with reduced diversity. *Crataegus azarolus L.* exhibited reduced variety. *A. niger* and *A. flavus* were the most prevalent, although *A. alternata* and *A. tenuissima* were encountered infrequently, with fungal counts of 4, 3, and 1 among samples.

Table 4: Fungal Species Identified from Medicinal Plant Samples.

Plant name	location	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Alternaria malorum*	*Alternaria brassicicola*	*Aspergillus terreus*	*Alternaria alternata*	*Alternaria tenuissima*	*Aspergillus oryzae*	*Fusarium brachygibbosum*	*Curvularia specifera*	*Alternaria destruens*	Fungal counts per sample
Urtica dioica	1	–	+	–	–	–	–	–	–	–	+	+	–	3
	2	–	+	–	–	–	–	–	–	–	+	–	–	2
	3	–	+	–	–	–	–	–	–	–	–	–	–	1
Prosopis farcta	1	+	–	+	–	–	–	–	–	–	–	–	–	2
	2	+	+	–	–	–	+	–	–	–	–	–	–	3
	3	+	+	–	–	–	+	–	–	–	–	–	–	3
Teucrium chamaedrys L.	1	+	+	+	–	–	–	–	–	–	–	+	–	4
	2	+	+	–	–	–	–	–	–	–	–	+	–	3
	3	–	+	–	–	–	–	–	–	–	–	–	–	1
Hypericum scabrum	1	+	+	+	–	–	–	–	–	–	–	–	+	4
	2	+	+	+	+	+	–	+	–	–	–	–	–	6
	3	+	+	+	–	–	–	–	–	–	–	–	–	3
Crataegus azarolus L.	1	+	+	–	–	–	–	–	+	+	–	–	–	4
	2	+	+	–	–	–	–	–	–	+	–	–	–	3
	3	+	–	–	–	–	–	–	–	–	–	–	–	1
Frequency of contamination in all samples		11	13	5	1	1	2	1	1	2	2	3	1	43

Agar Plate Method, fungal frequency and occurrence varied significantly across the three collection locations (Table 5). *Botrytrichum murorum* was the most common fungus found in *Crataegus azarolus L.*, appearing 67% of the time and occurring 13% in Location 3, and 47% for both frequency and occurrence in Location 1. *Chaetomium elatum* was detected in *Teucrium chamaedrys L.* at Location 3 with a frequency of 25% and an occurrence of 20%. *Aspergillus flavus* was dominant in *Urtica dioica* at Location 2, with a frequency of 35% and occurrence of 40%. *Aspergillus niger* was commonly found in *Urtica dioica* and *Prosopis farcta*, with frequencies of 10% and 61% in location 2, and a frequency of 7% and 39% in location 3. Various *Alternaria* species were recovered from *Hypericum scabrum*, *Teucrium chamaedrys L.*, and *Crataegus azarolus L.*, including *A. consortialis*, which was detected at Locations 2 and 3, with frequencies of 6% and 7%, and occurrences of 7% at both locations in *Urtica dioica* and *A. alternata* (frequency 11%, occurrence 7% in 7%T*eucrium chamaedrys L.*). *Aspergillus fumigatus* was found exclusively in *Hypericum scabrum*, with a frequency 38% occurrences (47%) in Location 2 and a frequency (15%) occurrences (13%) in Location 3. Rare isolates such as *Rhizomucor pusillus* were recovered from Prosopis farcta (frequency (11%), occurrences (13%) in Location 3). Several *Aspergillus* species (*A. chevalieri*, *A. versicolor*, *A. quadrilineatus,* and *A. amstelodami*) were distributed across *Hypericum scabrum*, *Teucrium chamaedrys L.*, and *Crataegus azarolus L.*

Aspergillus amstelodami was detected at Locations 1 and 3, with frequencies of 30% and 4%, and occurrences of 27% and 7% in *Hypericum scabrum*; in *Urtica dioica*, it occurred in location 3 with frequencies of 30% and occurrences of 27%, respectively, while *A. quadrilineatus* was found in *Crataegus azarolus L.* at frequencies (17%) and occurrences (7%) in Location 3. *Alternaria atra* was restricted to *Crataegus azarolus L.* (frequency (10%) occurrences (13%) in Location 1 and frequency (6%) occurrences (67%) in Location 2. These results highlight the ecological versatility of *Aspergillus* species and the

influence of host plants and environments on fungal community structure.

Table 5: Fungal Frequency and Occurrence according to Medicinal Plants and Locations

Plant Species	Fungi Species	Location 1		Location 2		Location 3	
		Frequency%	Occurrence%	Frequency%	Occurrence%	Frequency%	Occurrence%
Urtica dioica	*Penicillium chrysogenum*	17	7	-	-	-	-
	Botrytrichum murorum	33	7	-	-	-	-
	Aspergillus niger	-	-	10	13	7	7
	Alternaria consortialis	-	-	6	7	7	7
	Aspergillus flavus	-	-	35	40	-	-
	Aspergillus amstrodami	-	-	-	-	30	27
Prosopis farcta	*Aspergillus niger*	-	-	61	40	39	13
	Rhizomucor pusillus	-	-	-	-	11	13
	Penicillium griseofulvum	33	7	-	-	-	-
Teucrium chamaedrys	*Chaetomium elatum*	-	-	-	-	25	20
	Alternaria alternata	11	7	-	-	-	-
	Aspergillus versicolor	-	-	6	7	-	-
	Penicillium citrinum	-	-	-	-	7	17
Hypericum scabrum	*Penicillium chrysogenum*	-	-	10	13	4	67
	Alternaria ventricosa	-	-	5	7	-	-
	Aspergillus fumigatus	-	-	38	47	15	13
	Aspergillus chevalieri	5	7	29	33	7	13
	Alternaria consortialis	-	-	-	-	15	27
	Alternaria infectoria	5	7	-	-	-	-
	Aspergillus amstrodami	30	27	-	-	4	7
Crataegus azarolus	*Alternaria ventricosa*	-	-	8	7	-	-
	Botrytrichum murorum	47	40	22	20	67	13
	Aspergillus quadrilineatus	-	-	-	-	17	7
	Alternaria infectoria	-	-	14	13	-	-
	Alternaria atra	10	13	6	67	-	-

Molecular identification and phylogenetic analysis:

The qualifying and quantification findings indicated that the fungal DNA concentration of fungal isolates varied from 20 to 900 ng/µl, with a purity value ranging from 1.8 to 2.20. The sequencing findings of ITS revealed a variety of endophytic fungi in different medicinal plants. The blast analysis of ITS regions identified seven taxa of endophytic fungi (Table 6).

Table 6: Endophytes isolated from medicinal plants are classified by their genus or species, with identification based on the percentage of similarity found in the NCBI database.

Isolated endophytic fungi	accession numbers	Similarity%
Penicillium chrysogenum	PV636548	100%
Penicillium citrinum	PV636549	99.81%
Penicillium griseofulvum	PV636550	100%
Aspergillus niger	PV662136	100%
Aspergillus flavus	PV662129	100%
Aspergillus quadrilineatus	PV662130	98.01%
Aspergillus terreus	PV662133	100%
Aspergillus fumigatus	PV662132	100%
Aspergillus versicolor	PV662131	100%
Aspergillus oryzae	PV662134	100%
Aspergillus chevalieri	PV662135	100%
Aspergillus amstrodami	PV665663	100%
Rhizomucor pusillus	PV665498	100%
Chaetomium elatum	PV665499	100%
Botrytrichum murorum	PV665500	100%
Curvularia specifera	PV665501	100%
Fusarium brachygibbosum	PV665502	100%
Alternaria malorum	PV665729	99.80%

Alternaria tenuissima	PV665730	100%
Alternaria brassicicola	PV665731	100%
Alternaria destruens	PV665732	100%
Alternaria consortialis	PV665733	99.45%
Alternaria infectoria	PV665734	100%
Alternaria atra	PV665735	100%
Alternaria alternata	PV665736	100%
Alternaria ventricosa	PV665737	99.63%

The phylogenetic connections among various fungal species are depicted by several evolutionary groupings in the ITS region trees. Phylogenetic analysis was conducted to clarify the evolutionary connections among the fungal isolates derived from medicinal plant samples. The resultant tree (Figure 1) was generated utilizing molecular sequence data, with bootstrap values denoted at critical nodes to represent the statistical support for each branch.

The analysis revealed three major clusters corresponding to the genera *Aspergillus* and *Penicillium*, *Alternaria*, and other related genera. The first major clade included several *Aspergillus* species (*A. flavus*, *A. oryzae*, *A. niger*, *A. terreus*, *A. fumigatus*, *A. quadrilineatus*, *A. versicolor*, *A. chevalieri,* and *A. amstelodami*) and *Penicillium* species (*P. chrysogenum*, *P. citrinum, and P. griseofulvum*). These species are grouped with high bootstrap support (up to 100), reflecting their close taxonomic relationship within the order Eurotiales (Houbraken & Samson, 2011; Samson *et al*., 2014). *Fusarium brachygibbosum* formed a distinct branch outside this group, consistent with its classification in the order Hypocreales (O'Donnell *et al*., 2008). The second major cluster comprised *Chaetomium elatum*, *Botrytrichum murorum*, and *Curvularia specifera*, which are members of the Sordariales and Pleosporales, respectively. This group was also well supported by high bootstrap values (98–99) (Zhang *et al*., 2006). The third major clade consisted of various *Alternaria* species, including *A. malorum*, *A. infectoria*, *A. ventricosa*, *A. consortialis*, *A. atra*, *A. destruens*, *A. alternata*, *A. tenuissima*, and *A. brassicicola*. These species clustered together with strong bootstrap support (up to 100), confirming their close evolutionary relationships within the genus *Alternaria* (Woudenberg *et al.,* 2015*)*. Notably, *Rhizomucor pusillus* was positioned as a distinct outgroup, reflecting its classification in the phylum Mucoromycota (formerly Zygomycota), in contrast to the other isolates, which are Ascomycetes. The clear separation of *R. pusillus* from the other taxa was strongly supported (Benny *et al*., 2014).

Overall, the phylogenetic tree corroborates the molecular identification of the fungal isolates and highlights the diversity of fungal taxa associated with medicinal plants. The clustering patterns observed are consistent with established fungal taxonomy and demonstrate both the diversity and evolutionary relationships among the isolates recovered in this study.

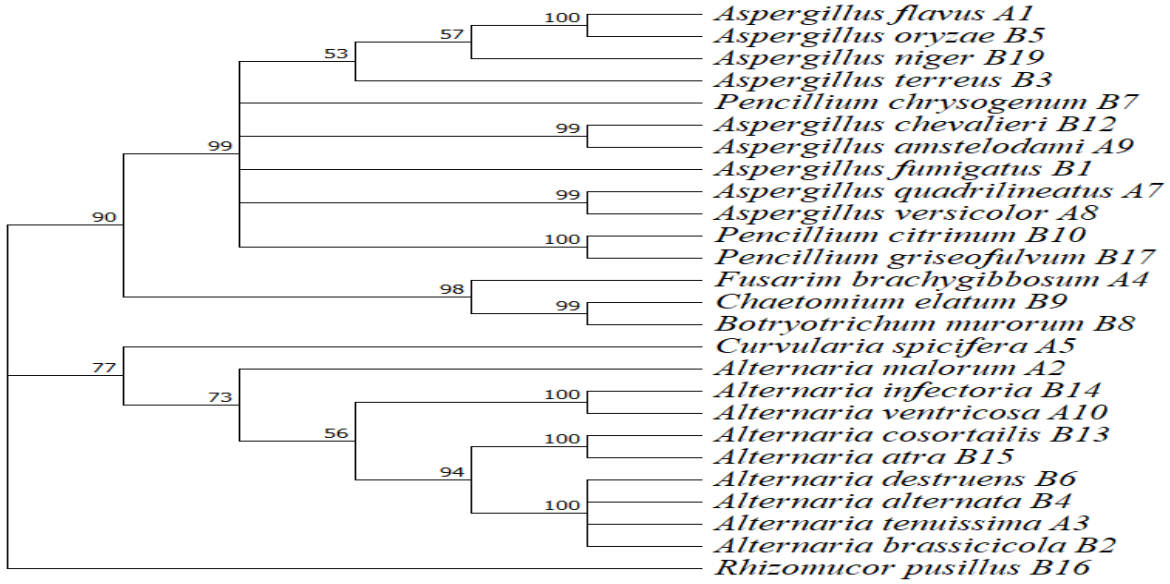

Figure 1: Phylogenetic tree of all the isolated fungi based on the ITS ribosomal DNA sequence.

4.DISCUSSION

The current study underscores the extensive taxonomic diversity of fungi associated with desiccated medicinal plants obtained from herbal shops in Duhok and Zakho, Iraq. We were able to isolate and identify a total of 26 species using a combination of morphological and molecular techniques, such as sequencing the internal transcribed spacer (ITS) rDNA region. The isolates were divided into four main groups: Eurotiomycetes, Dothideomycetes, Sordariomycetes, and Zygomycota. Ascomycetes were the most common type of fungus, which is consistent with previous research that showed this group is the largest fungal phylum and is well suited to colonies a wide range of substrates because they produce a lot of spores, can adapt to different environments, and grow quickly (Trail, 2007).

Our findings corroborate the persistent global observations identifying *Aspergillus, Penicillium*, and *Alternaria* as the predominant fungal contaminants of medicinal and aromatic plants (Klich, 2009). Traditional morphological identification, which relied on colony growth, pigmentation, and spore characteristics, provided initial taxonomic insights. However, Frisvad and Samson (2004) observed that morphological methods alone often fail to elucidate species-level diversity due to overlapping phenotypic traits. Consequently, ITS rDNA sequencing was utilized, which was recognized as the universal

fungal barcode for accurate identification (Schoch *et al*., 2012). The high similarity values (98–100%) between our isolates and NCBI reference strains confirmed that the identifications were correct.

The Agar Plate Method (APM) recovered a wider range of fungal taxa than the Standard Moist Blotter (SMB) method when comparing isolation techniques. APM was especially adept at finding both common and rare taxa. SMB, on the other hand, was better at isolating fast-growing fungi like *Aspergillus flavus* and *A. niger*. Toma and Abdulla (2013) made similar observations regarding Iraqi medicinal plants, noting that APM provides a clearer picture of fungal communities. Therefore, the selected isolation method significantly affects the identification of fungal diversity in herbal substrates.

The diversity and distribution of fungi exhibited considerable variation among plant species and collection sites, reflecting the influence of both ecological and geographical factors. For example, *Alternaria tenuissima* only grew on one host at one site, but *A. niger* grew on in *Prosopis farcta, Teucrium chamaedrys L., Hypericum scabrum and Crataegus azarolus L.*and at many different sites. This variation is consistent with what Porras-Alfaro and Bayman (2011) found: soil properties, moisture levels, and temperature gradients have a significant effect on the composition of fungal communities. The chemistry of the host plant, especially secondary metabolites, can also affect how fungi grow (Zagal *et al.*, 2024). These factors probably account for the uneven distribution patterns seen in our study.

A significant aspect is the identification of potentially toxigenic species, including *Aspergillus flavus, A. niger, Fusarium brachygibbosum*, and various *Alternaria* species. These fungi are recognized producers of toxic mycotoxins, such as aflatoxins, fumonisins, trichothecenes, and alternariol derivatives (Wang *et al.*, 2024). Aflatoxins are especially dangerous because they can damage the liver and cause cancer. This is especially true when they are found in herbal products (Bennett & Klich, 2003). This study did not directly evaluate mycotoxin production; however, the presence of these fungi highlights potential risks, particularly given Iraq's climatic conditions of elevated temperature and humidity, which facilitate toxin biosynthesis (Magan & Aldred, 2007).

The WHO, FAO, and European Pharmacopoeia are just a few of the many regulatory bodies around the world that have stressed the need to set clear limits on fungal contaminants and mycotoxins in herbal medicines (WHO, 2003). These kinds of rules are either nonexistent or inadequately enforced in Iraq. Abed *et al.* (2020) stressed that herbal products sold in Iraqi markets are often unregulated, increasing the risk of exposure to fungal metabolites. The current findings underscore the necessity of establishing national standards and executing systematic surveillance of medicinal plants for fungal contamination and toxin residues.

Additionally, the findings provide foundational data to inform subsequent toxicological investigations, especially to ascertain the true mycotoxin levels in Iraqi herbal products. Earlier research in Iraq has documented the existence of aflatoxins and ochratoxins in food products and spices (Abdullah *et al.*, 2009; Saadullah & Abdullah, 2018), indicating that analogous risks may be present in medicinal plants. To protect consumer health, it is important to use integrated methods that include sustainable agricultural practices, proper drying, hygienic storage, and regular testing in a lab.

This study indicates that medicinal plants in northern Iraq have a wide range of fungal communities, some of which may be toxic and are of global concern. We obtained dependable insights into the fungal spectrum by merging morphological and molecular identification techniques. These findings not only enhance the comprehension of fungal biodiversity in herbal materials but also underscore the pressing necessity for regulatory frameworks and quality control initiatives in Iraq to mitigate health risks linked to contaminated herbal products.

CONCLUSION

This study found significant variations in fungal diversity and distribution among medicinal plants gathered from three different Iraqi regions. The types of fungi isolated were influenced by both moist blotter and direct plate agar techniques, with dominant species such as *Aspergillus flavus* and *A. niger* being easier to identify. These results emphasize that to reduce fungal contamination and safeguard consumer health, Iraqi medicinal plants and dried foods must be properly monitored, handled, and stored.

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Ethical statement:

This study did not involve any experimentation on humans or animals and did not require ethical approval. Dried medicinal plants are collected from herbal shops without requiring special permission. All fungal isolations were handled in compliance with biosafety protocols.

Author Contributions:

Both authors K.R.A.; and R.A.H have reviewed the final version to be published and have agreed to be accountable for all aspects of the work, including the concept, design, acquisition, analysis, interpretation of data, writing and drafting the manuscript.

Conflict of Interest:

Both authors declare no competing interests.

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