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Antibacterial potential of extracts and metabolites isolated from the endophytic fungus *Chaetomium cochliodes* against phytopathogenic bacteria

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Summary Five fungal endophytes, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp., were isolated from an Egyptian herbaceous plant, *Tribulus terrestris*, and tested for their antibacterial activity against three phytopathogenic bacteria (*Pectobacterium carotovorum* subsp. *carotovorum, Ralstonia solanacearum, Pseudomonas syringae* pv. *syringae*). *Chaetomium* sp. showed the highest antibacterial activity. This strain was identified morphologically and molecularly as *Chaetomium cochliodes* MS03 (MW898133) based on the ITS1-5.8S rRNA-ITS2 genomic region. *Chaetomium cochliodes* caused 15 and 8 mm inhibition zones of *P. carotovorum* subsp. *carotovorum* and *R. solanacearum*, respectively. *Chaetomium cochliodes* isolate was fermented and extracted with ethyl acetate. The crude extract of *C. cochliodes* showed strong antibacterial activity against *P. carotovorum* subsp. *carotovorum* (inhibition zone = 27 mm). Bioassay guided isolation of the crude extract using silica gel column chromatography was conducted to isolate bioactive secondary metabolites. Minimum inhibitory concentrations (MICs) were 500, 32 and 4 mg/L for *C. cochliodes* extract, fraction 14 and fraction 15, respectively, against *P. carotovorum* subsp. *carotovorum*. Bioactive fractions were analyzed by GC/MS. The bioactive pure compound was identified as 9,12-octadecadienoic acid (Z,Z) and the chemical structure was confirmed by H^1 NMR and C^{13} NMR spectral analysis. The isolated compound showed a promising antibacterial activity against *P. carotovorum* subsp. *carotovorum* with MIC value of 32 mg/L.

Additional keywords: antibacterial activity, endophytic fungi, secondary metabolites, structure identification, *Tribulus terrestris*

Introduction

The interaction between microorganisms and their hosts is liable to the nature of the host and the surrounding environment; it can be symbiotic or mutualistic or pathogenic (Sahani and Hemalatha, 2018). Fungi are good examples of these relationships, they can be found as plant pathogens, and/or they can live as endophytes, asymptomatically without causing any signs of diseases, within the intracellular spaces of leaves, roots and stem tissues (Arnold *et al.,* 2000). When numerous microbial species exist in the same plant, their interaction may promote the secretion of some secondary metabolites by the endophytes or the host that obstruct the development of the harmful microbes (Kusari *et al.,* 2012).

Several endophytes are known for their ability to improve nutrient acquisition, promote growth, increase abiotic and biotic stress tolerance of the host plant, and enhance plant defense against many phytopathogens. Thus, endophytes are considered as efficient bio-control agents (Saad and Badry, 2020). Likewise the extracellular secondary metabolites produced by endophytic fungi occasionally possess biological activity against various plant pathogens (Mousa and Raizada, 2013). Different bioactive compounds with antimicrobial properties have been isolated from endophytes, such as alkaloids, flavonoids, peptides, polyketides, phenols, steroids, terpenoids, and quinones (Gunatilaka, 2006; Mousa and Raizada, 2013; Lugtenberg *et al.,* 2016).

Fatty acids are long, unbranched carbon chain carboxylic acids with saturated or unsaturated bonds. Fatty acids and their deriv-

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atives are major components of plant and fungal metabolites. They are playing a crucial role in host defense against pests having a broad potential of antibacterial (Kabara *et al.,* 1972), antifungal (Walters *et al.,* 2004), antimalarial (Carballeira, 2008), antifeedant, insecticidal and nematicidal (Stadler *et al.,* 1994) activities. Therefore, there is a continuous research interest in isolating natural compounds from endophytes which could serve as alternatives to synthetic pesticides.

Pectobacterium carotovorum subsp. *carotovorum* is a phytopathogenic, soil borne facultative anaerobic bacterium causing soft rot, blackleg or stem rot in many economically important crops, including vegetables, ornamental plants and fruits (Pérombelon and Kelman, 1980). Soft rot is a worldwide distributed disease affecting a variety of vegetable crops, such as potatoes (*Solanum tuberosum*), Chinese cabbage (*Brassica pekinensis*), carrots (*Daucus carota*), etc. It causes severe disease to vegetables during cultivation, post-harvest handling, and storage (Strange and Scott, 2005). *Ralstonia solanacearum* is a soil-borne bacterium causing the widespread disease known as bacterial wilt (Peeters *et al.*, 2013). *Pseudomonas syringae* pv. *syringae* constitutes a diverse group of bacterial strains that cause important diseases, such as bacterial canker of stone fruits, citrus blast, leaf blight of wheat and barley, sheath rot of rice, red streak of sugarcane and brown spot of bean (Bultreys and Kaluzna 2010; Dariush *et al.,* 2012).

Synthetic antibiotics, such as kasugamycin, gentamicin, streptomycin, oxolinic acid, oxytetracycline and validamycin are used to control pathogenic bacteria (Verhaegen *et al.*, 2024). However, due to their environmental hazards, their side effects on non-target organisms and the development of pathogen resistance, their use has become under restrictions. Thus the continuous efforts for discovering and developing new antimicrobial compounds from natural sources, including endophytic fungi, to overcome these difficulties, are very crucial and ever-increasing.

The present research aims to isolate and investigate the potential of endophytic fungi as a source of natural pesticides, focusing on endophytic fungi in plants native to Egypt for evaluation of their potential antibacterial activity, isolation and identification of the compounds responsible for such antibacterial activity. Thus, the isolation and the antibacterial activity assessment of five fungal isolates obtained from *Tribulus terrestris* L. (Zygophyllaceae), a plant known in ancient medicine for its diuretic, tonic, and aphrodisiac properties, were carried out. In addition, the antibacterial activity of extract, fractions and a pure compound from one of these fungi, *C. cochliodes*, was evaluated against *P. carotovorum* subsp. *carotovorum*.

Materials and methods

Sampling and isolation of endophytes

Apparently healthy and fresh leaves from specimens of *Tribulus terrestris* were collected from Shalalat garden, Alexandria (31°12′56.30″N, 29°57′18.97″E), Egypt. The plant was identified by Prof. FathAllah Zaitoon of Department of Plant Pathology, University of Alexandria. Fungal endophytes isolation process was performed within 24 h of sampling by a standardized surface sterilization method. Briefly, plant leaves were washed in running tap water, immersed in 70% ethanol for 1 min, then in 3% sodium hypochlorite solution for 2 min, and rinsed in sterile distilled water three times separately. The surface sterilized leaves were cut into 5 mm pieces with a sterile blade, inoculated onto Petri plates containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 mg/L), incubated at 28°C ± 2 for 14 days in the dark and checked every other day for emerging hyphae. Within the first week, the emerging hyphae were transferred to fresh PDA plates for sub-culturing several times to ensure pure isolates.

Morphological and molecular identification of fungal endophytes

Fungal cultures were maintained on PDA

at 28°C ± 2°C in the dark for 14 days, then visually examined for their morphological characterization to genus level based on macroscopic and microscopic features, such as colony color, growth rate, type of conidiophore and shape of conidia (Barnett and Hunter, 1998). Five fungal isolates (*Alternaria* sp.*, Aspergillus* sp.*, Chaetomium* sp.*, Rhizopus* sp. and *Curvularia* sp.) were identified and examined for their antibacterial activity as described in the following paragraph. The isolate (*Chaetomium* sp.) showing the highest activity was subjected to molecular identification to species level and phylogenetic analysis. Molecular identification using ITS-PCR amplification was conducted from a one-week-old fungal isolate in PDA culture. The fungal strain identification was performed based on sequencing analysis of the amplified ITS1-5.8S rRNA-ITS2 genomic region (White *et al.,* 1990). The amplified PCR product of ITS1- 5.8SrDNA-ITS2 was sequenced on both strands using the primer set: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), an automated ABI-Prism 377 DNA Sequencer (Applied Biosystems Inc., CA, USA) and a Taq FS Dye Terminator Sequencing Kit (ABI, USA). Sequence editing was carried out using Biology Work Bench 3.7 software. The sequence was compared to the available fungal sequences on the NCBI database using the Blast program (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers were obtained. This was achieved by generating a neighbor-joining distance-based tree using the software MEGA 6.

Antagonistic effects of the fungal isolates against phytopathogenic bacteria

Three phytopathogenic bacterial strains, *P. carotovorum* subsp. *carotovorum* (Jones, 1901) Hauben *et al.* 1999 (EMCC 1687), *P. syringae* pv. *syringae* Van Hall, 1904 (EMCC 1739), and *R. solanacearum* (Smith, 1896) Yabuuchi *et al.,* 1996 (EMCC 1274) were obtained from Microbiological Resource Centre (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacterial strains were cultured in Nutrient broth (NB) overnight at 30°C and their concentration was adjusted to 10^8 CFU ml^{-1}. The antagonistic activities of the five isolated endophytic fungi were tested as mycelium on agar plates against the three selected phytopathogenic bacteria, and were assessed by measuring the inhibition zone diameter. One milliliter of bacterial culture was scattered evenly onto nutrient agar plates using a sterile Drigalsky's handle, then a disk of 5 mm Ø of seven day-old mycelia of each endophytic fungus was placed in the central of the NA plate. Plates were incubated for approximately 24 h at 30°C and the diameter of the inhibition zones were measured in mm.

Fermentation and extraction of metabolites

Solid-state fermentation was carried for the most active antibacterial fungal strain isolated in this study, *Chaetomium cochliodes* (MW898133, as registered in NCBI, in this study). A pure colony of this isolate was inoculated in 1 L Erlenmeyer flasks containing autoclaved barley medium. For the barley medium, barley (100 g) was soaked in 150 mL of distilled water overnight then autoclaved at 121°C and pressure of 15 psi for 20 minutes. The inoculated flasks were incubated with shaking at 28°C for 30 days. Then the fungal *hyphae* and growth medium were extracted with ethyl acetate (EtOAc) and the extract was concentrated under vacuum. This crude extract was kept at 4°C for further investigations.

Primary antimicrobial evaluation of *C. cochliodes* extract

Preliminary antimicrobial screening of the *C. cochliodes* crude extract was carried out using the agar well diffusion method. The extract was tested against the *P. carotovorum* subsp. *carotovorum* strain EMCC 1687. Nutrient agar was poured into sterile Petri dishes and after solidification standardized concentration of an overnight culture of the *P. carotovorum* subsp. *carotovorum* strain was swabbed aseptically on the agar. Holes (5 mm diameter) were made in the agar plates using a sterilized cork bor-

er. Fifty microliters of entophyte extract solution (1000 mg/L) and DMSO (negative control) were put in each hole. Three replicates of treatment (endophyte extract) and control were arranged in each plate. The plates were incubated at 30°C for 24 hours and the inhibition zone diameters were measured.

Isolation of bioactive compound from fungal extract

Column fractionations of the EtOAc crude extract (2 g) were conducted using silica gel column chromatography two times. The first fractionation of the crude extract with column 3×50 cm filled with 70 g silica gel (200-300 mesh) and eluted with 1L of methylene chloride- methanol (0%, 0.25%, 0.5%, 1% and 5% MeOH/ CH_2Cl_2 v/v) to give fractions (1–15). The antibacterial activities of the 15 column fractions were tested using the 96-well plate bioassay. Fraction 14 (F14) (1% MeOH/CH_2Cl_2, 300 mg), which showed the highest activity followed by fraction 15 (F15), was subjected to further purification on silica gel column chromatography (1× 50 cm) eluted with hexane-ethyl acetate (100:0 – 0:100 v/v) to give a pure compound (170 mg).

GC/MS analysis of the bioactive fractions

The chemical composition analysis of the most bioactive column fractions (F14 and F15) was performed using Trace GC-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m x 0.25 mm x 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased to 250°C by 5°C /min and held for 2 min. The temperature was increased to 300°C by 30°C /min and held for 2 min. The temperatures of the injector and MS transfer line were kept at 270 and 260°C respectively. Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min and diluted samples of 1 µl were injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50–650 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 14 mass spectral database.

NMR of the isolated compound

The NMR spectra of the isolated compound, 9,12-octadecadienoic acid (Z,Z), were obtained on a Bruker BioSpin GmbH spectrometer (400 MHz). A sample (10 mg) of the compound was dissolved in 0.5 ml of $CDCl_3$. The ^{1}H and ^{13}C NMR spectra were taken at 25°C, and the chemical shift was expressed in parts per million.

Determination of minimum inhibitory concentrations (MICs) using micro-dilution assay

The antimicrobial activity of *C. cochliodes* crude extract, the column fractions and the isolated compound were tested in 96-well plates using nutrient broth as culture medium to determine the minimum inhibitory concentration (MIC). The bacterial inoculum of *P. carotovorum* subsp. *carotovorum* was prepared as described earlier. Stock solutions of the tested crude extract, the column fractions (15 fractions) and the isolated compound were prepared in dimethyl sulfoxide (DMSO). Apposite volumes of the stock solutions were transferred to 96-well plates contained the appropriate amount of NB to obtain final concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 mg/L. Then the bacterial inoculum (1.0×10^8 CFU ml^{-1}) was added and the 96-well plates (micro-dilution trays) were incubated at 30°C. After 24 h of incubation 20 µl of 2,3,5-triphenyltetrazolium chloride (TTC) was added and incubated for 30 min. at 30°C in the dark (Ellof, 1998). A change of color from colorless to pink indicated the reduction of TCC by the viable bacterial cells. The MIC was defined as the lowest concentration of the tested compounds that prevented this color change. DMSO was used as negative control and a reference antibiotic, Ampicillin, was

used as positive control.

Statistical analysis

Experimental results (diameter of inhibition zone) were the average values of three replicates. For the analysis of the data obtained from the fungal mycelial disk diffusion assay, data was subjected to one-way analysis of variance followed by Student-Newman Keuls test to determine significant differences between mean values at the probability level of 0.05.

Results

Isolation, morphological and molecular identification of endophytic fungi

A total of five fungal endophytes were isolated from healthy leaves of *T. terrestris*. which, after identification to the genus level (taxonomical morphological features including colony color, growth rate, type of conidiophore and shape of conidia), were classified in five genera: *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp. One of these strains, that was later determined as the most bioactive antibacterial strain against *P. carotovorum* subsp. *carotovorum*, was morphologically identified as *Chaetomium* sp. and was subjected to molecular identification. The fungal isolate was molecularly identified as *C. cochliodes* based on the ITS1-5.8S rRNA-ITS2 genomic region. The acquired sequence was submitted to the NCBI GenBank database and an accession number was obtained for *C. cochliodes* MS03 (MW898133). The sequence was analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The sequence was aligned using Align Sequences Nucleotide BLAST. The identification of the species was determined based on the best sequence alignment score. The DNA sequence was included in a phylogenetic study by means of comparative sequence analysis of the other rDNA sequences (Fig. 1).

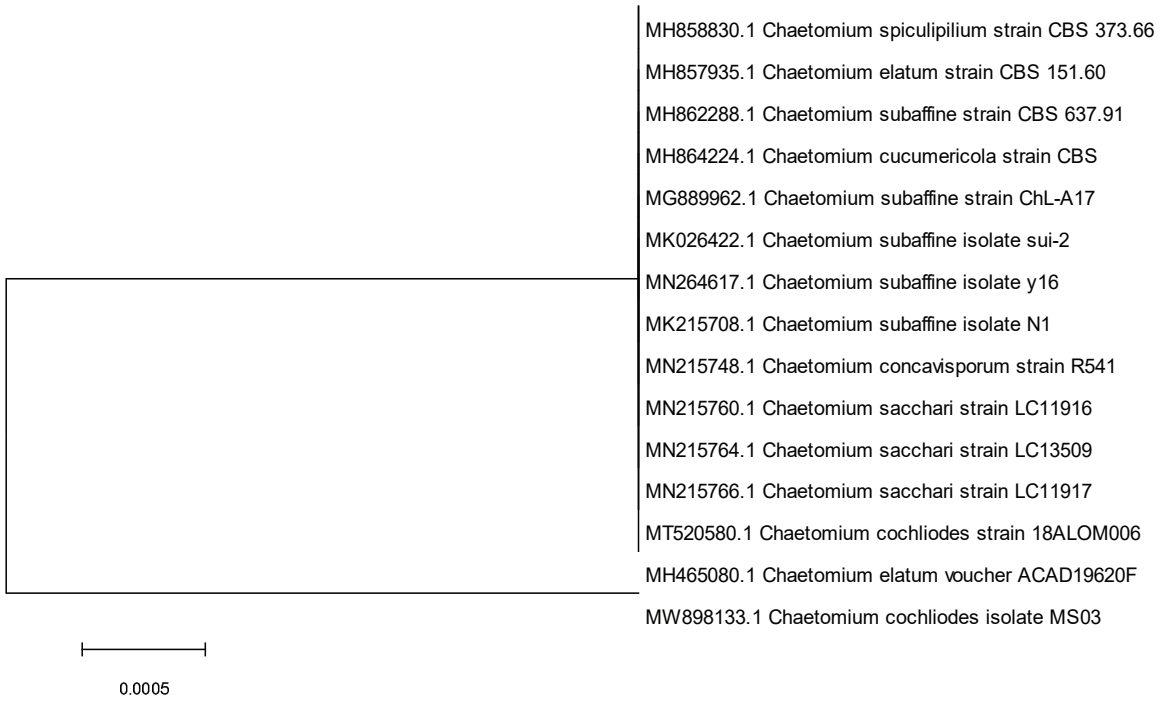

Figure 1. A neighbor-joining phylogenetic tree was constructed based on the alignment of the ITS1-5.8S rRNA-ITS2 genomic region sequences of *Chaetomium* genotypes derived from NCBI using MEGA 6 software.

Letters and numbers written in front of the scientific name are the GenBank accession numbers.

Antibacterial activities of endophytic fungi and *C. cochliodes* crude extract

The antagonistic activity of the five isolated endophytic fungi against the three selected phytopathogenic bacteria, based on the inhibition zone diameter on agar plates is presented in Table 1. *Chaetomium cochliodes* (MW898133) was the most active isolate against *P. carotovorum* subsp. *carotovorum* and *R. solanacerum* with 15 and 8 mm inhibition zones, respectively. In addition, *Aspergillus* sp. isolate showed weak antibacterial activity against *R. solanacerum* with 3 mm inhibition zone. All of the isolated endophytic fungi were not active against *P. syringae* pv. *syringae*. Based on the above-mentioned results, the EtOAc crude extract of *C. cochliodes* (MW898133) was tested for antibacterial activity against *P. carotovorum* subsp. *carotovorum* using the agar well diffusion method. This extract showed promising activity with inhibition zone 27 mm (Table 1).

GC-MS analysis of the crude extract of *C. cochliodes*

The GC–MS analysis of the most bioactive column fractions of the *C. cochliodes* (MW898133) crude extract led to the identification of nine (9) compounds in F14 and five (5) compounds in F15. The characterization of the compounds structures was obtained by comparison of mass spectra with the spectra present in those of WILEY 09 and NIST 14 mass spectral database. The characterized compounds and their retention time (RT), concentration (peak area %), molecular formula and molecular weight (MW) are presented in Tables 2 and 3.

Identification of a pure antibacterial compound

Fraction 14 of *C. cochliodes* extract that showed the highest antibacterial activity was further identified by using H^1NMR, C^{13}NMR spectral data and GC–MS as 9,12-octadecadienoic acid (Z,Z) (Fig. 2). Here are ^{1}H NMR and ^{13}C NMR spectral data of 9,12-octadecadienoic acid (Z,Z) ; ^{1}H NMR ($CDCl_3$): δ (ppm) 0.82 (3H, t, J = 7.2 Hz, Me-18), 1.18-1.25 (14H, m, H-4, H-5, H-6, H-7, H-15, H-16, H-17), 1.56 (2H, t, J = 6.4 Hz, H-3), 1.97 (2H, t, J = 7.2 Hz, H-14), 2.28 (2H, t, J = 7.2 Hz, H-2), 2.70 (2H, t, J = 6.0, H-11), 5.27-5.29 (4H, m, H-9, H-10, H-12, H-13); ^{13}C NMR ($CDCl_3$): δ (ppm) 14.1 (q, Me-18), 22.6 (t, C-17), 24.6 (t, C-3), 26.6 (t, C-11), 27.2 (t, C-8, C-14), 28.9-29.8 (t, C-4, C-5, C-6, C-7, C-15), 31.5 (t, C-16), 34.0 (t, C-2), 128.1, 128.8 (d, C-10, C-12), 130.0, 130.2 (C-9, C-13), 179.8 (s, C-1).

Antibacterial activity of *C. cochliodes* extract, fractions and isolated compound

Minimum inhibitory concentrations (MIC) of *C. cochliodes* crude extract, fractions and the isolated compound, 9,12-octadecadienoic acid (Z,Z), are shown in Table 4. The

Table 1. Antibacterial activity (zone of inhibition, mm) of five endophytic fungi and *Chaetomium cochliodes* crude extract.

Treatment	Inhibition zone (mm)		
	Ralstonia solanacerum	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pseudomonas syringae* pv. *syringae*
Alternaria sp.	0.00 c*	0.00 d	0.00
Aspergillus sp.	3.00 b	7.00 c	0.00
Chaetomium cochliodes	8.00 a	15.00 b	0.00
Rhizopus sp.	0.00 c	8.00 c	0.00
Curvularia sp.	0.00 c	0.00 d	0.00
Chaetomium cochliodes extract	-	27.00 a	-
DMSO (control)	0.00 c	0.00 d	0.00

* Different letters indicate significant differences among treatments within the same column according to least significant difference test (P ≤ 0.05).

Table 2. GC/MS analysis of fraction14 separated from ethyl acetate extract of the endophytic fungus *Chaetomium cochliodes* (MW898133).

No.	Retention time	Name	Area (%)	Molecular formula	Molecular weight
1	19.74	Butylated Hydroxytoluene	2.33	$C_{15}H_{24}O$	220
2	25.07	2H-Pyran-3-ol, tetrahydro-2,2,6-trimethyl-6-(4-methyl-3-cycloh exen-1-yl)-, [3S [3à,6à(R)]]-	1.03	$C_{15}H_{26}O_2$	238
3	28.67	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8 dione	0.67	$C_{17}H_{24}O_3$	276
4	29.63	n-Hexadecanoic acid	2.34	$C_{16}H_{32}O_2$	256
5	31.28	Palmitic Acid, TMS derivative	70.22	$C_{19}H_{40}O_2Si$	328
6	32.07	Methyl 9-cis,11-trans-octadecadienoate	1.80	$C_{19}H_34O_2$	294
7	32.94	9,12-Octadecadienoic acid (Z,Z)	15.41	$C_{18}H_{32}O_2$	280
8	34.25	9,12-Octadecadienoic acid (Z,Z)-trimethylsilyl ester	4.10	$C_{21}H_{40}O_2Si$	352
9	34.34	9-Octadecenoic acid, (E)-, TMS derivative	1.21	$C_{21}H_{42}O_2Si$	354

Table 3. GC/MS analysis of fraction 15 separated from ethyl acetate extract of the endophytic fungus *Chaetomium cochliodes* (MW898133).

No.	Retention time	Name	Area (%)	Molecular formula	Molecular weight
1	19.74	Butylated hydroxytoluene	1.19	$C_{15}H_{24}O$	220
2	31.28	Palmitic acid, TMS derivative	80.86	$C_{19}H_{40}O_2Si$	328
3	32.94	9,12-Octadecadienoic acid (Z,Z)	11.17	$C_{18}H_{32}O_2$	280
4	34.25	9,12-octadecadienoic acid (Z,Z)-, trimethylsilyl ester	5.23	$C_{21}H_{40}O_2Si$	352
5	34.34	9-Octadecenoic acid, (E)-, TMS derivative	1.55	$C_{21}H_{42}O_2Si$	354

crude extract displayed the lowest antibacterial activity with MIC value of 500 mg/L. The two fractions: F14 and F15 showed strong antibacterial activity with MIC values of 32 and 4 mg/L, respectively. Fraction 15 (MIC = 4 mg/L) was more active than a reference antibiotic, ampicillin (MIC = 16 mg/L). Furthermore, the pure compound, 9,12-octadecadienoic acid (Z,Z), revealed a promising antibacterial activity as its MIC value was 32 mg/L.

Discussion

Medicinal plants have been recognized as a rich source of endophytes with potential novel secondary metabolites of agricultural and pharmaceutical merit (Tan and Zou, 2001; Strobel *et al.,* 2004). In this study, five fungal endophytes, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp., have been isolated, for the first time, from the leaves of a well-known medicinal herb, *Tribulus terrestris* growing in Egypt. In agreement with our results, Sahani and Hemalatha (2018) isolated a total of 54 endophytic isolates including *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., and *Curvularia* sp. from different parts of these plants growing in Bengal. Similarly, the isolation of two fungal strains, *Curvularia aeria* MTCC-12847 and *Alternaria tenuissima*, have been previously reported from the leaf and stem of *T. terrestris* growing in Bengal and China, respectively (Wu *et al.,* 2014; Sahani *et al.,* 2019).

Of all isolated fungal endophytes, the

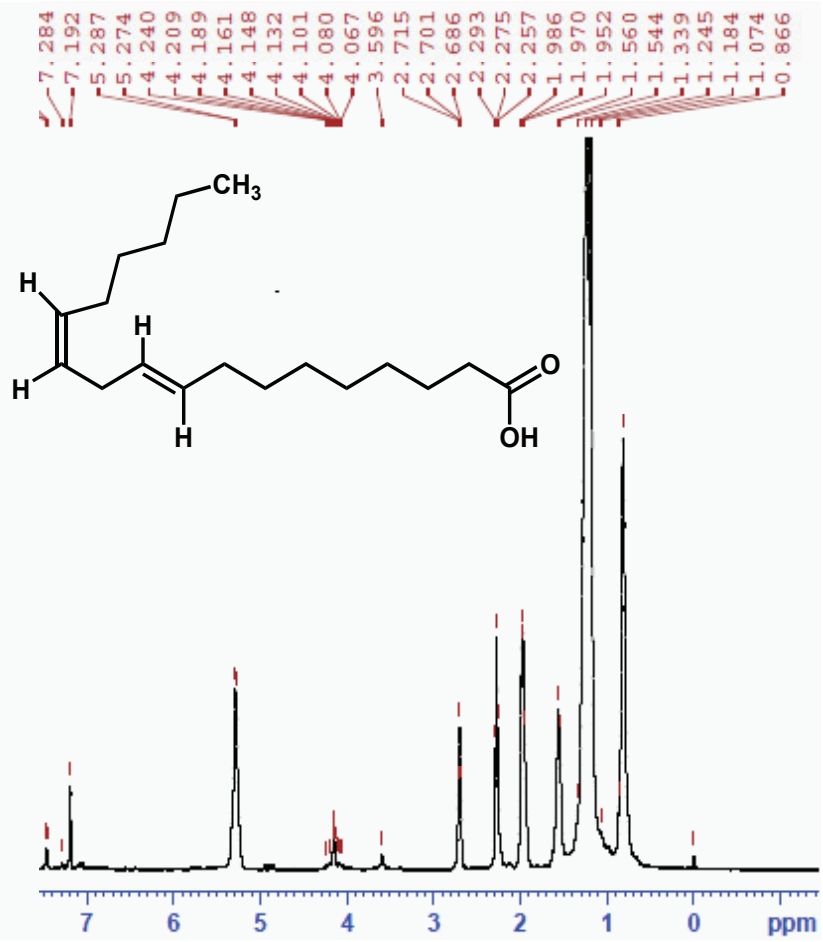

Figure 2. Chemical structure and ^{1}H NMR spectrum of 9,12-octadecadienoic acid (Z,Z) isolated from *C.haetomium cochliodes* (MW898133).

Table 4. Minimum inhibitory concentration (MIC) by the 96-well plate assay of *Chaetomium cochliodes* crude extract, column fractions and a pure isolated compound (µg/mL) against *Pectobacterium carotovorum* subsp. *carotovorum*.

Extract/compound	Minimum inhibitory concentration (MIC) mg/L
Chaetomium cochliodes extract	500
F14	32
F15	4
9,12- Octadecadienoic acid (Z,Z)	32
Ampicillin	16

extract, fractions and the isolated secondary metabolite from the endophytic fungus *C. cochliodes* (MW898133) showed strong antibacterial activity against *P. carotovorum* subsp. *carotovorum*. Similarly to our results, several fungal endophytes are known for their capacity to inhibit bacterial growth and produce compounds that have antibacterial activity (Hardoim *et al.,* 2015). For example, altersetin, an alkaloid isolated from the endophyte *Alternaria* spp., has been shown to display a strong antibacterial effect against many Gram positive bacteria (Hellwig *et al.,* 2002). An endophytic fungus, *Muscodor albus*, was reported to produce volatile compounds, such as aciphyllene, 2-butanone

and 2-methyl furan with antibiotic properties (Atmosukarto *et al.,* 2005). Furthermore, several endophytic fungi, such as *Pestalotiopsis mangiferae*, *Aspergillus* sp., *Nigrospora sphaerica* (URM-6060), *Pestalotiopsis maculans* (URM-6061) *Phomopsis* sp. and *Botryosphaeria* sp., have been described to produce secondary metabolites displaying antibacterial activity (Phongpaichit *et al.,* 2006; Pinheiro *et al.,* 2013; Subban *et al.,* 2013; Santos *et al*., 2015).

Regarding the presence of fatty acids in the *C. cochliodes* extract, fatty acids and their derivatives have been isolated by bioassay-guided fractionation from numerous plants and organisms as protectants against pathogenic bacteria (Han *et al.,* 2003; Desbois *et al.,* 2009 and 2010; Tanvir *et al.,* 2017 and 2018). According to Sumayo *et al*. (2014), linoleic acid was found to elicit induced systemic resistance (ISR) of tobacco against the bacterial soft rot pathogen, *P. carotovorum* subsp. *carotovorum*. Hexadecanoic acid ethyl ester showed antioxidant, nematicidal, pesticidal and antimicrobial activities (Farmer and Ryan, 1992; Blechert *et al.,* 1995). There are prior reports indicating the presence of fatty acids in endophytic fungi extracts and these fatty acids had been proven to be widely bioactive against gram-positive and gram-negative bacteria (Han *et al.,* 2003; Smith *et al.,* 2015; Malhadas *et al.,* 2017). Manganyi *et al*. (2019) stated that among 133 endophytic fungal strains isolated from *Pelargonium sidoides* only the isolate MHE 68, identified as *Alternaria* sp., had antibacterial activities against clinical bacteria strains, *Enterococcus faecium* and *E. gallinarum*. The chemical analysis of this *Alternaria* sp. extract indicated the presence of 9,12-octadecadienoic acid (Z,Z) and cyclodecasiloxane which, according to authors, could be accountable for the antibacterial activity.

Furthermore, many studies have exposed a relationship between the structure of fatty acids and their antimicrobial properties; unsaturated fatty acids are more effective than saturated fatty acids and the double bonds position is substantial for long chain fatty acids. (Kurihara *et al.,* 1999; Cerdeiras *et al.,* 2000; Zheng *et al.,* 2005; Won *et al.,* 2007; Hamel, 2009; Sado-Kamdem *et al.,* 2009). Likewise, our findings indicated that the most active fractions against *P. carotovorum* subsp. *carotovorum* were those containing the unsaturated fatty acids and their derivatives, while the fractions which contain saturated fatty acids were less active. This could also extend to the presence of methyl 9-*cis*,11-*trans*-octadecadienoate in F14, as the F14 had a more potent antibacterial activity compared to F15 that lacks methyl 9-*cis*,11-*trans*-octadecadienoate, given that esters have a remarkable effect on antimicrobial activity. The stereochemistry of unsaturated compounds has an important role in bioactivity as *cis*-isomers are more active than *trans*-isomers probably because the structures of *trans*-bonded unsaturated fatty acids resemble to the saturated acids and this was noticeable in our study since the active compound was a *cis* isomer (Wille and Kydonieus, 2003; Desbois and Smith, 2010).

Despite that the antibacterial mode of action of fatty acids is still poorly understood, several studies suggested that their prime target is the cell membrane; they disrupt the electron transport chain and oxidative phosphorylation. They also interfere with cellular energy production, inhibit enzyme activity, reduce nutrient uptake, causing peroxidation and auto-oxidation degradation or lysis of bacterial cells (Kurihara *et al.,* 1999; Zheng *et al.,* 2005; Won *et al.,* 2007; Hamel, 2009; Kenny *et al.,* 2009; Sado-Kamdem *et al.,* 2009).

In conclusion, the present study provides information on the isolation, identification and antagonistic activities of the five endophytic fungi (*Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. and *Curvularia* sp.), as mycelia, against three phytopathogenic bacteria (*P. carotovorum* subsp. *carotovorum*, *R. solanacearum* and *P. syringae* pv. *syringae*) with *Chaetomium* sp. being the most active against *P. carotovorum* subsp. *carotovorum*, *R. solanacearum*. In addition, the extract, fractions and a fatty acid, 9,12-octadecadienoic acid (Z,Z), isolat-

ed from *C. cochliodes* (MW898133) showed promising antibacterial activity against *P. carotovorum* subsp. *carotovorum*. The findings encourage further studies on the antibacterial mode of action and safety of endophytic fungi extracts and their secondary metabolites as a renewable source of bioactive compounds with possible application in agriculture and medicine.

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Αντιβακτηριακή δράση εκχυλισμάτων και μεταβολιτών που απομονώθηκαν από τον ενδοφυτικό μύκητα *Chaetomium cochliodes* έναντι φυτοπαθογόνων βακτηρίων

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Περίληψη Πέντε ενδοφυτικοί μύκητες, *Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Rhizopus* sp. και *Curvularia* sp., απομονώθηκαν από το Αιγυπτιακής προέλευσης ποώδες φυτό *Tribulus terrestris*, και ελέγχθηκαν ως προς την αντιβακτηριακή τους δράση έναντι τριών φυτοπαθογόνων βακτηρίων (*Pectobacterium carotovorum* subsp. *carotovorum, Ralstonia solanacearum, Pseudomonas syringae* pv. *syringae*). Ο μύκητας *Chaetomium* sp. παρουσίασε τη μεγαλύτερη αντιβακτηριακή δράση. Το στέλεχος αυτό αναγνωρίστηκε μορφολογικά και μοριακά ως *Chaetomium cochliodes* MS03 (MW898133) με βάση τη γονιδιωματική περιοχή ITS1-5.8S rRNA-ITS2. Ο μύκητας *C. cochliodes* προκάλεσε ζώνες αναστολής ανάπτυξης 15 και 8 mm του *P. carotovorum* subsp. *carotovorum* και του *R. solanacearum*, αντίστοιχα. Μετά από διεργασία ζύμωσης με τον μύκητα *C. cochliodes*, πραγματοποιήθηκε εκχύλιση με οξικό αιθυλεστέρα. Το ακατέργαστο εκχύλισμα του *C. cochliodes* έδειξε ισχυρή αντιβακτηριακή δράση έναντι του *P. carotovorum* subsp. *carotovorum* (ζώνη αναστολής ανάπτυξης= 27 mm). Βιοδραστικοί δευτερογενείς μεταβολίτες απομονώθηκαν από το ακατέργαστο εκχύλισμα με στήλη χρωματογραφίας διοξειδίου του πυριτίου (silica gel) και βιοδοκιμή. Οι ελάχιστες συγκεντρώσεις αναστολής της ανάπτυξης ήταν 500, 32 και 4 mg/L για το εκχύλισμα του *C. cochliodes*, το κλάσμα 14 και το κλάσμα 15, αντίστοιχα, έναντι του *P. carotovorum* subsp. *carotovorum*. Τα βιοδραστικά κλάσματα αναλύθηκαν με GC/MS. Η βιοδραστική καθαρή ένωση ταυτοποιήθηκε ως 9,12-οκταδεκαδιενοϊκό οξύ (Z, Z) και η χημική δομή επιβεβαιώθηκε με φασματική ανάλυση H^1 NMR και C^{13} NMR. Η ένωση που απομονώθηκε έδειξε ενθαρρυντικά αποτελέσματα για την αντιβακτηριακή δράση της έναντι του βακτηρίου *P. carotovorum* subsp. *carotovorum* με τιμή ελάχιστης συγκέντρωσης αναστολής ανάπτυξης 32 mg/L.

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