Research Article

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GC-MS profile of extracts of an endophytic fungus Alternaria and evaluation of its anticancer and antibacterial potentialities

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Abstract: Using microbial endophytes to produce bioactive compounds is a reliable scientific method. This investigation aimed to use the Acacia plant for isolating an endophytic fungal strain that has a bio-ability to produce a bio-crude extract. This study also encompassed the assessment of the extract's biological efficacy as an antibacterial and anticancer agent. Samples of the Acacia plant were collected from "Shuaib Huraymila," in Riyadh, Saudi Arabia. The isolation and identification of fungal endophytes was done, and then, the production of crude extract was performed using the isolated endophytes. The profile gas chromatography-mass spectroscopy of the extract was determined, followed by the assessment of its biological activity against drug-resistant infections and cancer cells through in vitro examination. The findings showed that the fungal endophyte was Alternaria (Alternaria sorghi), according to internal transcribed spacer sequencing and basic local alignment search tool analysis. The minimum inhibitory concentrations of the extract were 9.1 and 4.5 mg/mL for methicillin-resistant Staphylococcus aureus and drug-resistant Candida auris, respectively, and the IC50% values were 46.6 and 23.7 mg/mL for MCF-7 and A549, respectively. The findings showed that this strain had no antagonistic action against Culex pipiens. This study concluded that the fungal endophyte isolated from the Acacia plant has the bio-ability to produce antimicrobial and anticancer agents.

Keywords: Alternaria endophytes, A. sorghi, antimicrobial, anticancer, Cx. pipiens, Methicillin-resistant Staphylococcus aureus.

1 Introduction

Drug resistance and cancer diseases are global health problems that threaten the lives of millions in both developing and developed countries [1]. There are too many microbes, known as endophytes, that can inhabit the inner parts of healthy plants without causing any negative effects. The scientific term endophyte is defined as “in the plant” (in Greek, endon and phyton mean within and plant, respectively). Endophytes have a broad spectrum of applications, such as endophytic bacteria, fungi, algae, and insects [2]. Microbial endophytes have been reported to have various functional roles and the ability to biosynthesize numerous bioactive products. However, the extraction and purification of these bioactive metabolic compounds from endophytes have several problems. Bioactive endophytic compounds are very diverse, and they include anticancer, antioxidant, antibacterial, antifungal, viral, and antidiabetic agents, as well as other groups [3,4]. Approximately 20,000 organic derivatives have been produced using medicinal plant-associated microbial endophytes, and these products include derivatives of phenol, phenolic acids, indole, isocoumarin, lactones, poly-saccharides, amines and amides, phenylpropanoids, chlorinated metabolites, and xanthones [5].

Alternaria species are frequently detected and isolated from most plants as pathogenic and endophytic fungi. A lot of things go into isolating, identifying, and making bioactive products, such as choosing the right plant sources, isolating the right strains based on phenotypic and genotypic traits, and the type of symbiotic interactions [6]. It has
been reported that Alternaria endophytes isolated from medicinal plants could make a number of bioactive compounds, such as 3-nitropropionic acid and tenuazonic acid, which stop Mycobacterium tuberculosis (which causes tuberculosis), N-acetylgalactosamine, which fights diabetes, and altertoxins, which work against the HIV-1 virus [7].

The present investigation aimed to evaluate the biological activity of the crude extract produced using Alternaria endophytes isolated from the Acacia plant in Saudi Arabia against drug-resistant pathogens and to determine the gas chromatography-mass spectroscopy (GC-MS) profile of the crude extract. We also verified the inability of this fungal isolate to inhibit Culex pipiens.

2 Materials and methods

2.1 Sample collection

The plant samples were collected from “Shuaib Huraymila,” located 86 km north of Riyadh, Saudi Arabia. The 100 samples included the leaves and leaf stems of the Acacia plant. The samples were immediately transferred in sterile plastic boxes to the microbiology laboratory at King Saud University.

2.2 Preparation of samples

Each sample’s outer surface received a thorough wash with tap water and sterile water. Then, the outer surface was sterilized using a 70% ethanol solution for 5 min and washed three times using sterile water. The samples were placed inside sterile Petri dishes until the outer surfaces of the samples were completely dry.

2.3 Endophyte isolation, purification, and macroscopic and microscopic characteristics

A cross-cut was performed for every sample using sterile scalpels, and each piece was placed on the surface of a potato dextrose agar (PDA) plate. The PDA plate (Oxoid Ltd., Basingstoke, UK) was prepared according to the manufacturer’s instructions. Incubation was performed at 25 ± 1°C for 5 days. The fungal growth was observed daily, and each colony raised was picked up and then recultivated on a new PDA plate. The subcultivation method was used to obtain single colony cultures that could represent one fungus. The culture and microscopic features were tested to confirm whether each plate contained one fungus. Macroscopic and microscopic characteristics were observed. Studies on growth medium and the use of a microscopy test according to standard protocol were conducted to achieve this purpose [8].

2.4 Identification and basic local alignment search tool (BLAST) analysis

The DNeasy Plant Mini Kit (Qiagen) was used to extract the total DNA from fungal isolates according to the manufacturer’s instructions. The total DNA product was stored at −40°C for future analysis. The fungal ribosomal internal transcribed spacer (ITS) regions were amplified using the polymerase chain reaction (PCR) technique. A simple multiple-step PCR method was applied using the universal ITS forward and reverse primers (5′-TCGTAAGTGAACCTGCGG-3′ and 5′-GCTGCGTTCTTCATCGATGCG-3′) [9]. The PCR program was conducted in seven stages as follows: 95°C for 2 min in the first, 95°C for 30 s in the second, 62.4°C for 30 s in the third, 72°C for 60.0 s in the fourth, repeat of steps 2–4 more than 29 times in the fifth, 72°C for 5 min in the sixth, and 4°C continuously in the seventh.

The PCR amplicons were purified using a Montage PCR clean-up kit (EMD Millipore, Burlington, MA, USA). The pure PCR products were sequenced using a BigDye™ Terminator Cycle Sequencing Kit and a fully automated 96-capillary array DNA sequencer (3730XL) (Applied Biosystems, USA). BioEdit Sequence Alignment Editor software was used to edit the ITS sequencing. A BLAST analysis was conducted using the National Library of Medicine genetic sequence database (GenBank) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGETYPE=BLASTHome).

2.5 Bioactive compound production

Pure fungal isolates were screened to evaluate their ability to produce antimicrobial and anticancer agents using a specific broth medium. The growth medium was prepared for 1 L from 12.8, 6, 3, 1, and 1 g of Na2HPO4·7H2O, KH2PO4, NaCl, NH4Cl, and Bacto Soytone, respectively. The growth medium was sterilized using an autoclave at 121°C for 15 min and then left at room temperature to cool. One liter of the medium was inoculated with 0.1% (which is equivalent to 100 mL) of the fungal spores’ suspension (10⁶ spore/mL). The inoculated medium was incubated at 25 ± 1°C for 4 weeks. The fungal mycelia and spores were removed from the broth using the filtration method (membrane filter, 0.45 µm pore
The liquid–liquid extraction method was conducted using a combination of methylenechloride and ethylacetate (1:1) and 100 mL of the solvent mixture for 100 mL of fungal mycelia and spore-free broth. The extortion was repeated five times using a fresh solvent mixture prepared as described above. Filtration and evaporation were conducted using a membrane filter (0.22 µm pore size) and a rotary vacuum evaporator, respectively. The yield (mg/mL) of the crude extract was calculated and preserved at 7°C until further investigation.

2.6 GC–MS analysis

The biochemical profile of the crude extract was looked at with a Hewlett-Packard 5890 series II gas chromatograph and a VG Analytical 70-250S mass spectrometer. For this analysis, helium gas and a gas chromatograph with a fused silica capillary Elite-5MS column (Perkin Elmer, USA) were used. The stream rate applied was 1 mL/min. The temperature of the injection was set to 200°C, and the temperature of the oven was adjusted from 60°C (the holding time was 2 min) at 10°C/min to 300°C. In the final stage, the temperature was maintained isothermally for 20 min. The electron impact ionization system, with a typical energy of the electrons of 70 eV, was used in the detection stage. To cover the mass range of 35–600 m/z, a scan rate of 0.6 s (operation duration: 0.2 s) was applied.

2.7 Antimicrobial and anticancer testing

In this investigation, drug-resistant pathogenic microbial strains were used to evaluate the biological activity of crude extract as an antimicrobial agent. These strains included Methicillin-resistant Staphylococcus aureus (MRSA) and fluconazole-resistant Candida auris strain. Susceptibility tests for MRSA and C. auris were performed using the E-test method to confirm their resistance to standard antimicrobial drugs, in which the breakpoints applied in this stage were according to the Centers for Disease Control and Prevention (CDC) (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html) [10]. A minimal inhibitory concentration of the crude extract was determined using the microbroth two-fold dilution method, according to [11]. The 5% DMSO solvent was used as a negative control group; cefoxitin, oxacillin, and methicillin were used as positive control groups for MRSA, and fluconazole was used as a positive control group for C. auris.

The biological activity of the crude extract was evaluated using the colorimetric method (MTT assay) for determining the cell metabolic activity of cancer cells, including lung (A549) and breast (MCF-7) cancer cell lines. For this test, 24-well plates were used, where each well received 1,000 µL. The cancer cells (5 × 10^5 ± 10^5) incubated for 24 h were treated with the crude extract, with a serial concentration of 250–7.8 mg/mL, and then incubated for 48 h. The cancer cells treated with the crude extract were treated with 100 µL MTT reagent (its concentration was 5 mg/mL, 10% of the 1,000 µL volume that was used in 24-well plates), and the incubation was done for 2 h in a CO2 incubator at 37 ± 1°C. The treatment with an acidified isopropanol solution for 15 min was done. Afterward, the optical density of visible light at 570 nm was determined using a microplate reader (BioTek, USA) [12]. Cancer cell viability was calculated as a percentage using the following equation:

\[
\text{Cancer cell viability} \% = \frac{\text{OD of treated cancer cells} - \text{OD of untreated cells}}{\text{OD of treated cancer cells}} \times 100.
\]

The IC50% value was calculated using a probit regression table (EP17-A2) [13] according to the following linear equation:

\[
Y = XS \pm I,
\]

where \(Y = \text{probit value (5)}\), \(X = \log (\text{concentration, mg/mL})\), \(S = \text{slope}\), and \(I = \text{intercept}\).

2.8 Larva rearing and mosquito larvicidal bioassay

Eggs, larvae, pupae, and adults of Cx. pipiens were obtained from the stock culture at the Bio-Product Research Chair at King Saud University, Saudi Arabia, Riyadh. They were reared in the insectary at 28 ± 2°C under a 12-h photoperiod. The biological activity of the Alternaria isolate (10^2 spore/mL prepared using serial dilution) was calculated after 24, 48, and 72 h. The fungal spore suspensions were individually introduced to sterile 12-well plates (Corning Inc., NY, USA) (total volume, 4 mL) to test against the third instar of Cx. pipiens. The assay was performed in triplicate with 10 larvae per concentration.

2.9 Experimental design and statistical analysis

A complete random design was used to perform this work, and the data were analyzed using Originpro 2018 (OriginLab Corporation, USA).
3 Results

The findings from the isolation and purification process of endophytes isolated from *Acacia* samples collected from the “Shuaib Huraymila” region in Riyadh, Saudi Arabia, revealed that approximately 10% of the samples harbored bacterial endophytes, whereas fungal endophytes were found in just 3% of the samples. In order to identify the fungal isolates in their pure culture, the DNA’s ITS regions were subjected to sequencing, followed by an analysis utilizing BLAST. The results depicted in Figure 1 demonstrate that the fungal isolates under investigation can be classified under the genus *Alternaria*. Among the identified species, *Alternaria sorghi* exhibited the highest query cover and identification percentage. A query cover, E value, and percentage identification of BLAST analysis were greater than 92, 0, and 97, respectively. The BLAST analysis confirmed the macroscopic and microscopic characteristics of the genus *Alternaria*. The growth of the colonies was rapid, from black to dusky yellowish green-black or ashen color, and conidia were ovoid to ellipsoidal, with beaks having the shape of a cone to cylindrical shape. The color of the conidia was pale brown, with smooth walls.

The yield of the crude extract produced in this study was 10 mg/mL. The GC–MS profile of the crude extract showed some main compounds with an area of greater than 30%. These included erythro-pentitol derivatives, ω-altronic acid, D-ribo-hexitol, D-erythrose, D-glucitol, diisobutyl phthalate, indole, 3-(4-nitrophenylamino), oleic acid, octadecanoic acid, and methyl palmitate. As shown in Table 1, these components constituted more than 62% of the composition of the crude extract based on the GC–MS analysis (Figure 2). Moreover, indole and 3-(4-nitrophenylamino) accounted for 13% of the total composition of the crude extract.

The crude extract produced in this investigation showed biological activity against drug-resistant pathogens, including...
MRSA and fluconazole-resistant *C. auris*. The lowest concentrations of the crude extract that are shown in Figure 3 and Table 2 were 9.11 mg/mL for MRSA and 4.5 mg/mL for fluconazole-resistant *C. auris*. The analysis of variance showed that at the 0.05 level (*P* < 0.05), the means of the MICs against MRSA and fluconazole-resistant *C. auris* were not significantly different.

Figure 4 shows the biological activity of the crude extract against the lung (A549) and breast (MCF-7) cancer cell lines. The results showed that the crude extract had a good ability to inhibit the tested cancer cell lines based on the MTT method used in this study. The IC50% values of the crude extract were 46.69 mg/mL for MCF-7 and 23.71 mg/mL for A549. The mosquito larvae bioassay and larvae rearing results showed that the isolated *Alternaria* sp. did not have any biological activity as a bio-antagonistic factor against *Cx. pipiens* under the conditions used in this study. Table 3 shows that there are significant differences in cancer cell viability (%) between the different concentrations in this case of MCF-7 A and A549 at the 0.05 level.

### 4 Discussion

Scientific efforts must be made to address health problems that include insect-borne diseases or infections caused by drug-resistant pathogens. Endophytes are considered one of the natural sources of most microbes that may have biological activity to fight disease-carrying insects or to produce bioactive metabolites. This study aimed to isolate

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Max Score</th>
<th>Total Score</th>
<th>Query Cover</th>
<th>E value</th>
<th>Per. ident</th>
<th>Acc. Len</th>
<th>Accession</th>
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</thead>
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<tr>
<td><em>Alternaria</em> sp.</td>
<td>831</td>
<td>831</td>
<td>92%</td>
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<td>97.35</td>
<td>528</td>
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<tr>
<td><em>A. atra</em></td>
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<td>831</td>
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<td>97.35</td>
<td>500</td>
<td>MG025841.1</td>
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<td><em>A. sorghi</em></td>
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<td>896</td>
<td>100%</td>
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<td>97.35</td>
<td>604</td>
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<td>92%</td>
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<td>97.34</td>
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<tr>
<td><em>A. multifloris</em></td>
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<td>874</td>
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<td>96.98</td>
<td>573</td>
<td>MN077466.1</td>
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<tr>
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<td>874</td>
<td>100%</td>
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<td>96.98</td>
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**Figure 2:** GC–MS chart of the crude extract produced using *Alternaria* endophytic strain isolated from *Acacia* plant.

**Figure 3:** MIC (mg/mL) of the crude extract produced using *Alternaria* sp. isolated from healthy leaves and leaf stems of *Acacia* plant against MRSA and fluconazole-resistant *C. auris*. *There is no significant difference (*P* < 0.05) between MRSA and *C. auris*. 

**Figure 4:** Biological activity of *Alternaria* endophytic extract.
fungal endophytes from the *Acacia* plant collected from “Shuaib Huraymila” in Saudi Arabia and then evaluate their biological activity against drug-resistant pathogens (i.e., MRSA and fluconazole-resistant *C. auris*) and disease-carrying mosquitoes (*Cx. pipiens*).

Globally, many studies have isolated and investigated *Alternaria* endophytic isolated from several sources, such as grapevines [14], liverwort thallus, orchid roots, tobacco, strawberry, apple, rapeseed, soybean, rice, and citrus [6]. In Saudi Arabia, fungal and bacterial endophytes have been isolated and studied in many places and on many plants, such as the *Calotropis procera* plant [15], *Plectranthus tenuiflorus* plant [16], *Tamarix nilotica*, *Cressa cretica*, and *Penicillium chrysogenum* plants [17].

In this study, fungal endophytes were isolated from *Acacia* plants. This is the same as what was found in previous studies, which confirmed that these organisms live inside *Acacia* plants. For example, Hashem et al. [18] confirmed that fungal and bacterial endophytes have the ability to enhance the growth of *Acacia gerrardii* under environmental stress (salt stress conditions). Tran et al. [19] identified and investigated the biological activity of fungal endophytes isolated from *Acacia* spp. *Alternaria* endophytic isolates have been used in many scientific studies to make bioactive compounds, such as paclitaxel, which is used to treat cancer, as well as antimicrobial and antibiofilm agents [20,21]. Our findings agree with many previous studies reporting the antimicrobial and

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**Table 2:** The statistical analysis of means of minimum inhibitory concentrations (MICs) of the extract produced using *Alternaria* endophytic strain isolated from *Acacia* plant

<table>
<thead>
<tr>
<th></th>
<th>N analysis</th>
<th>N missing</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>SE of mean</th>
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</thead>
<tbody>
<tr>
<td>MRSA</td>
<td>3</td>
<td>0</td>
<td>9.1145</td>
<td>5.966</td>
<td>3.44499</td>
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<tr>
<td><em>C. auris</em></td>
<td>3</td>
<td>0</td>
<td>4.55729</td>
<td>2.98345</td>
<td>1.72249</td>
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</table>

**One way ANOVA**

<table>
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<tr>
<th></th>
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<th>Mean square</th>
<th>F value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>1</td>
<td>31.15336</td>
<td>31.15336</td>
<td>1.4</td>
<td>0.30224**</td>
</tr>
<tr>
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<td>89.0096</td>
<td>22.2524</td>
<td></td>
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</tr>
<tr>
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<td>120.16296</td>
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</tr>
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</table>

*The statistical analysis was done using Tukey test in one-way ANOVA at 0.05 level.

**Table 3:** The statistical analysis between different concentrations using one-way ANOVA (Tukey test at 0.05 level)

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>For MCF-7*</td>
<td>Model</td>
<td>5</td>
<td>6525.337</td>
<td>1305.0674</td>
<td>745752.8</td>
</tr>
<tr>
<td>Error</td>
<td>6</td>
<td>0.0105</td>
<td>0.00175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>6525.3475</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For A549*</td>
<td>Model</td>
<td>5</td>
<td>2745.33044</td>
<td>549.06609</td>
<td>52416.81034</td>
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<tr>
<td>Error</td>
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<td>0.06285</td>
<td>0.01048</td>
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<td></td>
</tr>
<tr>
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<td>2745.39329</td>
<td></td>
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</tbody>
</table>

*At the 0.05 level, the means of cancer cell viability (%) are significantly different between the different concentrations in the case MCF-7 and A549.

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![Cancer cell viability (%) of MCF-7 and A549 treated with several concentrations of the crude extract produced using *Alternaria* sp. isolated from healthy leaves and leaf stems of *Acacia* plants. IC50 of MCF-7 was 46.69 mg/mL and IC50 of A549 was 23.71 mg/mL.](image-url)
anticancer activities of extracts produced from *Alternaria* endophytic strains. The most important thing about the results of this study is that the crude extract made from the *Alternaria* endophytic isolates through a simple and quick process had good biological activity. The extract was very effective against MRSA and drug-resistant *C. auris*, which are two of the most drug-resistant pathogens. According to the statistical data reported by the CDC (https://www.cdc.gov/mrsa/community/index.html), 15% of patients in the United States carry MRSA on their skin or in their nose. *C. auris* is an emerging pathogenic fungus that is considered an urgent worldwide health threat. MRSA and drug-resistant *C. auris* infections have been diagnosed in several regions of Saudi Arabia [22–24].

GC–MS analysis is a useful method for screening the bioactive compounds of crude extracts that have the bioactivity to fight pathogenic microorganisms and cancer cells. The GC–MS profile of the crude extract made with the *Alternaria* endophytic strain isolated from the leaves and leaf stems of the *Acacia* plant showed many bioactive compounds that can stop pathogens from growing, such as diisobutyl phthalate. Shobi and Viswanathan [25] produced diisobutyl phthalate using *Begonia malabarica* and reported that this compound had activity as an antimicrobial and anticancer agent. Oleic acid has bioactivity for inhibiting microbes such as *S. aureus* and *Micrococcus kristinae* [26]. This report supports the present findings, as the concentration of oleic acid in the crude extract produced using the *Alternaria* endophytic strain reached 5.9%. Moreover, according to many laboratory experiments [27], oleic acid derivatives can inhibit MCF-7 (cancer of human breast) and HT-29 (cancer of the human colon), which is consistent with our findings.

The fact that the *Alternaria* endophytic strain isolated from the *Acacia* plant had the opposite effect on *Cx. pipiens* showed that this particular *Alternaria* isolate did not have any biological activity to fight malaria mosquitoes.

In sum, according to previous studies, *Alternaria* isolates can be pathogens for plants, animals, or humans. Others have been described as *Alternaria* endophytic strains with high bioactivity to biosynthesize many bioactive metabolites. Some have been shown to be able to fight plant pathogens.

### 5 Conclusion

The findings showed that the *Alternaria* endophytic strain could be isolated from the leaves and leaf stems of the *Acacia* plant. There are promising opportunities for using *Alternaria* endophytic strains to produce several bioactive compounds as antimicrobial and anticancer agents. The MICs of the crude extract produced using the *Alternaria* endophytic strain isolated from the *Acacia* plant were 9.1 and 4.5 mg/mL for MRSA and drug-resistant *C. auris*, respectively, and the IC50% values of the extract were 44.45 and 25.21 mg/mL for MCF-7 and AS49 cancer cells, respectively.

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**Author contributions:** All authors contributed materially to this scientific research. L.A. and F.A. planned and designed the work and wrote the manuscript. L. Al-Shuraym and F.A. performed the experiments and analysis and interpretation of the data. M.A. and M.W. revised and edited the manuscript. All authors agree to be accountable for all aspects of the work.

**Conflict of interest:** The authors declare that they have no competing interests.

**Ethical approval:** The conducted research is not related to either human or animal use

**Data availability statement:** All data generated or analyzed during this study are included in this published article.

### References


