Antifungal Potential of *Cladosporium* sp. (Endophytic fungi) Associated with *Olea europaea* L. Leaves

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Abstract

In the leaves of *Olea europaea* L. Olive trees an endophytic fungus was discovered. *Cladosporium* sp. was identified to be the fungus based on its morphological characteristics and nuclear ribosomal DNA ITS sequence analysis and was registered in NCBI as the *Cladosporium* genus has been registered under the number (0P939922.1) The species was not specified, and it was considered of unknown species after comparing it to global isolates. In comparison to olive leaf extract, *Cladosporium* sp. including total flavonoid, total phenolic, total terpenoid, and total saponins, Which were 121.9%, 198.1%, 89.13%, and 29.87 % respectively compared to its content in olive leaf extract, which was 61.54 %, 67.88 %, 17.1%, and 20.19% respectively. The *Cladosporium* sp. extract inhibited the growth of 27 isolates belonging to different species of *candida* which were *Candida albicans*, *C. lypolitica*, *C. tropicalis*, *C. sphaerica*, *C. krusei*, *C. guilliermondii*, *C. parapsilosis*, *C. norvegicus*, *C. glabrata*, and *C. kefyr*, the inhibition effects increased with increasing concentration to reach the highest level to suppress fungal growth when concentrated 30 mg/ml. This proves the antifungal potential of endophytic fungi in the future.

Keywords: Antifungal, *Candida*, *Cladosporium*, Endophytic, Olive trees.

Introduction

Endophytes are defined as microorganisms that live asymptotically in plant tissues, causing no damage to their hosts, and are isolated from surface-sterilized plant explants. The term endophytes means "in the plant" (from the Greek endon = within, phyton = plant), and most endophytes originated in the rhizosphere and phyllosphere. Endophytic fungi are regarded as the unseen inhabitants of the microbial world and represent an underutilized source of novel therapeutics and compounds. Fungi create secondary metabolites, which are characterized as low molecular weight molecules adapted for specialized tasks in nature but not necessary for growth. New, highly effective medications are desperately needed to treat diseases like cancer, bacterial drug resistance, and fungal infections, Numerous active chemicals with a wide range of biological characteristics are produced by endophytic fungus.

Plants with endophytic fungus have been classified separately. One of the most extensively sold and important botanical medicines is Olive and it is a rich source of secondary metabolites with broad-spectrum therapeutic properties. Anti-diabetic, anti-inflammatory, heart disease, respiratory and urinary tract infections, stomach and intestinal illnesses, antimicrobial for many fungal and bacterial pathogens, and more have all been demonstrated for it, and it has been used to treat these conditions since ancient times. There is little information...
regarding the endophytic fungal community inhabiting olive tree tissues. However, several endophytic fungi isolated from the olive plant were shown to have major pharmacological effects, such as *Phomopsis columnaris*, *Fusarium oxysporum*, and *Trichoderma gamsii* 12. *Penicillium commune*, *Penicillium canescens* and *Alternaria alternate* 13, *Alternaria sp.*, *Epicoccum sp.*, *Fusarium sp.*, *Aspergillus sp.*, *Anthrinium sp.*, *Chaetomium sp.*, *Diaporthe* sp., *Nigrospora sp.* 14,15. The purpose of this study is to determine the antifungal capability of endophytic fungus *Cladosporium sp.* associated with the leaves of *Olea europaea* L.

**Materials and Methods**

**Collection of samples**

Leaves of 16 distinct olive *Olea europaea* L. were collected from farms in Al-Duluiya city, Salah-AlDin Governorate, Iraq. The samples were obtained at random in April of 2022. They were transported to the lab in sterile polypropylene bags.

**Endophytic fungi isolation**

To eliminate dirt and dust, the olive leaves were sliced and thoroughly cleaned under running tap water for 20-30 seconds. The leaf fragments were sterilized three times with 75% ethanol for 2 minutes before being washed twice with sterile distilled water. Endophytic fungi were isolated from olive leaves using potato dextrose agar (PDA) media 16. The PDA plates were then incubated at 25 ± 2°C for seven days to allow endophytic fungi to proliferate. Purification of fungal isolates was accomplished by transferring a portion of the colony’s edge to a fresh PDA medium.

**Morphological and Molecular diagnosis of endophytic fungi**

Using the adhesive tape fixation technique and the lactophenol blue cotton stain, isolated endophytes were characterized microscopically, and observed with a compound microscope at 10X and 40X magnification. Observations were made regarding the shape, color, margin, elevation, spore structure, and other morphological characteristics of endophytic isolates 17.

The CTAB approach was used to extract DNA from *Cladosporium sp.* for the purposes of molecular screening and phylogenetic analysis 18. The ITS domain of rDNA was amplified with the universal primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4(50-TCCGCTGCTTATTGATATGC-30) as described by 19,20. With deionized water, the total volume was brought up to 50 mL. The ITS region was amplified in the following way: 95°C for 5 minutes, then 40 cycles of 94°C for 60 seconds, 50°C for 60 seconds, and 72°C for 60 seconds, with a final stretch at 72°C for 5 minutes. The PCR products were cleaned up with the Gel Extraction Kit (TianGen, China), attached to the sequencing vector pGEM-T easy vector, and read by Shanghai Sangon Biologic Engineering Technology and Service Co. Ltd. For a study of phylogeny BLAST from the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) was used to look at the ITS gene sequence of an isolated fungus. MEGA program version 4.0 was used to predict phylogenetic relationships 21.

**Quantitative evaluation of active compounds**

Total phenol content (TPC): The Folin-Ciocalteu method reported by 22 with some slight changes was used to determine the TPC of the fungal endophytes and olive leaves. Based on the Gallic acid calibration curve, the TPC was determined. The data was reported in terms of gallic acid equivalents (GAEs)(μg), which were calculated for each milligram of dry extract.

Total flavonoid content (TFC): used the NaNO2-Al(NO3)3-NaOH colorimetric test reported by 23. To calculate the TFC based on the Rutin calibration curve, the TFC was determined. The data was reported as the amount (μg) of Rutin Equivalents (RAEs) per milligram of dry extract.

Each extract solution was steeped in 9 mL of ethanol for 24 hours to determine its total terpenoid content as shown in 24. Using the formula (wi-wf/wi100), were able to calculate the yield (in percent) of total terpenoid contents.
Following the specified procedures, determined the total alkaloid content as shown in \(^{25}\), the percentage of bromothymol blue in a sample is calculated as \( Y\% = \frac{(NVM2)}{W} \times 100\% \), where \( N \) is the bromothymol blue solution concentration (mol/L), \( V \) is the volume of bromothymol blue solution eaten (L), \( M \) is the molecular weight of securinine (217.26), and \( W \) is the sampling weight (g). Measurements of total saponin content followed the guidelines established by \(^{25}\).

**Assay for antifungal activity**

Extracts of the endophytic fungus *Cladosporium* sp. were tested for their antifungal activity using the disk method. *Candida albicans* (6 samples), *Candida lypolitica* (2 samples), *Candida tropicalis* (4 samples), *Candida sphaerica* (3 samples), *Candida krusei* (3 samples), *Candida guillermondii* (2 samples), *Candida parapsilosis* (1 sample), *Candida norvegicus* (1 sample), *Candida glabrata* (4 samples), and *Candida kefyr* (1 sample) were used as indicator organisms. Identified and preserved isolates were obtained fungi laboratory in the College of Education for Pure Sciences, University of Tikrit.

**Results and Discussion**

**Morphological and Molecular diagnosis of endophytic fungi**

The result of isolating endophytic fungi from the olive tree was determined through microscopic and morphological analysis, which showed that all fungal isolates belonged to *Cladosporium* sp. Fungus. An examination of the rDNA ITS region and its relationship to other genes. The use of rDNA genes has proven effective in tracing the origins of previously unknown species \(^{27}\). The sequence was 99% identical to that of *Cladosporium* sp, according to a homology search against GenBank. (GenBank accession number 0P939922.1) Through the process of alignment, a phylogenetic connection was created as shown in Fig. 1. Molecular analysis placed the isolates within the genus *Cladosporium*, and linked them to the *Cladosporium* genus.
Figure 1. Phylogenetic tree demonstrating the relationship between *Cladosporium* sp. and other related fungi retrieved from GenBank based on ITS sequence homologies.

**Determination of secondary metabolites**

To determine the percentage yield, ethyl acetate used to extract the secondary metabolites from a fungal strain of *Cladosporium* sp. that was cultured in PD broth and Olive leaves as shown in Fig. 2. The results demonstrated that fungal extract had higher levels of secondary metabolites than olive leaf extract, with total flavonoid content rising to 121.9, a rise of 50.48% over the plant extract's 61.54, total phenolic content rising to 198.1, a rise of 34.27% over the plant extract's 67.88, and total terpenoid content rising to 89.13, compared 17.10 to olive leaf extract a rise of 19.19%, for total saponin an increase was 29.87% for fungal extract which was 67.6 compared to 20.19 in the olive leaf extract, as for alkaloids, the results show that their content in olive leaf extract was higher, and it was 2.67 mg.ml\(^{-1}\), compared to 1.3 mg.ml\(^{-1}\) for fungal extract.
Figure 2. Comparing the amount of secondary metabolites in an olive plant leaf extract and an endophytic fungus called *Cladosporium* sp.
According to the findings in Table 1, the 27 isolates of *candida* sp. from various species that were obtained from various clinical sources could not grow due to the endophytic fungus *Cladosporium* sp. As demonstrated in the Table below, all candida sp. isolates exhibited strong sensitivity to *Cladosporium* sp. extract, which increased with increasing concentration to reach the highest level to inhibit the fungal growth when concentrated 30 mg.ml\(^{-1}\). Fig. 3 illustrates, as an example, the inhibitory effect of *Cladosporium* sp. extracts on the growth of a *C.albicans* isolate.

### Table 1. Antifungal ability of Endophytic fungus *Cladosporium* sp. Extract against different isolated of *candida* sp.

<table>
<thead>
<tr>
<th>Isolate N.</th>
<th><em>candida</em> sp.</th>
<th>Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>0 mg.ml(^{-1})</td>
</tr>
<tr>
<td>1</td>
<td><em>C.albicans</em></td>
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</tr>
<tr>
<td>2</td>
<td><em>C.albicans</em></td>
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<tr>
<td>3</td>
<td><em>C.albicans</em></td>
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</tr>
<tr>
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<td><em>C.albicans</em></td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td><em>C.albicans</em></td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td><em>C.albicans</em></td>
<td>0</td>
</tr>
<tr>
<td>7</td>
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<td>27</td>
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P-value ≤ 0.05 (0.001)
Discussion

All higher plants are hosts to one or more endophytic microbes on this earth. Endophytic fungi are microbes that reside in living plant tissues without causing any immediate harm to their host. In this study, Cladosporium sp. was endophytic fungus that was isolated from olive plant leaves which were identified according to morphological characteristics and nuclear ribosomal DNA ITS sequence analysis. Endophytic fungi are microorganisms that reside in the tissues of living plants and do not cause any immediate damage to their host. Cladosporium species are extremely widespread around the globe, and it is possible to isolate them from a wide variety of organic materials. In comparison to the olive plant, which the fungus was isolated from, the results shown above indicate that Cladosporium extract is a rich source of secondary metabolites. These secondary metabolites include total flavonoid, total phenolic content, total terpenoid, and alkaloids. The antifungal activity of Cladosporium sp. was a reflection of its large content of secondary metabolites, each of which has its own unique set of biological features. Cladosporium sp. extract, in any concentration, was able to suppress the growth of numerous fungal isolates of Candida sp. that had been isolated from a variety of clinical sources. These potentially useful endophytes can be further utilized to expedite plant growth and amplification through the processes of isolating, genetically manipulating, and industrially scaling up their production. Additionally, the employment of endophytes as alternative sources of bioactive chemicals reduces the exploitation of the host plant, which results in the conservation of biodiversity.

Conclusion

The results indicate that the endophytic fungi isolated from Olea europaea L. leaves may have a bright future in medicine due to their capacity to generate physiologically active compounds with characteristics similar to those of the plant where the endophytic fungus was isolated. Therefore, further research is required to identify the active ingredients produced by endophytic fungi and investigate their diverse medicinal properties in an effort to find new sources of medications and therapies.

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Authors’ Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for republication, which is attached to the manuscript.

**Authors’ Contribution Statement**

All authors contributed to the completion of the research by participating in collecting plant samples, isolating and diagnosing endophytes, and writing and reviewing the manuscript. The research tasks were distributed equally among the three authors.

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التأثير المضاد للفطر Cladosporium sp. (فطريات داخلية) المعزول من أوراق نبات Olea europaea L.

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الخلاصة