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Antioxidant and antidiabetic activity of the endophytic fungus *Alternaria alternata* isolated from *Rosmarinus officinalis* L.

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Abstract

The results of the isolation of internal fungi showed that 70% of the leaves of the rosemary plant that were examined contained one isolation of the endophytic fungi by one fungal colony for each plant piece of rosemary plant leaves. The results of the phenotypic and microscopic examination of the internal fungus isolates showed that they belong to the same species: the fungus *Alternaria alternata*. The diagnosis was confirmed by PCR technique, and the isolates were compared with the global isolates registered in the gene bank (GenBank MF099865.1 and OQ764793.1), with which they matched 100%. The results of the evaluation of the biological efficacy of the extract of the internal fungi *A. alternata* showed that it possesses vital properties of an antioxidant by inhibiting the activity of free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) The extract internal fungi recorded a high percentage of inhibitory activity of free radicals and was 93.5% at a concentration of 500 mg, we note that the inhibitory effect increased with increasing concentration of the extract. The results of the current study also showed that the extract of the internal fungi *A. alternata*. It showed efficiency in inhibiting the activity of the alpha-amylase enzyme, and therefore, it works as an antidiabetic, as it achieved an inhibition rate of alpha-amylase activity of 75.55%.

Keywords

Alternaria alternata; antidiabetic; antioxidant; indoor fungi; rosemary plant

Introduction

Endophytic fungi establish a relationship between certain species of fungi and plants where fungi coexist within plant tissues without causing harm (1,2). They grow within or between plant cells and spend their life or part of their life cycle without causing any damage or phenotypic changes in plant cells (3). Many of the compounds produced by endogenous fungi have diverse pharmaceutical applications and are used to make antibacterial, antifungal, antiviral, antioxidant, antidiabetic, anti-inflammatory, and many other compounds (4).

Endophytic fungi are transmitted from soil to plant but are often transmitted through seeds (5). According to the hypothesis of the coevolution of internal plant fungi proposed by (6), which shows that endophytic fungi help the plant in chemical defense against various pathogens by producing biologically active secondary metabolic compounds, it was also found that these substances are similar to those produced by the plant (7).

Rosmarinas officinalis L. is a plant belonging to the Dicotyledon Order - Lamiales, Family - Lamiaceae, an aromatic plant widely used in foods, endemic to the Mediterranean regions of Europe and North Africa, and cultivated in Spain, Italy, France, Algeria, Morocco and Portugal (8). The rosemary plant contains many phenolic compounds, some of which are effective antioxidants due to the presence of their phenolic hydroxyl groups. However, they also have many other beneficial effects, such as antimicrobial, antiviral, anti-inflammatory, and anti-cancer activities, and are also known to be an effective chemopreventive agent (9). Rosemary is also widely used as a culinary spice, and its fragrance is used in soap and cosmetics. Rosemary leaves contain 1.0-2.5% essential oils, and essential oil is characterized by being colorless and sometimes has a pale yellow color with a distinctive refreshing smell (10).

Therefore, this study aimed to isolate and diagnose internal fungi from the rosemary *Rosmarinas officinalis* L. leaves and study their biological characteristics, which are of great importance.

Materials and Methods

This study was conducted in the Mycology Laboratory and the Plant Cell and Tissue Culture Laboratory of the Department of Life Sciences at the College of Education for Pure Sciences, University of Diyala, Iraq, from 1/1/2023 to 1/7/2023.

Plant material

Rosmarinus officinalis L. rosemary plants were obtained from Baquba city nurseries and placed in plastic bags to isolate the endophytic fungi.

Isolation of internal fungi.

To isolate the internal fungi from the leaves of the rosemary plant, the healthy leaves of five plant samples were taken, and from each sample, ten leaves were taken from different places of plants and washed with running water to remove the suspended dust, then they were sterilized superficially by immersing them in ethanol 70% for a minute. After this, they were immersed in sodium hypochlorite solution at a concentration of 2% for 4 minutes. The leaves were then washed with distilled water three times, cut into pieces with a length of 0.5 cm, and transferred to the container Petri dishes. On the medium of PDA, the dishes were placed in the incubator at 25 ± 2 °C until the emergence of fungal growths (11). After the appearance of fungal growth, a swab was taken by the fungal colony and transferred to a Petri dish containing the medium PDA. The dishes were incubated at 25 ± 2 ° C for a week with follow-up growth to purify fungal isolates and obtain pure colonies to diagnose later. The isolates were purified by placing part of the edge of the fungal colony in tubes containing PDA medium, and the tubes were incubated in the incubator at a temperature of 25 ± 2 ° C for a week. After that, the tubes were kept in the refrigerator (12).

Phenotypic diagnosis of Endophytic fungi

The fungus was phenotypically diagnosed by observing the growth of colonies on the PDA medium. The phenotypic characteristics of fungal colonies, such as color, shape, texture, and size, were observed, and the shape of the colony was observed from the opposite side of the dish (13,14).

Microscopic and molecular diagnosis of endogenous fungi

A sample of the fungal colony was taken by a loop vector, placed on a microscopic slide containing blue lactophenol dye, blended well, fixed by passing the glass slide over the flame several times, and examined with a microscope at the magnification of 10X and 40X to observe the hyphae (14). Microscopic characteristics of fungal filaments' shape, conidia size, and conidiophores' branches were observed. The diagnostic key was consulted, and the fungus was identified as *Alternaria alternata* through the characteristics of the carrier conidia and fungus conides depending on (15-18). DNA extraction from fungi colony samples was performed using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit from the Korean company Bioneer. These were used for molecular testing and evolutionary analysis. The rDNA ITS site was amplified using the primer ITS1 (F 5 ̃TCCGTAGGTGAACCTGCGG3 ̀) and ITS4 R 5´ TCCTCCGCTTATTGATATGC3 ̀) prefixes with deionized water, the total volume was raised to 50 ml. The ITS (region) sequence was amplified in the following way: 95°C for 5 minutes, then 35 cycles of 95°C for 30 seconds. 55°C for 30 seconds, 72°C for 1 second, with final stretching at 72°C for 1 second.

Preparation of internal fungus filtrate *Alternaria alternata*

Alternaria alternata was grown on liquid dextrose potato medium after a 250 ml flask was prepared and sterilized by a sealant device. Each flask was inoculated with several tablets with a diameter of 0.5 mm of fungal colonies, and the decanters were incubated in a vibrating incubator at a temperature of 25 ± 2 °C for 28 days with continuous follow -up fungal growth. After this, the filtrate was prepared by passing the liquid culture medium through Whatman no.1 filter paper using a vacuum pump (19). After filtering, ethyl acetate was added to the culture as an organic solvent to obtain a fungal extract of the same size as the medium. Then, it was mixed for ten minutes and set aside until two layers were formed. The top layer contains the extracted active compounds separated by the separation funnel. A rotary evaporative separator was then used to obtain a dry or concentrated extract for subsequent experiments, and the dry extract was kept in an opaque glass bottle away from sunlight in the refrigerator (20).

Biological efficacy of the extract as an antioxidant

The ability of the fungi extracts to capture free radicals DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the method described in (21) was followed by dissolving 2 ml of DPPH in 100 ml of methanol, knowing that the concentration of DPPH is 400 µg / ml. To prepare the

standard solution, 0.5 g of the sample was taken and mixed with 100 ml of methanol and distilled water. The standard solution concentration was (5000 ppm) and using the dilution law, other concentrations were prepared for the studied samples (30, 60, 120, 250, and 500 ppm). The mixture was shaken vigorously and left at room temperature for 30 minutes. The absorbance was then measured at a wavelength of 517 nm using the UV-VIS Shimadzu spectrophotometer (Shimadzu), and the IC value of 50 of the sample, which is the sample concentration required for inhibition of 50% of free radicals DPPH, was calculated using a logarithmic dose inhibition curve Low absorption of the reaction mixture indicates higher free radical activity. The free radical sniping activity was expressed by the percentage inhibition equation calculated as follows:

Inhibition percentage (%) = (A0 – A1 / A0) × 100

A0 means the absorbency value of the control sample without extract.

A1 represents the absorbency value of the abstract sample.

Biological efficacy of the extract as an antidiabetic

The ability of the fungal extract as an antidiabetic was revealed by studying its ability to inhibit the activity of the alpha-amylase enzyme. The reaction mixture was prepared by adding 40 µL of the extract in 200 µL of Phosphate Buffer and 40 µL (24 ml^{-1} unit) of pancreatic amylase. The mixture was kept in the incubator at 37 °C for 10 minutes, followed by adding 50 µL of starch (1%), and was incubated at 37 °C for 20 minutes. The reaction was terminated by adding 0.5 ml of 3,5 dinitro salicylic (DNSA), after which the samples were placed in a water bath at boiling point for 5 minutes. The samples were then cooled at room temperature. The reaction was stopped by adding 200 µl of Potassium sodium tartrate tetrahydrate, known as Rochelle salt, at a concentration of 40% with 2 ml distilled water to all samples. The absorption was read using an optical spectrometer at a wavelength of 540 nm (22). The alpha-amylase inhibition activity was expressed by the percentage inhibition equation calculated as follows:

Alpha Amylase activity (%) = (A0 – A1 / A0) × 100

A0 means the absorbency value of the control sample without the extract.

A1 represents the absorbency value of the extracted sample.

Results and Discussion

Isolation of internal fungi from rosemary leaves

Table 1 shows the results of the isolation of internal fungi from random samples of rosemary plants *Rosmarinus officinalis* L. That 70% of the samples examined contained one isolation of the endophytic fungi, by one fungal colony per plant piece of rosemary leaves. The results show that the total number of colonies was 35 fungal colonies ranging from 2 to 8 fungal colonies, and all these colonies are apparently due to the fungus itself, which will be presented later.

Table 1. Total Numbers of Colonies of Indoor Fungi Isolated from *Rosmarinus officinalis* L.

N	Total number of colonies	%
1	5	50
2	6	60
3	8	80
4	7	70
5	4	40
6	0	0
7	2	20
8	3	30
9	0	0
10	0	0
T	35	

• Note that the number represents the total colonies in 10 plant plots

The results of the current study confirm that many fungi cannot grow rosemary plant tissues because of this plant's chemical properties and antimicrobial activity. It is understood that it coexists with one or two types of organisms, and this is confirmed by one of the studies that showed that there is one fungal isolation that can colonize the internal tissues of the rosemary plant in a coexisting manner, and this fungal isolation was due to the fungus *Trichoderma harzianum* (23). Another study (24) showed that two internal fungi were isolated from the rosemary plant *Rosmarinus officinalis*. *Aspergillus flavus* and sp. *Columnaris*.

Phenotypic and microscopic diagnosis

The phenotypic and microscopic examination results in Fig. (1 A.B) showed that the isolate is *Alternaria alternata*. Fungus belongs to the genus *Alternaria* and is a widespread fungus in the environment. Fig. 1 shows that the fungal colonies were olive and tended to be black due to melanin pigment. The diameter of the colony ranges between 5 to 7 cm after a week of planting on the medium of PDA, depending on the environmental conditions where the dorsal qualities of this fungus are affected by the difference in the type of medium, temperature, and pH agree with (25). Fig. 1 also shows the fungal hyphae, the lengths of the conidial chains and their branches, the methods of arranging these conidia in the chains, in addition to the shape of the single conidium, its longitudinal and transverse barriers, the length of the beak and other essential characteristics in the diagnosis. Fig. 1 also shows fungi conidia, characterized by mace carried on short conidial stands in chains or individually (15).

Molecular diagnostics

The results of the PCR serial examination showed the identity of the studied sample, and these samples belong to the species *Alternaria alternata*. The sequencing results indicated no differences (in DNA sequences) in both samples examined compared to retrograde genetic sequences. These results are consistent with the phenotypic diagnosis for all fungi samples studied. It was inferred from the evolutionary tree that the DNA sequence

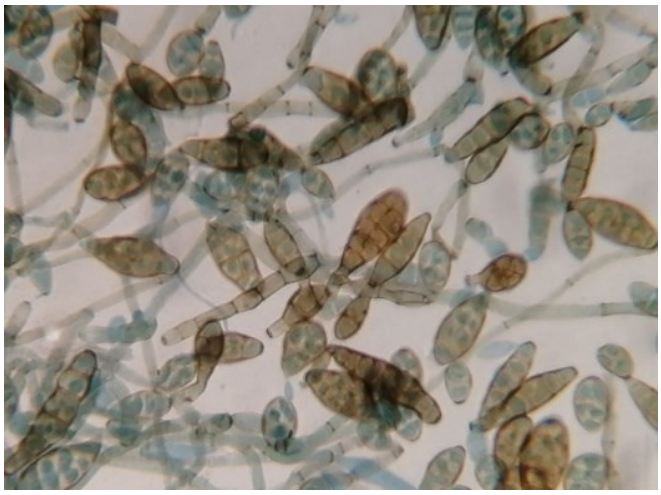

Fig. 1. *Altertnaria alternata*, (A) the image to the right shows the shape of the fungal colony on the center of the PDA, while (B) the image to the left represents the fungus conides under light microscopy at a magnification of ×40.

of *Alternaria alternata* showed significant affinity with two strains isolated from Iraq in the GenBank (MF099865.1 and OQ764793.1).

Chain reactions indicated the exact identity after performing a PCR chain reaction in both samples S1 and S2 and the convergence of the whole sequence of the isolated fungi studied with the genetic sequences of the fungus *Alternaria alternata* (GenBank acc. OQ764793.1). These results agree with (26) and (27), who indicated that specialized initiators could amplify specific sites of specific genes for *Alternaria alternata* fungus by PCR device that would provide a rapid and reliable diagnosis of *Alternaria alternata* isolates. The results also indicate the success of the initiator designed by (26) in the diagnosis of *A.alternata,* isolates according to this source. These results agreed with the results of the researcher above that this initiator is designed in a specialized manner to detect the isolates of this fungi and the success of this initiator in his specialization towards the isolates of the fungus *A. alternate* in its precise design where he targeted a single piece of the 18S rDNA gene.

These results support the phenotypic diagnostic results given for *Alternaria alternata*. The specific site design of the gene was identified in the NCBI program. Its locations within the most homogeneous target were confirmed, as shown in Fig. 2. The results of the ribosome samples examined revealed no DNA variants in the analyzed samples compared to the more closely related genetic retro DNA sequences, as in Fig. 3. The ITS gene was diagnosed by the polymerase chain reaction (PCR) of the isolated fungus, where the results of the polymerase reaction were when DNA amplification (the appearance of polymerase product packets) as in Fig. 2.

The diagnosis of fungi based on phenotypic traits is insufficient to reach species belonging to the genus *Alternaria*. Due to the diversity and similarity of different species, phenotypic characteristics are not sufficiently defined, and a more accurate molecular method appears to be necessary to distinguish between species of the same genus (28). Rezaei-Matehkolaei et al. (29) have also pointed out that using state-of-the-art technologies such as polymerase chain reaction (PCR), followed by

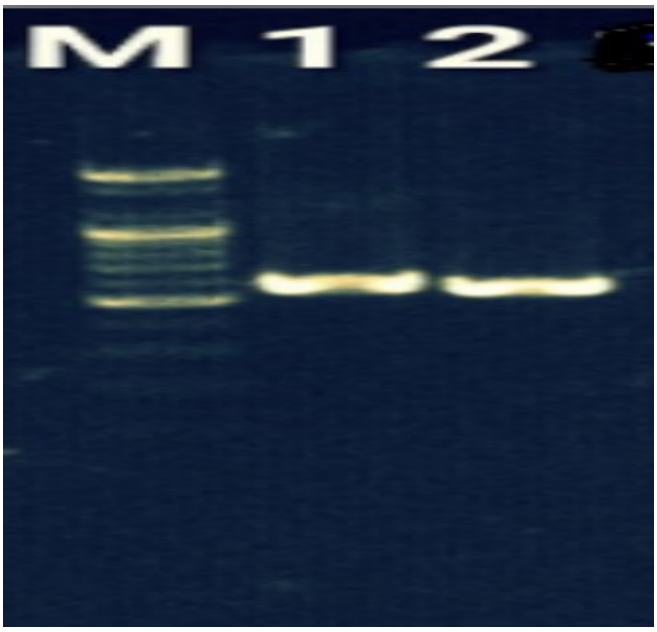

Fig. 2. Electrical relay of DNA on the lacarose gel of two isolates of the internal fungus *Alternaria alternata* isolated from the rosemary plant.

nucleotide sequencing of specific genes and obtaining data on the genetic makeup and diversity of fungi, has important implications in determining the spread of fungi.

The possibility of relying on the ITS region on a large scale in classification and molecular diagnosis due to its ease of amplification and having a wide range of variation even in highly related species, where amplification of the special ITS region rDNA was used to diagnose isolated fungi during this study because it gives appropriate taxonomic accuracy for most fungi (30). Moreover, there are many sequences for this region in the GenBank, which allows for comparing the sequences obtained. The ITS region has ITS1 and ITS2 sequences and is considered a conserved region in DNA sequencing (31). Preserved regions indicate that sequencing has been preserved by natural selection, a highly maintained and protected sequence without any change to safeguard the phenotypic evolutionary tree across many generations, and DNA sequences in the genome gradually change over time due to random changes such as mutations, deletions, and regrouping of many sequences or Some of them may be omitted as a result of chromosomal rearrangement.

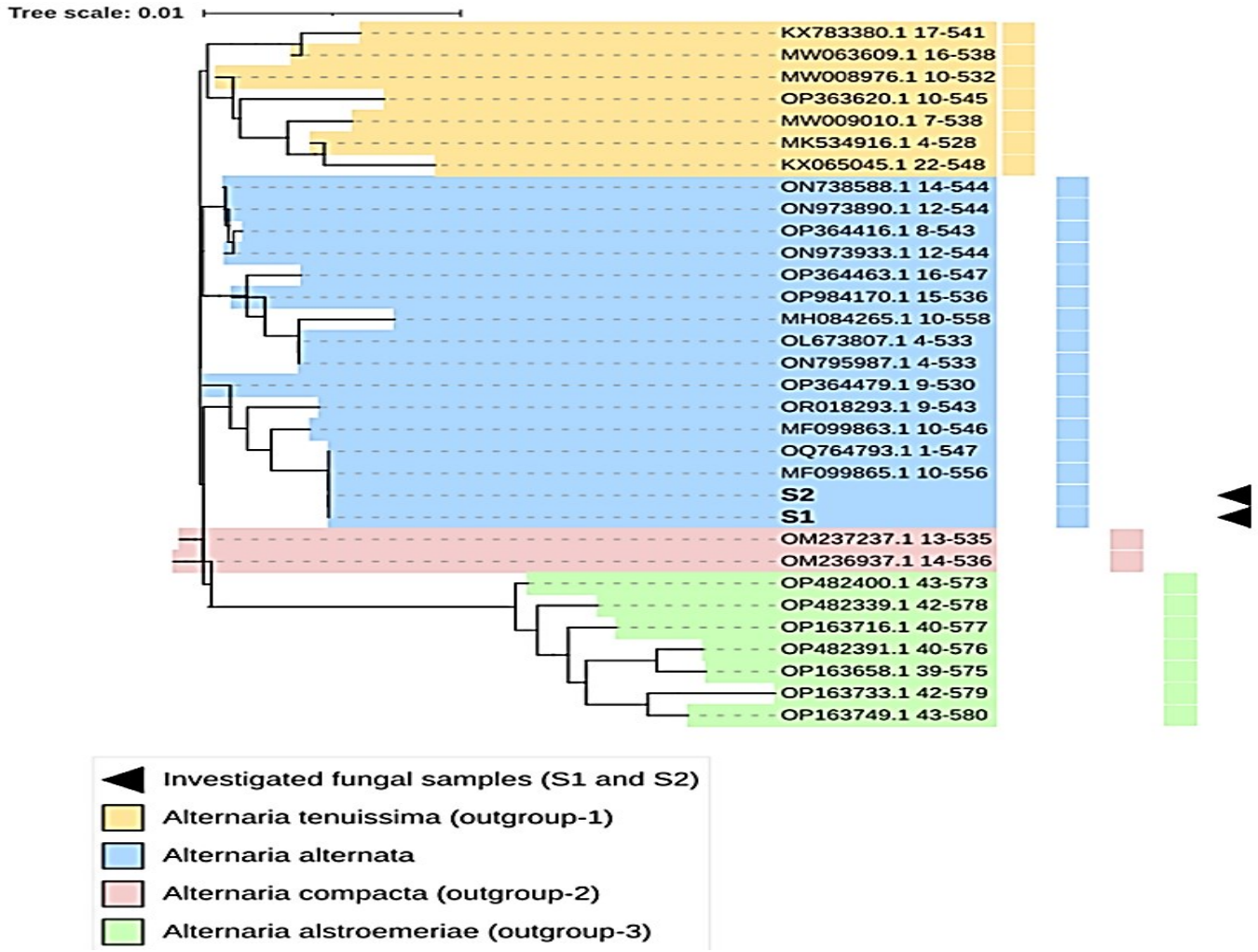

Fig. 3. An evolutionary tree of *Alternaria alternata* isolates shows the degree of convergence between the diagnosed species and those in the gene

Preserved regions are sequences that resist any forces to change their sequence, such as mutations and other effects, to maintain their position in the genome.

This study created A comprehensive evolution tree in Fig. 3 according to DNA changes observed in the ITS1-ITS2 inflated ribosome of the initiator of *A. alternate*. This evolutionary tree contains our 1S and 2S samples and other relative DNA sequences.

The tree currently built is made up of the region of origin and branched into several branches in this evolutionary tree. The branches were merged to provide a retrograde point for determining the relationships between the inner varieties of *Alternaria alternative* sequences. The inner group refers to the group of organisms or species that is the primary focus of the study, while the outer group refers to closely related taxa or taxa that are known to be outside the inner group. Including an external group in genetic analysis helps determine the tree's point of origin, providing a point of comparison that allows researchers to determine the direction of evolutionary changes. By comparing the varieties of the inner group of *A. alternata* with the outer group of other related sequences, it becomes possible to deduce ancestral characteristics and evolutionary relationships.

Evaluation of the Biological Efficacy of *Alternaria alternata* Fungi Extract for Rosemary Plant

Antioxidant

The results of Table 2 showed the role of *Alternaria alternata* fungi extract for rosemary plant as an antioxidant DPPH, where the results show that the effect of *A. alternata* fungi extract in suppressing the impact of free radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH) was 68.798%. We can also see from the Table that the inhibition effect increased with increasing concentration of the extract, which reached its highest level at a concentration of 500 mg.

The formation of free radicals in cells is one of the most important causes of many diseases, as free radicals are chemically unstable after the atom loses an electron, so the cell becomes unbalanced, which leads to cell damage. Therefore, when exposed to oxidative stress, cells suffer from a wide range of diseases, including chronic complications in humans and serious diseases (32).

The results of the current study showed the vital effect of the extract of the internal fungus *A. alternata* of the rosemary plant in suppressing the impact of free radicals and their role as antioxidants, and this can be attributed to the high content of active compounds such as phenols, alkaloids, terpenes, and rosmaric acid, which are characterized by their properties as antioxidants. In a study conducted by (33) on evaluating the role of the effective endophytic fungi as antioxidants and antimicrobials, they found that extracts of fungi isolated from within the plant *Alternaria alternata* contained

Table 2. Biological Efficacy of *Alternaria alternata* Fungi Extract as an Antioxidant.

Average	500 mg	250 mg	120 mg	60 mg	30 mg	AA %
44.504	74.88	62.59	40.58	25.89	18.59	VIT C
68.798	93.5	85.03	71.83	52.6	41.03	Fungi extract

secondary metabolites such as flavonoids, which the study proved to have an essential role in suppressing free radicals and thus act as antioxidants.

Antidiabetic

Table 3 shows the role of *A. alternata* fungi extract isolated from rosemary plant as an antidiabetic by measuring its ability to inhibit the activity of the alpha-amylase enzyme, where the results show that the extract of the fungus *A. alternata* effectively inhibited enzymatic activity. The inhibition rate increased by increasing the concentration of the extract, and the inhibition rate reached 75.55% at a concentration of 0.5 mg.

This finding can be attributed to the fact that diabetes complications occur due to oxidative stress due to forming free radicals with glucose oxidation (34). Therefore, it is often recommended to use antioxidants with antidiabetic drugs to avoid such complications. This is consistent with the results of the current study, which

Table 3. Biological efficacy of *Alternaria alternata* fungi extract as an antidiabetic.

Average	0.5 mg	0.4 mg	0.2 mg	0.1 mg	AAI %
52.5	75.55	65.25	41.35	27.85	Fungi extract

confirmed that the extract of the internal fungus *A. alternata* of the rosemary plant has a role in suppressing free radicals resulting from oxidative activity, so it is considered an effective antioxidant.

Conclusion

The results of the current study confirm the promising future of endophytic fungi in the production of secondary metabolic compounds with various therapeutic properties compared with medicinal plants from which endophytic fungi were isolated. The study showed that the extract of the fungus *Alternaria alternata* possesses effective biological properties as an antioxidant and antidiabetic, confirming the role. The important thing is that the endophytic fungus extract is isolated from the rosemary plant.

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Authors' contributions

RMA participated in the study design and statistical analysis. Also, conceived the study, participated in its design and coordination, and conducted the research and tests on biological effectiveness. BMA carried out the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

Ethical issues: None

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