

LC-MS and GC-MS -based phytochemical perspectives and antimicrobial effects of Endophytic Fungus *Chaetomium ovatoascomatis* Isolated from *Euphorbia milii*

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Abstract

Antimicrobial activity of endophytic fungi isolated from *Euphorbia milli* was evaluated against Gram positive, Gram negative bacteria, unicellular yeast and filamentous fungi. The most active strain was selected and identified morphologically and genetically as *Chaetomium ovatoascomatis* NRC. The total ethyl acetate extract of *C. ovatoascomatis* NRC exhibited a significant antimicrobial activity against Gram negative; *Escherichia coli*, *Salmonella enteric* and fungi; *Aspergillus niger* with MIC of 62.5 ug/ml. While *n*-hexane fraction demonstrated broader activity against Gram positive; *Bacillus subtilis*, *Lactobacillus cereus*, Gram negative; *Escherichia coli* and *Salmonella enteric*, fungi; *Candida albicans* and *F. solani*. Phytochemical analysis of ethyl acetate strain extract was performed by LCMSMS analysis and *n*-hexane fraction was analyzed by GCMS. LCMSMS determined five major metabolites with potential antimicrobial activities including coumarin glycoside; esculin and flavonoids. GCMS analysis of *n*-hexane fraction tentatively identified 30 compounds, where 9,12-octadecadienoic acid methyl ester was the major compound.

Introduction

Several studies revealed that secondary metabolites of microbial origin represent unexplored sources for new bioactive compounds, where endophytes have the ability to synthesize a novel lead molecules which can be used as therapeutic agents and drug discovery (Falade et al. 2021, Abdul-Razek et al., 2020, Gunatilaka 2006, Okoye et al. 2015). Plants that contain endophytes are much healthier than those free of endophytes, since endophytes provide growth and protection for the host plant by different mechanisms (Busby et al. 2016, Rachel et al. 2022). Fungi are plant-like organisms that lack chlorophyll and are represented by several fungal species. There are 1.5 million species approximately; of these, about 74,000 are known (Hawksworth 2001). Endophytic fungi are found in all kinds of plants including trees, grasses, algae and herbaceous plants (Hyde and Soyong 2008, Bacon and white 2000). Antimicrobial activities including promising antifungal agents, as well as antioxidant of endophytic fungi attributed to synthesis of a number of secondary metabolites including phenolics, alkaloids, quinones, lignans, peptides, flavonoids, steroids, isocoumarins and flavonoids (Khalil et al., 2021, Talukdar et al., 2021). Several compounds with antimicrobial, antimalarial and anticancer potential were isolated from endophytic fungi (Hridoy et al. 2022, Dramaee et al. 2002, Li et al. 2018). Therefore, endophytes are a huge reservoir for accumulated wealth of unique chemical structures that could be exploited for treatment of numerous diseases.

Euphorbiaceae family is one of the largest families with over 330 genera and 8,000 species. Several *Euphorbia* plants have been used since ancient times as traditional medicinal herbs in India and China for treatment of different ailments as asthma, cough, tumors, and antiviral with variety chemical classes of secondary metabolites (Diop et al 2018, Ernst 2015, Olounladé 2017, Zengin 2017).

Euphorbia milli has been used in folks medicine for treating hepatitis, warts and cancer and further report including antiviral and cytotoxic activities (Chaman et al 2019, Delgado et al 2003) as well as antifungal, antinociceptive and molluscicidal properties (Opferkuch and Hecker 1982, Rauf et al 2014). The antiproliferation potential of *E. milli* using an exquisite combination of phytopharmacological and advanced computational techniques revealed that chloroform fraction of methanol extract has the highest antioxidant and a significant cytotoxicity (Talha et al 2020). Phytochemical studies revealed the presence of sterols, sesquiterpenes, triterpenes, phenolic acids, flavonoids, coumarin, alkaloid and ellagic acid (Hammad et al 2019, Hemalatha et al 2015, Marie and Ronnie 2018).

The previous studies of endophytic fungi isolated from *Euphorbia* species demonstrated that *Chaetomium strumarium* isolated from *E. hirta* (Anitha and Mythili 2017), *Fusarium* sp. from *E. peginensis* (Dai et al 2008) and laccase enzyme producing fungi from *E. milli* (Rao et al 2018).

Endophytic fungi isolated from medicinal plants are still unexplored in the discovery of bioactive metabolites and limited numbers of endophytes from medicinal plants have been reported (Uzma et al., 2019). Therefore, scientists of different disciplines are focused on the biological evaluation of secondary metabolites produced by fungi. There is only one report including the laccase enzyme production ability of endophytic fungi from *E. milli* (Rao et al 2018) however, antimicrobial and identification of corresponding metabolites weren't previously reported. Hence, the present study aimed to evaluate the antimicrobial potential of endophytic fungus strains isolated from *E. milli* and to identify the main secondary metabolites by GCMS and LCMSMS techniques.

Materials And Methods

Plant material

The plant sample of *Euphorbia milli* was collected from Orman Garden (30°1'45"N, 31°12'47"E) Giza, Egypt. Samples were placed in polyethylene bags, labeled, transported to the laboratory, and refrigerated at 4 °C.

Isolation and purification of endophytic fungi

Fresh, healthy parts of leaves and stem of *Euphorbia milli* have been cut into pieces and placed in Potato dextrose agar (PDA). After a week of incubation at 28°C temperature, when fungal mycelium tips were observable, each hyphal tip was transferred onto a fresh PDA slant. For purification of the fungal strains, this step was repeated several times until the colony was deemed uniform. All pure isolates were numbered and stored on the surface of PDA slants at 4° C (Guiraud 1998, Hanlin 2000). Fungal characterizations were made according to their macroscopic and microscopic characteristics. The cultural and morphological characteristics of the isolate were compared with species descriptions reported in the standard

taxonomic manuals to identify the fungal genera and species. Light micrographs were captured using an Olympus CX40 RF100 light microscope coupled to a Canon A620 digital camera (Hanlin 2000). Based on the antagonistic screening; the most active fungal isolate was selected for biological activity studies.

Genotypic identification of the most active fungal isolates

Sequencing and molecular phylogenetics

Based on the preliminary screening; the most potent fungal isolate representing the highest antimicrobial activity was inoculated on PDA and colony morphology was recorded after 48 h of incubation at 25 °C. Microscopic observations were made on the fungus stained with lactophenol cotton blue under the microscope. The molecular characterization was performed through isolation of 18S rDNA. The ITS1–5.8S–ITS2 genomic region was amplified from genomic DNA, using the forward primer ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and the reverse primer ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Hamed et al. 2015; White et al. 1990). In short, the reaction conditions for polymerase chain reaction are performed with 2 µl of 10 X Taq PCR buffer, 1.6 µl from 2.5 mM dNTP mixture, 1 µl of both forward and reverse primers (10 pmol/µl), 2 µl template genomic DNA (20 ng/µl), 0.2 µl from KOMATaq. (2.5 U/µl), and distilled water (HPLC grade) up to 20 µl. The reaction mixture was as follows: initial denaturation at 95 °C for 1 min, 30 cycles denat. 95 °C for 30 s, annealing 55 °C for 2 min, extension 68 °C for 1.5 min, final extension 68 °C for 10 min for 1 cycle. After completion, the PCR products were electrophoresed on 1% agarose gels, containing ethidium bromide (10 mg/ml), to ensure that a fragment of the correct size had been amplified. The amplification products were purified by Montage PCR clean up kit (Millipore). The purified PCR products were sequenced, using the 2 primers that were used before in the PCR reaction. Sequencing was performed by big Dye Terminator Cycle Sequencing kit V.3.1 (Applied Biosystems, USA). PCR products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied Biosystems, USA) at the Macrogen, INC, Seoul Korea.

Phylogenetic data were obtained by aligning the nucleotides of different 18S rRNA retrieved from BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), MEGA6 BLAST and fast minimum evolution method were used for tree formation. The further parameters in the tree were inserted through MEGA-X software, and the sequence obtained was submitted to NCBI database

Fermentation and crude extract preparation

The most active fungal endophyte was cultivated on PDA medium and incubated at 28°C, statically. After 21 days of incubation, secondary metabolites were extracted by soaking in methanol 70% overnight. The cultures were filtered under vacuum through filter paper (Whatman No. 4; Brentford, UK). The extraction process by ethyl acetate was carried out three times for complete extraction. The organic phase was concentrated to dryness under reduced pressure using a rotary evaporator to obtain the crude fungal extract. A part of crude ethyl acetate extract was partitioned using *n*-hexane and the other part was fractionated on column chromatography using silica gel to afford six sub-fractions (F1-F6). The six fractions were obtained by applying ethyl acetate extract on silica gel column using *n*-hexane/ethyl acetate in the order of increasing polarity; 5, 10, 20, 30, 40, and 50 % respectively. TLC was used to collect the similar fractions and detection was performed under UV lamp (254 and 356 nm) as well as spraying reagents.

In vitro antibacterial and antifungal assay

Antimicrobial activity was measured by an agar diffusion method against Gram positive bacteria (*Bacillus subtilis* ATCC 6633), Gram negative bacteria (*Escherichia coli* ATCC 25922), unicellular yeast fungi (*Candida albicans* ATCC 10321), and filamentous fungi (*F. solani* NRC15 & *F. oxysporum*). Bacteria and yeast strains were obtained from the American Type Culture Collection while, fungi were obtained from the culture collection of the Chemistry of Natural and Microbial Products Department, National Research Centre, Egypt. Thiophenicol (Thiamphenicol, Sanofiaventis, France) and Treflucan (Fluconazole, Egyptian Pharmaceutical Industries Company EIPICO) were used as antibacterial and antifungal positive controls, respectively, in a concentration of 100µg/disc and DMSO was used as a negative control. The discs (1mg/5 µl) were applied on inoculated nutrient agar and PDA media plates and incubated for 24 h at 30 °C for bacteria and 72 h at 28 °C for fungal strains, respectively. The zone of inhibition was recorded and compared with the positive control treatments.

MIC of endophytic strains and their fractions.

Endophytic strain *C. ovatoascomatis* NRC, *n*-hexane fraction, and sub-fractions F1-F6, were screened *in vitro* against Gram positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC29213 & *Lactobacillus cereus* ATCC14579), Gram negative bacteria (*Escherichia coli* ATCC 25922 & *Salmonella enteric* ATCC 25566), unicellular yeast fungi (*Candida albicans* ATCC 10321), and filamentous fungi (*Aspergillus niger* NRC53, *F. solani* NRC15 & *F. oxysporum*). The minimum inhibitory concentration of each sample against tested microorganisms was determined by microtitre broth dilution method: MIC was performed on the extracts, fractions and isolated compounds by serial dilution as previously described (Eloff 1998, Teh et al 2017) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The serial dilutions from the stock solution were made ranges 500, 250, 125 and 62.5 using Nutrient broth and Potato dextrose broth for Bacteria and fungi respectively in 96-well microplates. The bacterial suspension containing approximately 5×10^5 colony-forming units/mL was prepared from a 24 h culture plate. From this suspension, 100 µl was inoculated into each well. A sterility control well and a growth control well were also studied for each strain. Thiophenicol and Treflucan were used as positive controls for antibacterial and antifungal activity in a concentration of 500, 250, 125 and 62.5 µg. The microtiter plates were incubated at 37 °C,

24 h for bacteria and 48 h for fungi. After incubation; the MIC values visually determined. The lowest concentration of each extract displaying no visible growth was recorded as the minimum inhibitory concentration.

GCMS analysis

GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperature was set at 280 °C. The oven temperature was programmed at an initial temperature 50 °C (hold 2 min) to 150 °C at an increasing rate of 7 °C/min. then to 270 at an increasing rate 5 °C/min (hold 2min) then to 310 as a final temperature at an increasing rate of 3.5 °C/min (hold 10 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA), WILLY library data of the GC/MS system.

LC-ESI-TOF-MS Analysis

Sample Preparation

A stock solution of the extract was prepared from 50 mg of the lyophilized ethyl acetate extract dissolved in 1000 µL of the solvent mixture composed of water: methanol: acetonitrile (H₂O:MeOH: ACN) in a ratio of 2:1:1. Complete solubility of stock solution was obtained by sample vortexed and ultra-sonication at 30 kHz for 10 min. An aliquot, 20 µL of the stock solution was again diluted with 1000 µL of the H₂O: MeOH: ACN (2:1:1) and centrifuged at 10,000 rpm for 5 min, and 10 µL (1 µg/mL) was used for injection. The LC-MS analysis was also performed for blank and quality control samples/internal standard (IS) for confidence in the experiment. The sample was injected in negative mode.

Instruments and Acquisition Method

Small molecules were separated on an ExionLC system (AB Sciex, Framingham, MA, USA) connected with an autosampler system, an in-line filter disks pre-column (0.5 µm x 3.0 mm, Phenomenex, Torrance, CA, USA), and an Xbridge C18 (3.5 µm, 2.1 x 50 mm) column (Waters Corporation, Milford, MA, USA) was maintained at 40 °C, and at a flow rate of 300 µL/min was utilized. The mobile phase consisted of solution (A): 5 mM ammonium formate in 1% methanol, pH adjusted to 8.0 by using sodium hydroxide, and solution (B): consisting of acetonitrile (100%). The gradient elution was performed with the following program: 0–20 min, 10% B; 21–25 min, 90% B; 25.01–28 min, 10% B; and then 90% B for equilibration of the column. The mass spectrometry (MS) was performed on a Triple TOF 5600+ system equipped with a Duo-Spray source operating in the ESI mode (AB SCIEX, Concord, ON, Canada). The sprayer capillary and declustering potential voltages were -4500 and -80 V. The source temperature was set at 600° C, the curtain gas was 25 psi, and gas 1 and gas 2 were 40 psi. The collision energy -35 V (negative mode) with CE spreading 20 V and the ion tolerance for 10 ppm were used. The TripleTOF5600+ was operated using an information dependent acquisition (IDA) protocol. Batches for MS and MS/MS data collection were created using Analyst-TF 1.7.1. The IDA method was used to collect full-scan MS and MS/MS information simultaneously. The method consisted of high-resolution survey spectra from 50 to 1100 m/z and the mass spectrometer was operated in a pattern where a 50-ms survey scan was detected. Subsequently, the top 15 intense ions were selected for acquiring MS/MS fragmentation spectra after each scan (Fayek et al 2019).

LC-MS Data Processing

MS-DIAL 3.70 open-source software (Tsugawa et al 2015) was used for non-targeting, small molecule comprehensive analysis of the sample. According to the acquisition mode, ReSpec negative (1573 records) databases were used as reference databases. The search parameters were set as MS1 and MS2 mass tolerance: 0.01 Da and 0.05 Da for data collection, for peak detection; minimum peak height: 100 amplitude, mass slice width: 0.05 Da, smoothing level: 2 scans, minimum peak width: 6 scans, for identification MS1 and MS2 tolerance: 0.2Da/each, for alignment; retention time tolerance: 0.05 min and MS1 tolerance: 0.25 Da. The MS-DIAL output was used to run again on PeakView 2.2 with the MasterView 1.1 package (AB SCIEX) for feature (peaks) confirmation from Total Ion Chromatogram (TIC) based on the criteria; aligned features having Signal-to-Noise ratio greater than 5 and intensities of the sample: blank greater than 5.

Results And Discussion

Microorganisms isolation and antimicrobial screening

A total of five fungal isolates belonging to genera; *Alternaria*, *Aspergillus*, *Fusarium*, and *Chaetomium* were isolated from *Euphorbia milii*. Antimicrobial assays of microorganism's fungal isolates are shown on table 1. The ethyl acetate extract of fungal isolate coded (MP2) *Chaetomium ovatoascomatis* revealed a promising activity against Gram negative *E. coli* ATCC25922, and Gram positive *B. subtilis* with inhibition zones 15 and 22 mm respectively and showed a moderate activity against tested yeast *C. tropicalis* with inhibition zone 7 mm as well as considerable effect against tested fungal strains *F. solani* and *F. oxsporum* with inhibition zone 7 and 13 mm, respectively. Based on these obtained results of the screening process the strain *Chaetomium ovatoascomatis* was selected for phytochemical investigation using LCMS and GCMS analysis.

Table 1: Antimicrobial assays of microorganism's fungal isolates

Pathogenic	Inhibition zone diameter (mm)				
	G+ bacteria	G- bacteria	Yeast	Fungi	
	<i>B. subtilis</i>	<i>E. coli</i>	<i>C. tropicalis</i>	<i>F. solani</i>	<i>F. oxysporium</i>
Isolate Code					
MP1*	7	15	7	8	7
MP1(WB**)	7	19	6	8	10
MP2*	22	15	7	8	13
MP2 (WB**)	21	12	7	22	7
MP3*	9	10	N. A.	13	7
MP3 (WB**)	7	13	N. A.	8	6
PP1*	6	6	8	15	6
PP1 (WB**)	7	15	7	8	6
PP2*	7	12	7	7	6
PP2(WB**)	7	13	6	N. A.	7
PC	29	10	14	15	11
Notes:					
* Refers to that the fungal extract from fungi cultivated on PDA					
** Refers to that the fungal extract from fungi cultivated on Media Contain wheat bran as carbon source					
The inhibition zone diameter expressed in (mm).					
Thiophenicol and Treflucan were used as positive controls at a concentration of 100 µg/disc, DMSO was used as negative control.					

Identification of the most active isolates based on molecular phylogenetic analysis

Morphological identification illustrated the formation of colonies on PDA fast growing, reaching around 7 cm diam. in 7 days after incubation at 25 °C, colony sparse, rough, raised with entire edge, thinly hairy, entire, well defined at margin; colony from above; rough, sparse, whitish; from below, pale brown,. Sporulate on PDA these morphological characteristics agreed with (Raza et. al., 2019). The phylogenetic analysis of the isolated fungal strain MP2 *Chaetomium ovatoascomatis* revealed 98% identity with ITS sequences of rRNA genes of related species using BLAST programs (Fig. 1C). (Selim et al., 2014).

Phytochemical analysis

Endophytic fungi are a rich source for secondary metabolites such as coumarins, terpenoids, glycosides, flavonoids, with numerous antifungal, anticancer and antibacterial compounds. Terpenoids showed several pharmacological effects including antibacterial, antitumor, antiviral, anti-inflammatory as well as cardiovascular diseases treatment (Yang et al. 2020). Flavonoids possess antifungal activity through different mechanisms as mitochondria and plasma membrane destruction (Al Aboody, and Mickymaray 2020). Antibacterial effect of flavonoids occurred through harming the cytoplasmic membrane, hindering the building of nucleic acids (Zhenyou et al. 2022). A number of coumarins with antioxidant, antifungal, anticancer and antibacterial activities were produced from endophytic fungi (Singh et al., 2021). A phytochemical analysis of ethyl acetate extract of *Chaetomium ovatoascomatis* NRC and its non polar hexane fraction was performed by LCMSMS and GCMS respectively.

LCMSMS of *Chaetomium ovatoascomatis* NRC

Ethyl acetate extract of *Chaetomium ovatoascomatis* NRC was analyzed by LCMSMS (Fig.2) where, 36 metabolites were identified (Table 2). Coumarine glycoside represented by esculin compound was the major constituent with 31.8% followed by flavonoids with 28.1%. Apigenin was the major flavonoid with 14.3% followed by naringenin with 5% and luteolin with 4% while catechine was 3.3% and acacetin was 1.5%. Gibberellic acid diterpene was detected by 5.5% and hydroxycinnamic acid glycosides represented by 1-*O-D*-glucopyranosyl sinapate (1.2%) was determined. Further compounds were identified in lower concentration are shown in table 2. Mass spectra of the major identified compounds are shown in figure 3.

Table 2: LCMSMS of *Chaetomium ovatoascomatis* NRC, Adduct ion as [M-H]⁻

	RT (minute)	Area %	Molecular Formula (Precursor m/z)	Error (ppm)	Compound Identify
1	1.08	0.56	C ₈ H ₁₄ O ₄ (173.082)	-0.7	Suberic acid
2	1.13	0.18	C ₄ H ₄ O ₄ (115.0025)	4.1	Maleic acid
3	1.20	0.23	C ₇ H ₆ O ₃ (137.023)	1.8	Salicylic acid
4	1.32	0.52	C ₆ H ₁₄ O ₆ (181.0716)	0.3	Galactitol
5	1.36	0.1	C ₇ H ₆ O ₄ (167.032)	8.9	3,4-Dihydroxyphenylacetic acid
6	4.09	0.04	C ₁₀ H ₇ NO ₃ (188.0373)	-7.8	Kynurenic acid
7	5.27	0.27	C ₁₁ H ₂₀ N ₂ O ₆ (275.1044)	-0.9	L-Saccharopine
8	5.90	1.5	C ₇ H ₁₂ N ₂ O ₃ (171.102)	2.1	Glycyl-L-proline
9	6.66	1.2	C ₁₇ H ₂₂ O ₁₀ (385.0583)	-0.9	1-O-b-D-glucopyranosyl sinapate
10	6.97	0.15	C ₂₀ H ₁₈ O ₁₁ (433.0175)	9.3	Quercetin-3-D-xyloside
11	7.23	0.03	C ₁₀ H ₁₄ N ₂ O ₅ (241.0567)	-5.4	Thymidine
12	7.33	0.4	C ₂₁ H ₂₀ O ₁₁ (447.0908)	3.7	Quercitrin
13	7.62	0.07	C ₉ H ₁₃ N ₃ O ₅ (242.1765)	-0.5	Cytidine
14	7.50	0.78	C ₂₁ H ₂₀ O ₁₀ (431.095)	3.2	Kaempferol-3-O-alpha-L-rhamnoside
15	7.73	0.3	C ₁₆ H ₁₂ O ₇ (315.0169)	-2.3	3'-Methoxy-4',5,7-Trihydroxyflavonol
16	8.02	0.03	C ₁₀ H ₉ NO ₂ (174.0531)	14.1	1-methoxyindole-3-carbaldehyde
17	8.05	0.05	C ₉ H ₆ O ₄ (177.09)	11.7	6,7-DIHYDROXYCOUMARIN
18	8.04	0.05	C ₁₀ H ₁₂ N ₄ O ₅ (267.0323)	20.0	Inosine
19	8.11	0.75	C ₇ H ₁₅ NO ₂ (144.0433)	6.1	L-beta-Homoisoleucine
20	8.37	0.11	C ₆ H ₅ NO ₃ (138.0168)	11.2	<i>P</i> -Nitrophenol
21	8.52	0.02	C ₉ H ₈ O ₄ (179.0349)	-0.4	Caffeic acid
22	8.58	0.27	C ₁₈ H ₃₀ O ₂ (277.0596)	40.9	gamma-Linolenic acid
23	8.89	0.04	C ₂₇ H ₃₀ O ₁₇ (625.1782)	6.1	Quercetin-3,4'-O-di-beta-glucopyranoside
24	9.2	4.0	C ₁₅ H ₁₀ O ₆ (285.0399)	1.1	Luteolin
25	9.32	0.18	C ₉ H ₁₁ NO ₄ (196.0946)	14.0	3,4-Dihydroxy-L-phenylalanine
26	9.49	0.16	C ₁₅ H ₁₀ O ₇ (301.0347)	0.7	Quercetin
27	9.84	0.038	C ₄₂ H ₆₂ O ₁₆ (821.2292)	1.0	Glycyrrhizate
28	10.14	5.5	C ₁₅ H ₁₂ O ₅ (271.0251)	-0.9	Naringenin
29	10.35	0.37	C ₁₈ H ₁₆ O ₈ (359.1476)	3.7	rosmarinic acid
30	10.51	14.3	C ₁₅ H ₁₀ O ₅ (269.046)	-0.9	Apigenin
31	11.86	2.95	C ₁₂ H ₂₄ O ₁₁ (343.1548)	0.9	Maltitol
32	12.8	3.3	C ₁₅ H ₁₄ O ₆ (289.1434)	0.9	Catechin
33	12.88	0.09	C ₁₆ H ₁₄ O ₆ (301.1837)	0.1	Hesperetin
34	12.99	31.8	C ₁₅ H ₁₆ O ₉ (339.0492)	4.9	Esculin
35	13.42	5.5	C ₁₉ H ₂₂ O ₆ (345.1719)	-1.0	Gibberelin A3

36	14.05	1.5	C ₁₆ H ₁₂ O ₅ (283.062)	-1.0	Acacetin
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GCMS analysis

GCMS analysis of the non polar, hexane fraction was tentatively identified for 30 compounds (Table 3) where 9,12-octadecadienoic acid methyl ester was the major compound (35.6%) followed by 9-octadecenoic acid methyl ester (16.1%) then hexadecanoic acid methyl ester (13.9%). Further compounds were tentatively identified as 2-ethyl-4-methyl-2,5-dihydrooxazole, l-Limonene, 2-Methyl-2(1,4-dimethylcyclohexyl) cyclopentanone and Octadecanoic acid, methyl ester in the range of 4.94 to 3.10%.

Table 3: GCMS of *n*-hexane fraction

	RT	Probability	Compound Name	Area %	Mol. Wt	Formula
1	5.18	4.61	2-ethyl-4-methyl-2,5-dihydrooxazole	4.94	113	C ₆ H ₁₁ NO
2	5.82	90.07	2,5-Dibromo-1,4-dihexadecylbenzene	0.11	682	C ₃₉ H ₆₉ Br ₂
3	9.18	22.95	l-Limonene	4.39	136	C ₁₀ H ₁₆
4	22.35	40.39	Phenol,2,4-bis(1,1-dimethylethyl)	0.30	206	C ₁₄ H ₂₂ O
5	24.40	12.38	3-Butenamide	0.16	85	C ₄ H ₇ NO
6	27.40	47.00	2-Methyl-2-(1,4-dimethylcyclohexyl)cyclopentanone	3.98	208	C ₁₄ H ₂₄ O
7	28.19	11.06	1-Heptadecene	0.16	238	C ₁₇ H ₃₄
8	28.66	9.86	2-Pyridinecarboxylic acid, 2-acetylhydrazide	0.14	179	C ₉ H ₉ N ₃ O ₂
9	28.94	16.06	Pentadecanoic acid methyl ester	0.28	256	C ₁₆ H ₃₂ O ₂
10	29.89	16.64	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl)ester	0.26	278	C ₁₆ H ₂₂ O ₄
11	30.46	8.68	10-Undecenoic acid methyl ester	0.16	198	C ₁₂ H ₂₂ O ₂
12	30.91	41.85	Hexadecanoic acid methyl ester	13.90	270	C ₁₇ H ₃₄ O ₂
13	31.73	5.07	1,2-Benzenedicarboxylic acid, monobutyl ester	0.51	222	C ₁₂ H ₁₄ O ₄
14	32.09	21.28	4-oxo, 2-hexenal-7	0.50	112	C ₆ H ₉ O ₂
15	32.19	21.66	Pentadecanoic acid, 4,6,10,14-tetramethyl, methyl ester	0.21	312	C ₂₀ H ₄₀ O ₂
16	32.35	8.39	9-Dodecenoic acid, methyl ester,	0.18	212	C ₁₃ H ₂₄ O ₂
17	32.79	36.35	Heptadecanoic acid methyl ester	0.90	284	C ₁₉ H ₃₆ O ₂
18	33.63	3.36	17-Pentatriacontene	0.13	490	C ₃₅ H ₇
19	33.87	9.74	1-Heptadecanol	0.18	256	C ₁₇ H ₃₆ O
20	34.08	31.32	9,12-Octadecadienoic acid (Z,Z) methyl ester	35.62	294	C ₁₉ H ₃₄ O ₂
21	34.16	10.84	9-Octadecenoic acid (Z),methyl ester	16.09	296	C ₁₉ H ₃₆ O ₂
22	34.59	29.00	Octadecanoic acid, methyl ester	3.10	298	C ₁₉ H ₃₉ O ₂
23	35.29	95.12	Cyclohexane 1,3,5-trimethyl-2-octadecyl	0.12	378	C ₂₇ H ₅₄
24	40.16	93.16	Tetratertbutyl 2,6-di(3-propenyl) 3,7-dimethoxybicyclo[3.3.0]octa-3,7-diene-2,4,6,8-dicarboxylate	0.13	646	C ₃₆ H ₅₄ O ₁₀
25	40.56	4.70	1-Hexanol,2-ethyl	0.22	130	C ₈ H ₁₈ O
26	41.49	31.23	Di(2-ethylhexyl)phthalate	9.70	390	C ₂₄ H ₃₈ O ₄
27	42.05	4.55	Undecane	0.11	156	C ₁₁ H ₂₄
28	43.49	5.25	Docosane	0.12	310	C ₂₂ H ₄₆
29	47.02	5.71	Octanal	0.15	128	C ₈ H ₁₆ O
30	47.30	3.43	1-Decanol,2-hexyl	0.14	242	C ₁₆ H ₃₄ O

Antibacterial activity

Endophyte fungi represent interesting sources of novel bioactive compounds which can be utilized for pharmaceutical, medicinal and agricultural applications (Manganyi and Ateba 2020). Several studies on fungal endophytes have been reported significant antimicrobial activity due to the

presence of different classes of bioactive molecules (Rao et al. 2015, Rodriguez et al. 2009). In the present study, ethyl acetate extract of *Chaetomium ovatoascomatis* NRC, *n*-hexane fraction and sub-fractions (1F-5F) were evaluated against Gram positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC29213& *Lactobacillus* sp.), Gram negative bacteria (*Escherichia coli* ATCC 25922 & *Salmonella enteric* ATCC 25566), unicellular yeast fungi (*Candida albicans* ATCC 10321), and filamentous fungi (*Aspergillus niger* NRC53, *F. solani* NRC15 & *F. oxysporum*). Antibacterial results (Table 4) revealed a promising activity of ethyl acetate extract of *Chaetomium ovatoascomatis* against Gram negative (*E. coli* ATCC25922, *Salmonella enteric* ATCC 25566) and *Aspergillus niger* NRC53 with MIC of 62.5ug and considerable effect against Gram positive (*Staphylococcus aureus* ATCC29213, *Lactobacillus cereus* ATCC14579) and *Candida albicans* ATCC 10321 with MIC value of 125.0ug. The most significant activity was recorded for *n*-hexane and F2 fractions against Gram positive, Gram negative, yeast and filamentous fungi with MIC of 62.5 ug for both fractions. Whereas *n*-hexane has higher activity against *F. solani* NRC15 while F2 is more effective against *Aspergillus niger* NRC53. F4 fraction showed its activity against two of Gram positive (*Bacillus subtilis* ATCC 6633, *Lactobacillus cereus* ATCC14579.), two Gram negative (*Escherichia coli* ATCC 25922, *Salmonella enteric* ATCC 25566) and *Aspergillus niger* NRC53. While F1 exhibited antibacterial activity against Gram positive (*Staphylococcus aureus* ATCC29213, *Bacillus subtilis* ATCC 6633), and Gram negative (*Escherichia coli* ATCC 25922, *Salmonella enteric* ATCC 25566). F3, F5 and F6 showed lower considerable antimicrobial activities than the previous fractions. The antimicrobial activity of ethyl acetate extract of *Chaetomium ovatoascomatis* is due to the presence of phenolic compounds represented by esculin (31.8%) and flavonoids (28.1%) as major chemical constituents. These in consistent with the previous effective antibacterial activity of coumarin and isocoumarin derivatives against *E. coli* (Ding et al., 2019, Singh et al., 2021) as well as significant antimicrobial activities of flavonoids (Zhenyou et al. 2022, Al Aboody, and Mickymaray 2020). While antimicrobial results of *n*-hexane could attributed to the presence of 9,12-octadecadienoic acid methyl ester and 9-octadecenoic acid methyl as major constituents which are in consistent with the previous reported activity of fatty acid methyl esters (Chandrasekaran et al., 2008, Agoramoorthy et al., 2007). Currently, chromatographic identification of the obtained fractions is proceeding to determine the chemical constituents and rationalize their antimicrobial activities.

Table 4: MIC of fungal endophyte strain and their fractions

	G+ve			G-ve		Yeast	Fungi		
	<i>Staphylococcus aureus</i> ATCC29213	<i>Bacillus subtilis</i> ATCC 6633	<i>Lactobacillus</i> sp.	<i>Escherichia coli</i> ATCC 25922	<i>Salmonella enteric</i> ATCC 25566	<i>Candida albicans</i> ATCC 10321	<i>Aspergillus niger</i> NRC53	<i>F. solani</i> NRC15	<i>F. oxysporum</i>
<i>C.ovatoascomatis</i>	125	250	125	62.5	62.5	125	62.5	250	250
Ethyl acetate									
<i>n</i> -hexane	250	62.5	62.5	62.5	62.5	62.5	125	62.5	62.5
F1	62.5	62.5	125	62.5	62.5	125	500	125	125
F2	250	62.5	62.5	62.5	62.5	62.5	62.5	125	62.5
F3	125	NA	NA	125	NA	250	125	NA	NA
F4	250	62.5	62.5	62.5	62.5	250	62.5	125	125
F5	250	62.5	NA	62.5		500	62.5	NA	NA
F6	125	NA	NA	62.5	NA	250	250	NA	NA
PC	125	125	125	125	125	125	125	125	125
*NA Not Detected									
PC: Positive Control									

Conclusion

Chaetomium ovatoascomatis NRC strain isolated herein for the first time from *E.milli* and showed a promising antimicrobial activity against tested pathogens. *n*-hexane and sub-fractions of the strain exhibited to broader activity against Gram positive and Gram negative bacteria, yeast and filamentous fungi with MIC value more than the positive control. LCMSMS of ethyl acetate extract of *Chaetomium ovatoascomatis* revealed the presence of coumarin glycoside; esculin as a major phenolic with potential antimicrobial activity. GCMS of *n*-hexane showed 9,12-octadecadienoic acid methyl ester as a mjr component with expected antimicrobial activity. The obtained promising antimicrobial activity of *Chaetomium ovatoascomatis* and its fractions encourage for further *in-vivo* study to be applied in pharmaceuticals.

Abbreviations

E. milli
Euphorbia milli

GCMS
Gas Chromatography/Mass spectrometry
LCMS/MS
Liquid Chromatography with tandem mass spectrometry
MIC
Minimum Inhibition Concentration
NRC
National Research Centre

Declarations

The authors have no competing interests to declare that are relevant to the content of this article.

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Author Contributions

*"All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by **Kamel H. Shaker, Heba-tollah M. Sweelam, Amal Zaki, Moustafa M. Zohair and Warda E. Ashour**. The first draft of the manuscript was written by **Kamel H. Shaker** and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."*

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request."

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Figures

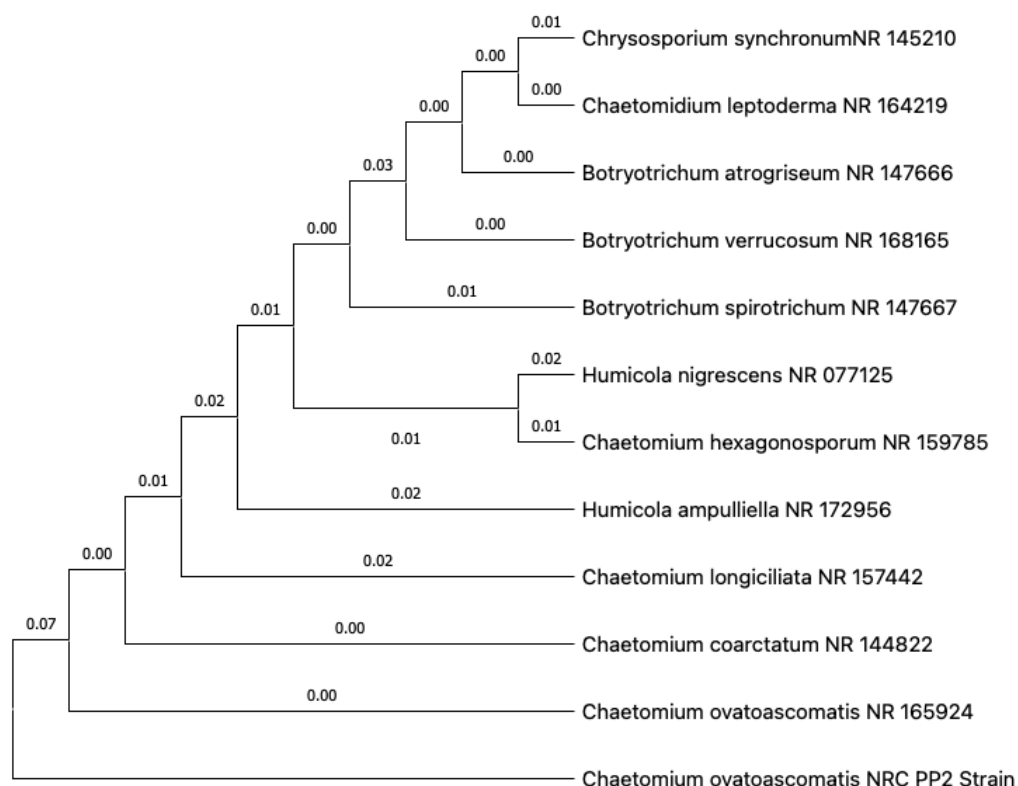


Figure 1

Phylogenetic analysis of fungal strains

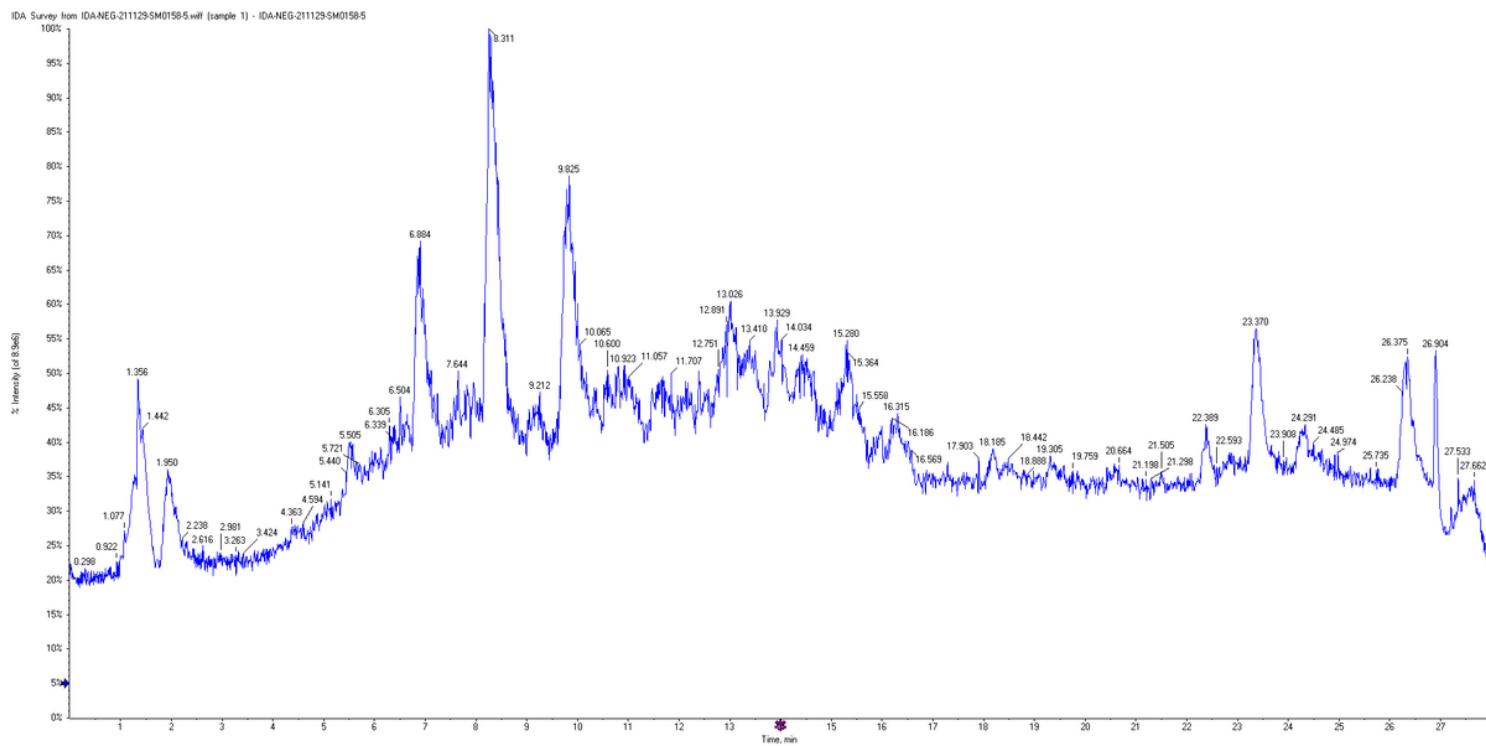


Figure 2

LCMSMS of ethyl acetate extract of *Chaetomium ovatoascomatis* NRC

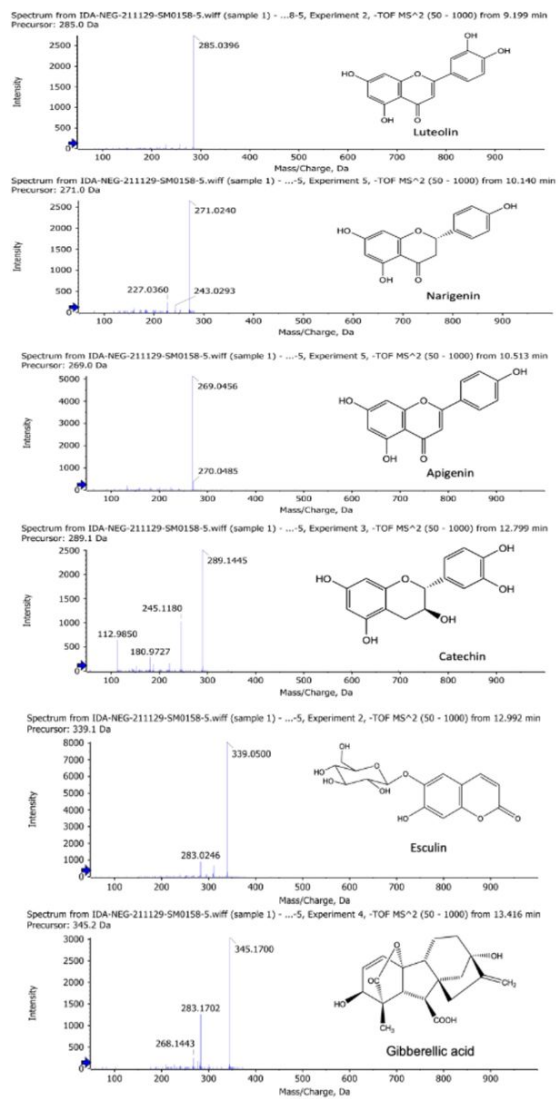


Figure 3

LCMSMS of major identified compounds