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Original Article

Biological Activity and GC-MS/MS Analysis of Extracts of Endophytic Fungi Isolated from *Eichhornia crassipes* (Mart.) Solms

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Abstract

Introduction: Endophytic fungi are good sources of bioactive compounds that are exclusive to their hosts. *Eichhornia crassipes* plant produces different bioactive compounds. The aim of the present study is to isolate and identify endophytic fungi that reside in *Eichhornia crassipes* tissues, and to evaluate the biological activities of their extracts.

Materials and Methods: Endophytic fungal spp. were isolated from leaves and petioles of *Eichhornia crassipes*, identified and then extracted. The ethyl acetate extracts were tested against bacteria, fungi, hepatitis B virus and *Schistosoma mansoni* cercariae. The chemical composition of these extracts was determined by Gas chromatography-mass spectrometry (GC- MS/MS) analysis.

Results: We found that four fungal spp. were dominant in *Eichhornia crassipes*; they were molecularly identified as *Aspergillus flavus* OM758315*, Aspergillus fumigatus OM688980, Aspergillus welwitschiae* OM758326 and *Corynascus sepedonium* OM688206, with *A. flavus* as the most frequent. The ethyl acetate extract of the four fungal spp. showed pronounced antimicrobial effects, whereas the highest antiviral effect on hepatitis B virus was that of *A. flavus* followed by *A. fumigatus* extracts. All the tested extracts were cercaricidal to *Schistosoma mansoni* cercariae, where *A. flavus* was the most effective. GC- MS/MS analysis indicated the presence of various bioactive compounds.

Conclusions: *Aspergillus flavus, Aspergillus fumigatus, Aspergillus welwitschiae* and *Corynascus sepedonium* as endophytes of *Eichhornia crassipes* showed promising antimicrobial, antiviral and cercaricidal properties.

Keywords: Antiviral*, Aspergillus,* cercaricidal, *Eichhornia crassipes*, Endophytes, GC-MS/MS

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Introduction

Eichhornia crassipes (Mart.) Solms (water hyacinth) is a ubiquitous aquatic free floating herb that is classified under family Pontederiaceae.[1] Despite being a very productive plant, it is known to be the worst aquatic weed.[2] It spread from Brazil to tropical and subtropical regions,[3] and reached Africa where its high densities resulted in many problems. Its mat blocks waterways and interferes with irrigation and agriculture activities,[4] and hence adversely affects aquatic systems, economic projects and eventually humans.[5] Nevertheless, water hyacinth was found to act as a bio-filter, heavy metals adsorbent,[6,7] and bioactive metabolites producer.[8] It produces a variety of compounds which showed antimicrobial,[9-11] wound healing,[12,13] antioxidant,[5,14,15] antitumour,[16] and larvicidal activities.[17] Phytochemical studies showed that the plant contains tannins, flavonoids, alkaloids and saponins.[18]

Endophytic fungi belong to mitosporic and meiosporic ascomycetes that asymptomatically reside in the internal tissues of plants beneath the epidermal cell layer, where they colonize healthy and living tissue via quiescent infections.[19] They are very promising sources of novel biologically active compounds that are exclusive to those of their host plants,[20] and play a role in helping both endophytic fungi and their host plants to tolerate biotic and abiotic stresses. These bioactive secondary metabolites can be investigated and applied as antiparasitic and antimicrobial agents.[21-23] This continuous need to search for new substances of biological origin that can act as antibacterial, antifungal, and antiviral agents[24,25] is a consequence of the ability of pathogenic microorganisms to develop new mechanisms to resist the effect of antibiotics. In addition, many studies have been carried out to investigate the potentiality of new compounds from natural sources to act as larvicidal or antiparasitic agents for combating infectious and endemic diseases such as schistosomiasis.[25-27] So, the aim of the present work is to isolate and identify some of the endophytic fungi of

Eichhornia crassipes, investigate the biological activities of these fungal extracts, and determine the major compounds in the most effective extracts.

Materials and Methods

Isolation of Endophytic Fungi

Eichhornia crassipes (Mart.) Solms plant was collected from watercourses in Gharbeya Governorate, Egypt. To ensure that the resulting fungi are the plant endophytes, healthy ones were selected for the study. Leaves and petioles were separated, firstly washed with tap water, and secondly surface sterilized with 70% ethanol, 4% sodium hypochlorite, and 70% ethanol for one, three and one min, respectively. Finally, they were rinsed twice, dried, and cut into smaller pieces under sterile conditions,[28] and then placed onto malt extract agar media containing chloramphenicol to prevent bacterial growth. Each Petri dish contained four pieces of the plant parts distributed in the four corners of the plate, so that fungi can grow freely and can be separated easily. All Petri dishes were incubated for one week at 28 °C, and when the outgrowth of endophytic fungi from the explants was noted, the detected outgrowths were transferred to malt extract agar plates to get pure colonies. The isolated pure fungi were kept in slants at 4 °C.

Identification of Fungi

Morphological Identification

Taxonomic identification depending on morphological characteristics was carried out according to identification keys using specific media; Czapek Agar for *Aspergillus*,[29,30] potato carrot agar for dematiaceous hyphomycetes;[31,32] Czapek yeast extract Agar, potato dextrose agar and malt extract agar for miscellaneous fungi[33-35] and ascomycetes.[36]

Molecular Identification

Total genomic DNA was extracted using DNeasy kit (Qiagen) according to Pamphile and Azevedo,[37] and then both the concentration and the integrity of DNA were investigated by electrophoresis using a High DNA Mass Ladder (Invitrogen, California, USA) as the standard DNA molecular weight. Then, the DNA was visualized using the transilluminator of the gel documentation system (BIO-RAD, Gel Doc 2000). *Taq* polymerase chain reaction (PCR) Master mix (Qiagene™) was used to amplify or synthesize DNA fragments using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt.

The DNA fragments of interest (PCR products and linear plasmid) were purified by agarose gel electrophoresis. The location of the desired band was determined by using an appropriate ladder on the UV transilluminator. The gel slice containing the desired band was then removed from the gel to a dialysis bag, which was filled with a mixture of 1x Tris/Borate EDTA (TBE) and 0.5% SDS for elution, and then placed again in an electrophoretic marine. Using the same electric current, the band was moved from the gel slice to the surrounding buffer, then the buffer was transferred to a 1.5 ml tube, and an equal volume of phenol chloroform isoamyl alcohol (PCI) was added to extract the DNA. After 5 min of centrifugation, the upper layer was removed in a new 1.5 ml tube; 2 ml of absolute ethanol was added to precipitate the DNA, which was re-suspended in an appropriate volume of TE buffer.

Sequencing of plasmid and amplified PCR fragments was carried out by Cy5/Cy5.5 Dye Primer Sequencing kit from Visible Genetics Inc. for use with the Open Gene automated DNA sequencing system[38,39] at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt. The primer sequences; ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used for identifying 18s in the present work. The resulting sequences were then compared with those available online in the public databases of National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST).[40]

Extraction of Fungal Secondary Metabolites

Each fungal isolate was cultivated on malt extract agar medium (MEA) for 7 days, then discs of fungal growth were inoculated into potato broth media, incubated in a shaking incubator for 10 days, and then the mycelial mat was separated from the media containing the fungal metabolites by filtration.[41,42] Filtrates were then extracted with ethyl acetate for three successive times till exhaustion. The organic fractions were collected together and dried under vacuum to get crude fungal extracts.[43]

Antimicrobial Assay

Antimicrobial activities of endophytic fungal extracts were assayed on some pathogenic microorganisms using well diffusion method.[44] A volume of 100 µl of 20 mg/ml tested sample was put in a 6 mm well. The tested fungal species were *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Candida albicans* ATCC 10231. The tested Gram positive bacterial species were *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. While Gram negative bacterial species were *Escherichia coli* ATCC 25922 and *Proteus vulgaris* ATCC 13315. Malt extract agar media was used for fungal tests, while Mueller-Hinton agar medium was used for bacterial tests. Fungal cultures were incubated at 25 °C for 3 days, while bacterial cultures were incubated at 37 °C for 24 h. Ketoconazole was used as a standard antibiotic for fungi, while gentamycin was used as a bacterial standard antibiotic. These standards were used as positive controls at a concentration of 20 mg/ml, while dimethyl sulfoxide (DMSO) was used as a

negative control. After the incubation periods, the diameters of inhibition zones were measured in millimeters.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC values of endophytic fungal extracts were estimated using well diffusion method[44] according to NCCLS recommendations.[45] Bifold serial dilutions of fungal extracts were prepared using DMSO. Mueller-Hinton agar media were seeded by bacterial suspensions then poured in plates and left to solidify. Wells of 6 mm diameter were made in plates using a sterile Cork borer. Each well contained 100 µl of each fungal extract concentration. The plates were incubated at 37 °C for 24 h. MIC value was determined as the lowest concentration of the extract that prevented the visible microbial growth after the incubation period.

In vitro Antiviral Assay on Hepatitis A Virus

The Cell Culture and The Virus

For the antiviral assay, we used Vero cells (CCL-81; American Type Culture Collection, USA) from African green monkey kidney maintained in maintenance medium (MEM) containing L-glutamine (2 nM), fetal calf serum (FCS: 10% v/v), streptomycin (100 µg/ml), and penicillin (100 U/ml). Vero cells were propagated for four weeks, and then they were incubated at 37 °C in a humidified atmosphere. Hepatitis A virus (HAV) was kindly provided by the Virology laboratory, Department of Microbiology, Faculty of Medicine, Al-Azhar University, Egypt.

Virus Stock Preparation

In 75 cm^2 culture flasks, Vero cells were infected by each HAV virus, giving it one hour to be absorbed. Using 2% MEM, non-adherent particles were removed. On the other hand, the infected cells were covered with 2% MEM (20 ml) and incubated till observing a complete cytopathological effect (CPE) for 4-6 continuous days. After repeating the previous step for two times, we determined the challenge dose of the virus using plaque formation assay,[46,47] and the virus was kept at -20 °C till being used.

The Maximum Nontoxic Concentration of the Tested Extracts on Vero Cells

To determine the maximum nontoxic concentration (MNTC) of each extract, two-fold dilutions of the fungal extracts were prepared in maintenance medium (MEM). Then, Vero cells were treated with 0.1 ml of each dilution in a 96- well plate (Falcon, Corning, USA), where three wells containing only maintenance medium were considered control. The plates were incubated at 37 °C. The cells were examined frequently up to two days using a microscope to detect the minimum concentration that can cause adverse effects on cell morphology as a physical sign of toxicity. The MTT colorimetric assay was performed as follows; MTT solution was prepared (5 mg/ml in PBS) (Bio Basic Canada INC), then 20 µl of MTT solution were pipetted in each well, and then put on a shaker (150 rpm) for 5 min to ensure that the MTT was mixed with the media, then the plate was incubated for 4 h (37 °C and 5% CO_2) to allow the MTT to be degraded, and finally the media were removed. Formazan which is a MTT metabolic product was resuspended in 200 µl DMSO, put on a shaker (150 rpm) for 5 min to mix the formazan with the solvent. The optical density was read at 560 nm and the background was subtracted at 620 nm. The resulted optical density was directly proportional to cell quantity. The MNTC of each extract was determined to be applied in the antiviral test.[48]

Antiviral Test

In a 96 well plate, 200 µl media was put in each well, and then 10,000 cells were added. Three control wells were left empty to act as blank. The plate was incubated (at 37 °C and 5% CO_2) overnight, then 1:1 v/v of non-lethal dilution of tested extract and the virus suspension were incubated for one hour. 100 µl from viral/sample suspension were then added, and the plate was put on a shaker (150 rpm for 5 min). The plate was incubated (at 37 °C, 5% CO_2) for one day to allow virus exposure to the extracts. Two ml of 5mg/ml MTT solution/96 well plate was prepared in PBS, then 20 µl MTT solution was added to each well. After that, the previously mentioned steps in determining MNTC were repeated from mixing the MTT with the media till measuring the optical density at 560 nm.

Cercaricidal Effect

100 freshly shed *Schistosoma mansoni* cercariae in 5 ml water were mixed with 5 ml of 100, 200, and 300 mg/L of each fungal extract in a well plate. Dechlorinated water (10 ml) containing 100 cercariae were kept as control. The cercariae were observed each 15 min from 0 time to 105 min under a dissecting microscope to observe their motility. Dead cercariae that stopped moving were counted.[49]

Gas Chromatography-Mass Spectrometry (GC-MS/MS)

GC-MS/MS spectrometry was carried out using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). The system is an electron ionization one (70 ev) and the carrier gas is helium (flow rate of 1 ml/min). The injector and MS transfer line temperature was adjusted at 280 °C. The oven temperature was set to be 50 °C as an initial temperature (hold 2 min) to 150 °C with a rate of 7 °C/min increase, followed by 270 °C at an increasing rate of 5 °C/min (hold 2 min), and then to 310 °C as a final temperature at an increasing rate of 3.5 °C/min (hold 10 min). All the identified components were quantified according to

their relative peak areas. The compounds were identified tentatively by comparing their irrelative retention time and mass spectra with those of the National Institute of Standards and Technology (NIST), Wiley library data of the GC-MS/MS system.

Statistical Analysis

The results of antimicrobial assay were shown as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA using SPSS statistical software. The differences between mean values were analyzed at $p \leq 0.05$ with Duncan's multiple range test.

Results

Endophytic Fungi

Four fungal species were the most dominant from the petioles and leaves of *Eichhornia crassipes*. The most frequent was *Aspergillus flavus*, as 10 samples were isolated from the leaves, followed by *Aspergillus welwitschiae*; 7 samples from the leaves. On the other hand, four samples of *Aspergillus fumigatus*, and two samples of *Corynascus sepedonium* were isolated from the petioles. Table 1 shows that *Aspergillus flavus* was the most dominant species represented by 43.48% frequency, while *Corynascus sepedonium* was the least dominant with 8.70% frequency.

The identification of the isolated fungi was confirmed using molecular techniques. Accession numbers of the identified fungi were obtained as OM758315 for *Aspergillus flavus*, OM688980 for *Aspergillus fumigatus*, OM758326 for *Aspergillus welwitschiae* and OM688206 for *Corynascus sepedonium*.

Table 1. Endophytic Fungal Species Isolated from *Eichhornia crassipes*

Fungal Species	Frequency (%)
Aspergillus flavus	43.48
Aspergillus welwitschiae	30.43
Aspergillus fumigatus	17.39
Corynascus sepedonium	8.70
Total count	100

Table 2. Antimicrobial Activities of the Endophytic Fungal Extracts

Tested Microorganism	Endophytic Fungal Extract				Control
	Aspergillus flavus	*Aspergillus fumigatus*	*Aspergillus welwitschiae*	*Corynascus sepedonium*	
Fungi					**Ketoconazole**
Aspergillus flavus	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	16 ± 2^{ef}
Aspergillus fumigatus	0 ± 0^{a}	0 ± 0^{a}	15 ± 1^{de}	0 ± 0^{a}	17 ± 1^{f}
Aspergillus niger	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	15 ± 0^{de}
Candida albicans ATCC 10231	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	20 ± 1^{g}
Gram positive bacteria					**Gentamycin**
Staphylococcus aureus ATCC 25923	25 ± 1^{jk}	27 ± 0^{lm}	26 ± 2^{kl}	24 ± 1^{ij}	24 ± 1^{ij}
Enterococcus faecalis ATCC 29212	23 ± 1^{h}	28 ± 2^{m}	27 ± 1^{lm}	26 ± 1^{kl}	26 ± 2^{kl}
Gram negatvie bacteria					**Gentamycin**
Escherichia coli ATCC 25922	20 ± 0^{g}	26 ± 1^{kl}	23 ± 0^{h}	25 ± 2^{jk}	30 ± 2^{n}
Proteus vulgaris ATCC 13315	13 ± 2^{c}	11 ± 2^{b}	13 ± 1^{c}	14 ± 1^{cd}	25 ± 2^{jk}

The values are the diameters of inhibition zones (mm); Each value is the mean of triplicate readings (Mean ± SD).
Mean values with different letters in the same row are significantly different at $p \leq 0.05$.

Table 3. The Minimum Inhibitory Concentrations (MIC) Values (µg/ml) of Endophytic Fungal Extract

Tested Microorganism	Endophytic fungal extract			
	Aspergillus flavus	*Aspergillus fumigatus*	*Aspergillus welwitschiae*	*Corynascus sepedonium*
Gram positive bacteria				
Staphylococcus aureus ATCC 25923	312.5	2500	1250	312.5
Enterococcus faecalis ATCC 29212	156.25	1250	156.25	26
Gram negatvie bacteria				
Escherichia coli ATCC 25922	312.5	1250	625	156.25
Proteus vulgaris ATCC 13315	5000	10000	5000	78.14

Antimicrobial Assay and Minimum Inhibitory Concentration (MIC)

As shown in table 2, all tested fungal species were resistant to endophytic fungal extracts except *Aspergillus fumigatus* that was sensitive to *Aspergillus welwitschiae* extract. All tested bacterial species, either Gram positive or Gram negative, were sensitive to all fungal extracts, whereas *Proteus vulgaris* was the most resistant. *Aspergillus fumigatus* extract was the most effective on *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli*, while *Corynascus sepedonium* extract was the most potent to *Proteus vulgaris*. The best minimum inhibitory concentration was that of *Corynascus sepedonium* extract against *Enterococcus faecalis* (Table 3).

Antiviral Effect

Table 4 shows that the maximum nontoxic concentration (MNTC) of *A. flavus*, *A. fumigatus*, and *Corynascus sepedonium* extracts was 62.5 µg/ml, while that of *A. welwitschiae* was 31.25 µg/ml. In addition, the highest antiviral effect was that of *Aspergillus flavus* followed by *A. fumigatus* extracts, whereas the lowest antiviral effect was that of *Corynascus sepedonium* extract (Table 5).

Cercaricidal Effect

Treatment of cercariae with ethyl acetate extract of *A. flavus*

Table 4. Cytotoxicity and Maximum Nontoxic Concentration (MNTC) of Fungal Extracts on Vero Cells

ID	Concentration (µg/ml)	O.D.			Mean O.D.	SD	Viability %	Toxicity %	IC_{50} (µg/ml)	MNTC (µg/ml)
Vero cell	---	0.431	0.386	0.398	0.405	0.013	100	0		
Aspergillus flavus extract	1000	0.024	0.031	0.027	0.027	0.002	6.749	93.251	201.65	62.5
	500	0.063	0.048	0.055	0.055	0.004	13.662	86.337		
	250	0.156	0.172	0.188	0.172	0.009	42.469	57.531		
	125	0.231	0.209	0.233	0.224	0.008	55.391	44.609		
	62.5	0.365	0.398	0.387	0.383	0.01	94.65	5.35		
	31.25	0.399	0.402	0.383	0.395	0.006	97.449	2.551		
Aspergillus fumigatus extract	1000	0.025	0.019	0.022	0.022	0.002	5.432	94.568	221.65	62.5
	500	0.055	0.072	0.046	0.058	0.008	14.239	85.761		
	250	0.146	0.173	0.159	0.159	0.008	39.342	60.658		
	125	0.328	0.341	0.338	0.336	0.004	82.881	17.119		
	62.5	0.407	0.385	0.389	0.394	0.007	97.202	2.798		
	31.25	0.41	0.396	0.4	0.402	0.004	99.259	0.741		
Aspergillus welwitschiae extract	1000	0.02	0.018	0.019	0.019	0.001	4.691	95.308	74.73	31.25
	500	0.019	0.024	0.022	0.022	0.001	5.35	94.65		
	250	0.043	0.028	0.05	0.04	0.006	9.959	90.041		
	125	0.042	0.067	0.073	0.061	0.009	14.979	85.02		
	62.5	0.176	0.189	0.157	0.174	0.009	42.963	57.037		
	31.25	0.398	0.378	0.396	0.391	0.006	96.461	3.539		
Corynascus sepedonium extract	1000	0.065	0.078	0.084	0.076	0.006	18.683	81.317	388.73	62.5
	500	0.123	0.115	0.136	0.125	0.006	30.782	69.218		
	250	0.321	0.289	0.309	0.306	0.009	75.638	24.362		
	125	0.367	0.352	0.387	0.369	0.01	91.029	8.9712		
	62.5	0.401	0.4	0.388	0.396	0.004	97.86	2.14		
	31.25	0.408	0.392	0.405	0.402	0.005	99.177	0.823		

O.D.: Optical density; SD: Standard deviation; IC_{50}: The concentration of the extract required for 50% inhibition of Vero cells *in vitro*; MNTC: The maximum nontoxic concentration.

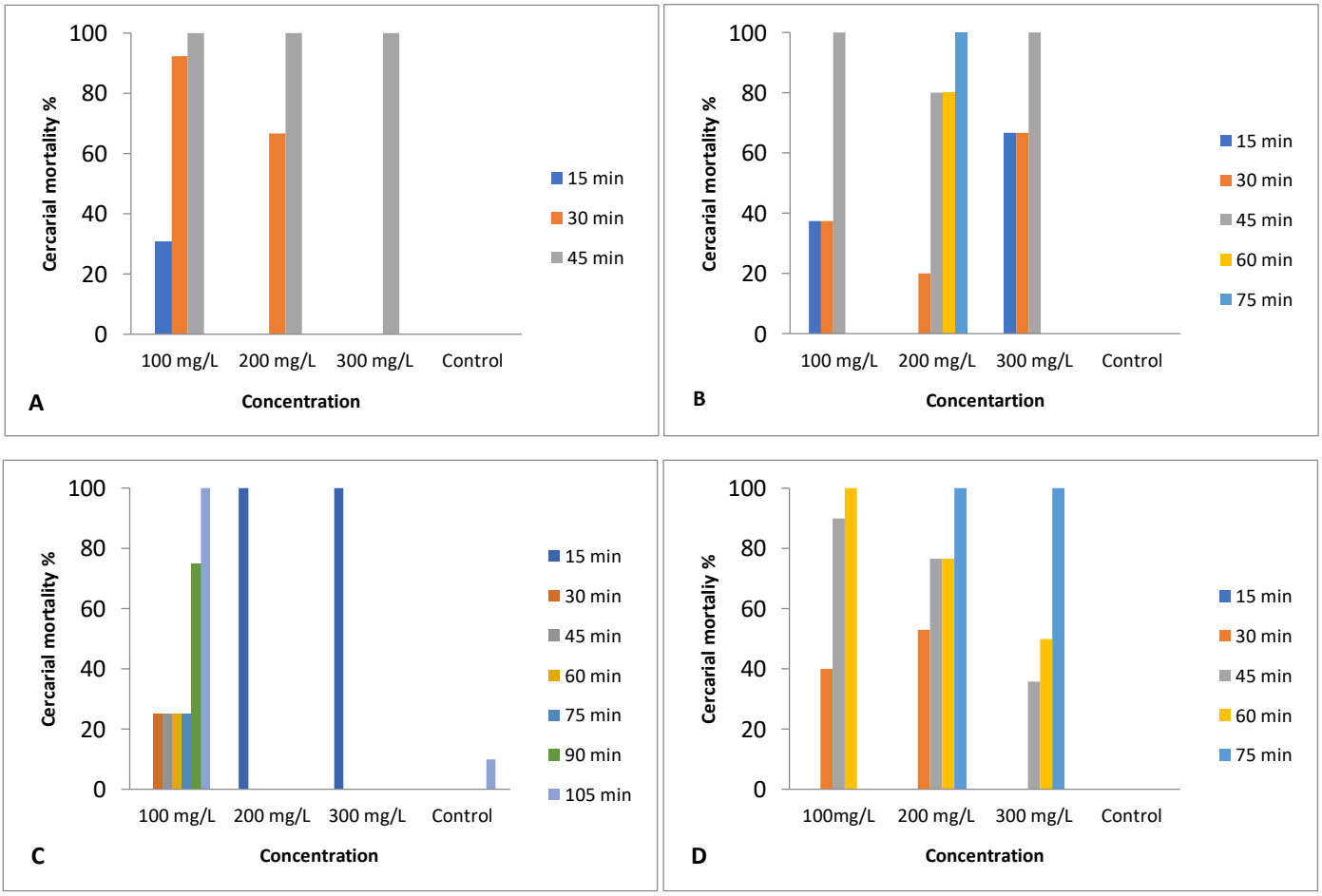


Figure 1. Cercaricidal Effect of **(A)** *Aspergillus flavus*, **(B)** *Aspergillus fumigatus*, **(C)** *Aspergillus welwitschiae*, and **(D)** *Corynascus sepedonium* Extracts.

Table 5. The Antiviral Effect (%) of the Tested Fungal Extracts on Hepatitis A Virus (HAV)

Test	Concentration (µg/ml)	O.D.			Mean O.D.	SD	Viability	Toxicity	Viral activity %	Antiviral effect %
Control Vero cell		0.438	0.421	0.449	0.436	0.007	100	0		
HAV		0.208	0.196	0.215	0.206	0.142	47.32	52.68	100	0
Aspergillus flavus extract	62.5	0.325	0.287	0.291	0.301	0.01	69.04	30.96	58.78	41.22
Aspergillus fumigatus extract	62.5	0.261	0.289	0.255	0.268	0.009	61.54	38.46	73	26.99
Aspergillus welwitschiae extract	31.25	0.256	0.248	0.287	0.264	0.01	60.47	39.53	75.04	24.96
Corynascus sepedonium extract	62.5	0.22	0.237	0.246	0.234	0.007	53.75	46.25	87.81	12.19

O.D.: Optical density; SD: Standard deviation.

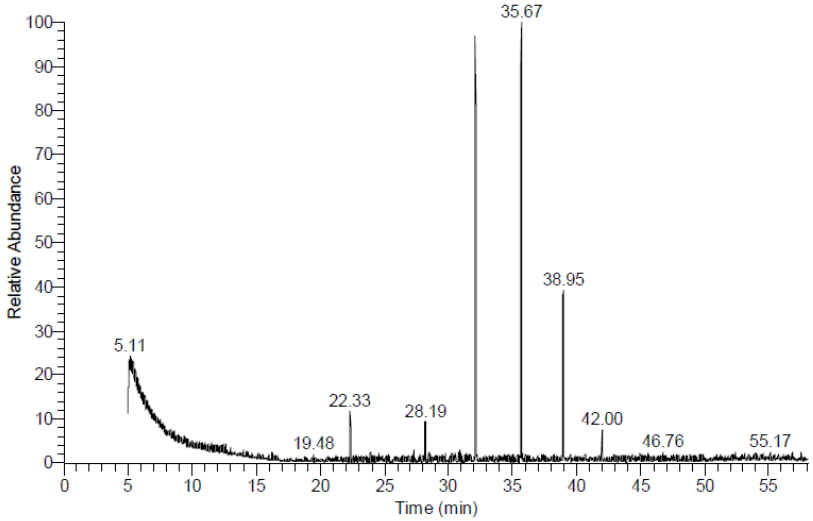
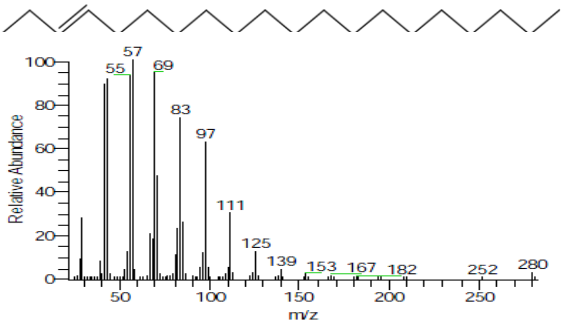
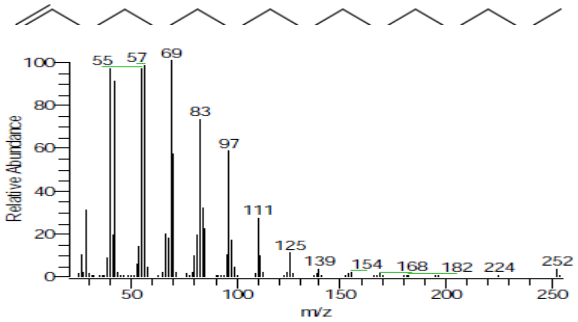


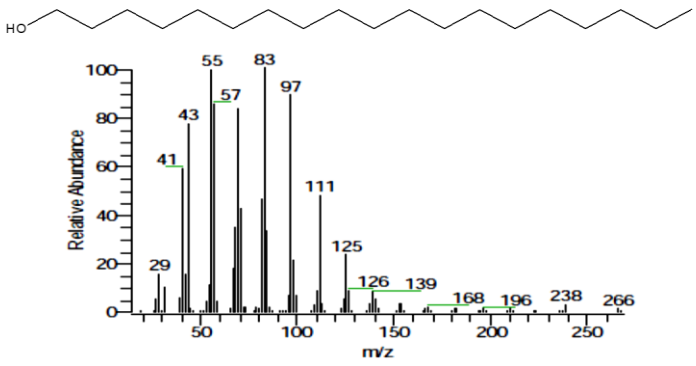
Figure 2. GC-MS/MS Chromatogram of all Identified Compounds in *Aspergillus flavus* Extract.



3-Eicosene, E



3-Octadecene, E



n-Nonadecanol-1

Figure 3. GC-MS/MS Chromatograms of the Major Identified Compounds in *Aspergillus flavus* Extract.

Table 6. GC-MS/MS Analysis of *Aspergillus flavus* Extract

NO.	MW	MF	Area %	RT	Compound Name
1	704	$C_{33}H_{36}O_{17}$	0.48	8.5	6-CXylosyl-8-Cglucosy lapigeninpermethylated derivative
2	240	$C_{16}H_{16}O_2$	0.43	10.55	Spiro[2-methylcyclohex-3-en-5-one1,2'-tetralone]
3	206	$C_{13}H_{18}O_2$	3.14	22.34	3,4-Dihydro-2-H-1,5-(3"tbutyl)benzodioxepine
4	470	$C_{32}H_{54}O_2$	0.52	25.33	A'-[(5'aCholestan-3'6yl)methyl]- 3-butyrolacetone
5	138	$C_9H_{14}O$	0.42	29.76	4-(CYCLOPENTYLIDENE)-2-BUTANONE
6	298	$C_{19}H_{38}O_2$	0.47	30.12	Octadecanoicacid,methyl ester
7	220	$C_{16}H_{28}$	0.46	30.54	4-HEXADECEN-6-YNE, E
8	310	$C_{22}H_{46}$	0.6	30.78	Docosane
9	94	C_6H_6O	0.47	31.36	4-Methylidenecyclo-2-pentenone
10	252	$C_{18}H_{36}$	28.03	32.1	3-Octadecene,E
11	708	$C_{44}H_{36}O_9$	0.47	33.7	3,5-Diphenyl3,5-(9,10-phenanthylene)tricyclo[5.2.1.0]decane-4-one-8-exo -9-endo-dicarboxylic acid diacetoxymethylester
12	760	$C_{48}H_{56}O_8$	0.47	34.07	OxylateMethoxychromeneprecocene tetramer
13	128	C_9H_{20}	0.58	34.68	Heptane, 2,2-dimethyl
14	280	$C_{20}H_{40}$	28.61	35.67	3-Eicosene, E
15	284	$C_{19}H_{40}O$	12.06	38.95	n-Nonadecanol-1
16	540	$C_{39}H_{72}$	0.58	39.45	Benzene, (1-hexadecylheptadecyl)
17	266	$C_{19}H_{38}$	2.36	42	1-Nonadecene
18	648	$C_{41}H_{44}O_7$	0.45	43.09	5"-(1,1-Dimethylethyl)2,2',2",2"',2""-pentamethoxy[1,1':3',1":3",1''':3"'.1""-quinquephenyl]-3,3""-dimethanol
19	658	$C_{44}H_{34}O_6$	0.46	43.95	(P,P,S)Dimethyl5,5'dihydroxy-1,1',12,12'-tetramethyl[6,6']bi(benzo[c]phenanthrenyl)-8,8'-dicarboxylate
20	552	$C_{40}H_{56}O$	0.53	46.76	Lycoxanthin
Oxygenated compounds			20.37%		
Deoxygenated compounds			61.22%		
Isoprene compounds			1.47%		
Monoterpenes compounds			3.14%		
Sesquiterpenes compounds			43.81%		
Diterpenes compounds			29.21%		
Triterpene compounds			1%		
Sesquarterterpene compounds			0.58%		
Tetraterpenes compounds			0.53%		
Polyterpenes compounds			1.85%		
Total identified compounds			81.59 %		

MW: Molecular weight; MF: Molecular formula; RT: Retention time.

Table 7. GC-MS/MS Analysis of *Aspergillus fumigatus* Extract

NO.	MW	MF	Area %	RT	Compound Name
1	152	$C_{10}H_{16}O$	0.47	6.33	Arthemiseole
2	152	$C_{10}H_{16}O$	2.21	9.08	Ariemisia Ketone
3	154	$C_{11}H_{22}$	0.75	10.59	Cis-1,2-Di(1,1-dimethylethyl)cyclopropane
4	182	$C_{11}H_{18}O_2$	2.62	20.75	36-Nonadien1ylacetate
5	206	$C_{13}H_{18}O_2$	5.57	22.34	3,4-Dihydro-2H-1,5-(3"-t-butyl) benzodioxepine
6	124	C_9H_{16}	0.75	23.51	3,3,5,5-Tetramethylcyclopentene
7	240	$C_{16}H_{16}O_2$	0.53	24.97	2-Methylene1,3-diphenyl1,3-propanediol
8	154	$C_{10}H_{18}O$	0.7	26.33	4-Hepten-3-one,5-ethyl-2-methyl
9	180	$C_{12}H_{20}O$	0.53	26.7	8-Decen-2-one,9-methyl-5-methylene
10	262	$C_{19}H_{34}$	0.36	27.62	Z,Z,Z-4,6,9-Nonadecatriene
11	252	$C_{18}H_{36}$	16.74	28.2	3-Octadecene,E
12	138	$C_9H_{14}O$	1.51	28.71	Ketone,1,5-dimethylbicyclo[2.1.0]pent-5-ylmethyl
13	336	$C_{22}H_{40}O_2$	0.45	29.07	2-HPyran,2-(7-heptadecynyloxy)tetrahydro
14	166	$C_{10}H1_4O_2$	1.3	29.48	25-HFuranone,5-(2-methyl-3-methylene-4-butyl)
15	196	$C_{12}H_{20}O_2$	0.81	29.72	Tetrahydrofuran-2ol,3,4-di[1-butenyl]
16	184	$C_{11}H_{20}O_2$	1.9	29.8	3-Heptyne-2,5-diol,6-methyl-5-(1-methylethyl)-
17	194	$C_{13}H_{22}O$	0.64	30.43	4-(2,2-Dimethyl-6-methylenecyclohexyl)butanal
18	166	$C_{10}H_{14}O_2$	0.38	30.64	2-(5-H)Furanone,5(2-methyl-3-methylene-4-butyl)
19	270	$C_{24}H_{48}O_2$	0.99	30.9	Tricosanoicacid,methylester
20	350	$C_{25}H_{50}$	0.52	31.07	Heptadecane,9-(2cyclohexylethyl)
21	280	$C_{20}H_{40}$	16.45	32.11	5-Eicosene,E
Deoxygenated compounds			35.57%		
Oxygenated compounds			20.61%		
Isoprene compounds			2.26%		
Monoterpens compounds			15.26%		
Sesquiterpenes compounds			17.63%		
Diterpens compounds			17.89%		
Sestraterpens compounds			0.52%		
Total identified compounds			56.18%		

resulted in 100% mortality after 45 min in all tested concentrations. In case of *A. fumigatus* extract, 100 mg/L and 300 mg/L resulted in 100 % mortality of cercariae after 45 min. Regarding cercariae treated with *A. welwitschiae* extract, 100% mortality was recorded after 60 min of exposure to 200 and 300 mg/L, while at 100 mg/L, this was achieved after 105 min. Although all cercariae died after 60 min of exposure to 100 mg/L of *Corynascus sepedonium* extract, 75 min were enough to cause the mortality of all cercariae treated with 200 and 300 mg/L (Figure 1).

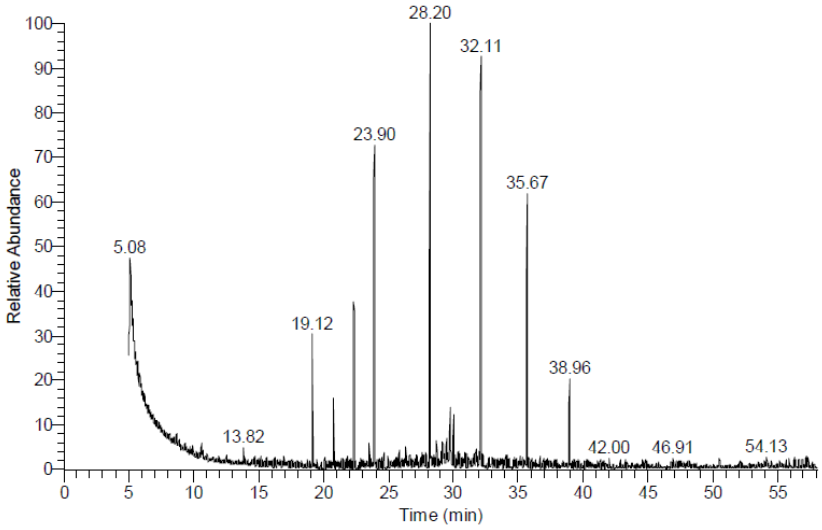


Figure 4. GC-MS/MS Chromatogram of all Identified Compounds in *Aspergillus fumigatus* Extract.

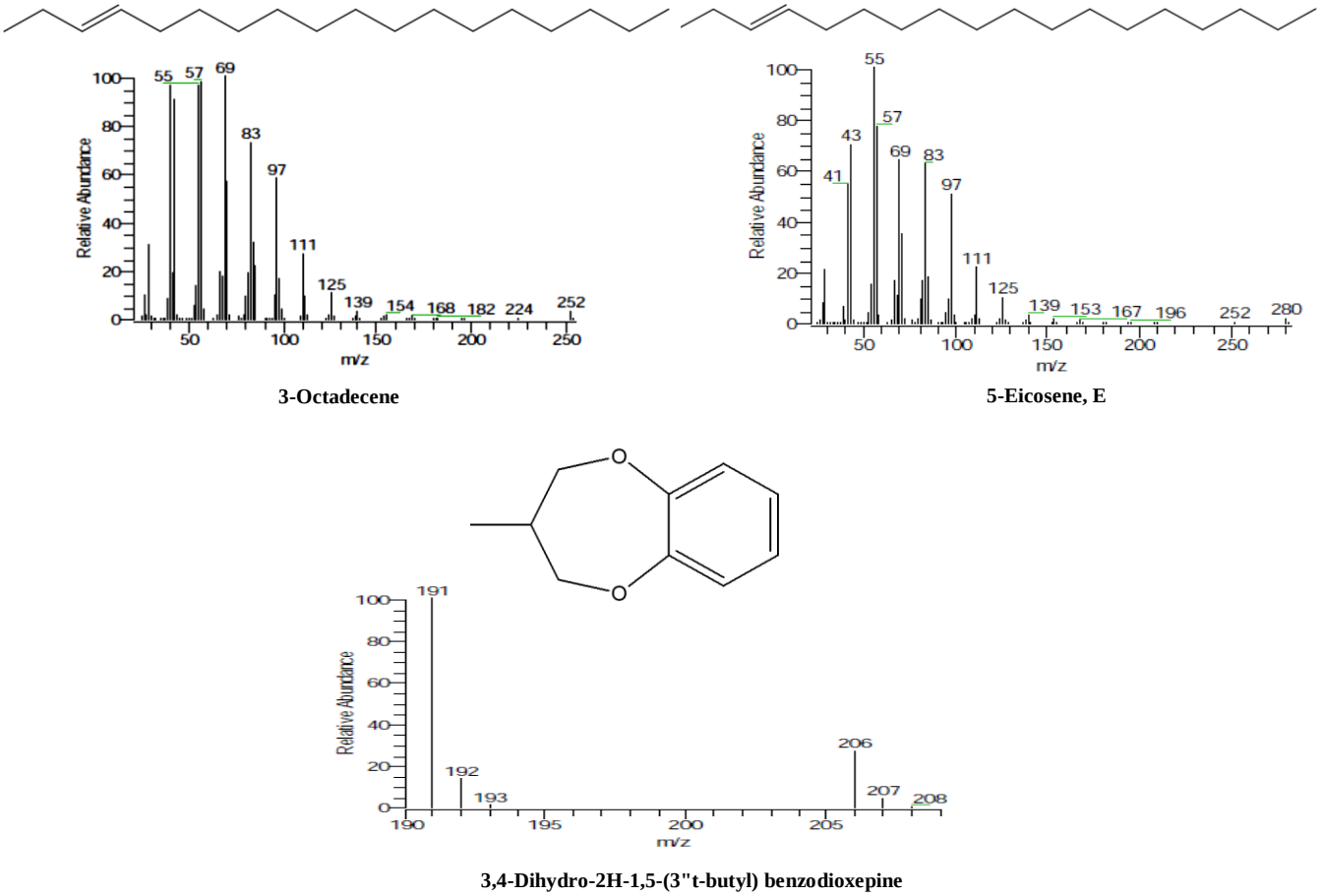


Figure 5. GC-MS/MS Chromatograms of the Major Identified Compounds in *Aspergillus fumigatus* Extract.

Gas Chromatography-Mass Spectrometry (GC-MS/MS) Analysis

GC-MS/MS analysis of *Aspergillus flavus* extract presented in Figure 2 led to identification of 20 compounds representing 81.59% of the total composition. The tentatively identified compounds are listed in table 6, as the major ones were 3-Eicosene, E (28.61%), 3-Octadecene, E (28.03%), and n-Nonadecanol-1 (12.06%) (Figure 3). On the other hand, the analysis of *Aspergillus fumigatus* extract resulted in the identification of 21 compounds representing 56.18% of the total composition (Figure 4). These compounds are listed in table 7, where the major ones were 3-Octadecene, E (16.74%), 5-Eicosene, E (16.45%), and 3,4-Dihydro-2H-1,5-(3"-t-butyl) benzodioxepine (5.57%) (Figure 5).

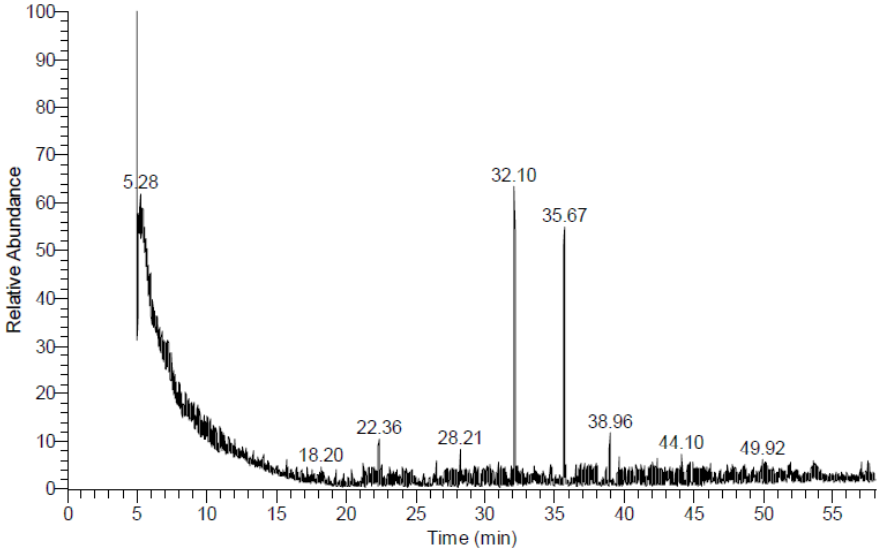


Figure 6. GC-MS/MS Chromatogram of all Identified Compounds in *Aspergillus welwitschiae* Extract.

Table 8. GC-MS/MS Analysis of *Aspergillus welwitschiae* Extract

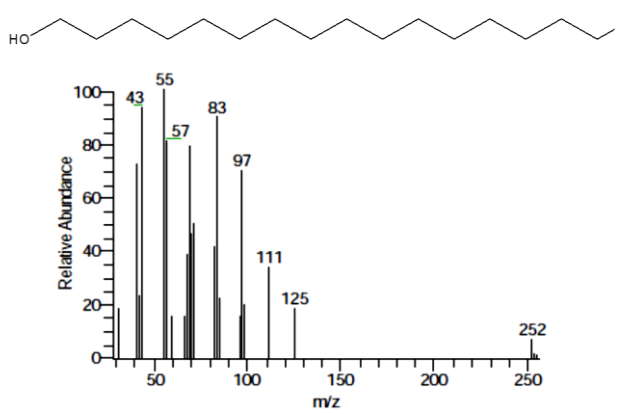
NO.	MW	MF	Area %	RT	Compound Name
1	116	$C_5H_8O_3$	1.03	7.72	(R)Dihydro5(hydroxymethyl)2(3H)furanonehydroxym3H)furanone
2	648	$C_{44}H_{56}O_4$	1.2	8.04	5,11,17,23-Tetratbutyl-25,26,27,28-tetrahydroxycalix-4-arene
3	40	C_3H_4	1.1	9.31	1,2-Propadiene
4	206	$C_{12}H_{14}O_3$	2.63	22.35	8-Dimethoxy-4-methyl-4-Hchromene
5	224	$C_{16}H_{32}$	1.14	28.2	Cyclohexadecane
6	616	$C_{32}H_{40}O_{12}$	1.14	29.75	Anodendroside E 2,monoacetate
7	696	$C_{40}H_{56}O10$	1.45	31.14	Nephthoside 1,2',3',4'Tetraacetate
8	270	$C_{18}H_{38}O$	18.5	32.1	1-Octadecanol
9	542	$C_{33}H_{34}O7$	1.05	34.72	1,8-diisopropyl-2,3,9,10-tetramethoxy-6-H,13-H-5,12-dioxadibenzo[b,i] pyrene-7-carbaldehyde
10	266	$C_{19}H_{38}$	16.07	35.67	(cis)-2-Nonadecene
11	256	$C_{17}H_{36}O$	5.05	38.96	1-Heptadecanol
12	610	$C_{36}H_{50}O_8$	1.22	39.99	2,13,21,28,35,42-Hexaoxa-1,14 dioxo[14.8.8](2,1,4)cyclophane
13	676	$C_{41}H_{40}O_9$	0.99	46.22	5"(1,1Dimethylethyl)2,2',2",2"',2""-pentamethoxy[1,1':3',1":3",1''':3"'.1""-quinquephenyl-3,3"" dicarboxylic acid
14	686	$C_{41}H_{66}O_8$	1.44	48.68	(2R)-8,13Epoxy2,2-(8',13'-epoxy-2'-bmethoxy-3'-oxolabdane-1-oxolabdane-1'a,2'-adiyldioxy)1-ahydroxylabdan-3
15	684	$C_{44}H_{76}O_5$	1.36	50.55	Lipo-3-episapelinA
16	692	$C_{44}H_{44}N_4O_4$	2	51.23	N,N'-Dicyclohexyl-1,7-dipyrrolidinylperylene-3,4:9,10-tetracarboxylic acid bisimide-9
17	602	$C_{36}H_{42}O_8$	1.27	11. 19	1,2-Bis(4(2,3-dimethoxybenzyl)-2,3-dimethoxyphenyl)ethane
Oxygenated compounds			40.33%		
Deoxygenated compounds			15.31%		
Isoprene compounds			1.03%		
Monoterpene compounds			2.63%		
Sesquiterpenes compounds			40.76%		
Triterpene compounds			2.19%		
Polyterpenes compounds			6.99%		
Others			1.1%		
Total identified compounds			58.64%		

MW: Molecular weight; MF: Molecular formula; RT: Retention time.

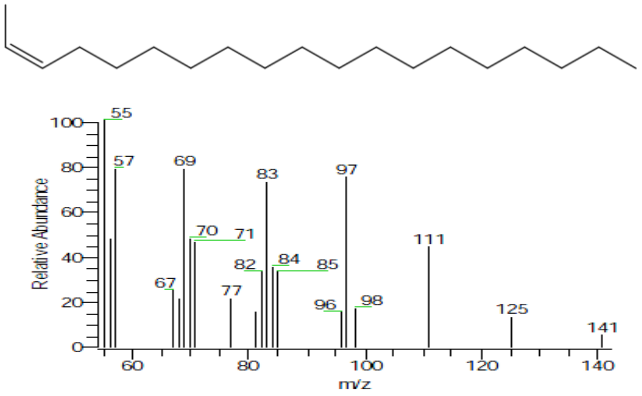
Moreover, *Aspergillus welwitschiae* extract contained 17 identified compounds representing 58.64% of the total composition (Figure 6). The tentatively identified compounds are listed in table 8, with1-Octadecanol (18.5%), (cis)-2-nonadecene (16.07%), and 1-Heptadecanol (5.05%) as the major compounds (Figure 7). Additionally, the analysis of *Corynascus sepedonium* extract led to identification of 40 compounds representing 97.79% of the total composition (Figure 8). The tentatively identified compounds are listed in table 9, as 3-Methyl-2-butenoic acid, oct-3-en-2-ylester (19.31%), Camphenilone (18.73%), 2,5-Dimethylcyclohexano (14.37%), 1-Tetradecanol (9.8%), n-Nonadecanol-1 (9.18%), and Z,Z,Z-1,4,6,9-Nonadecatetraene (5.09%) were the major ones (Figure 9).

Discussion

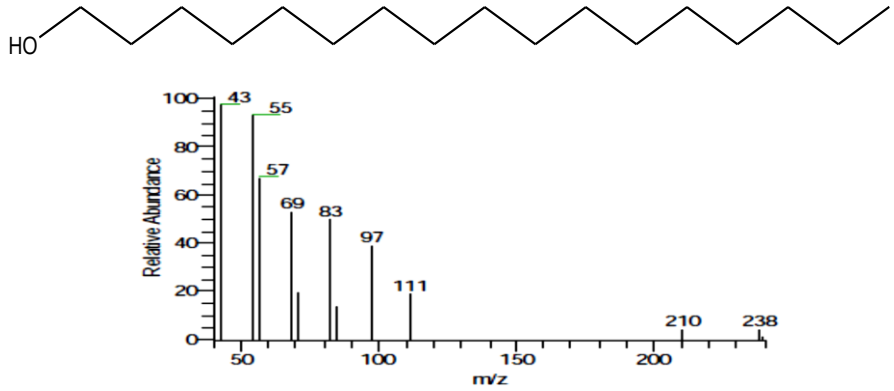
The present study demonstrated that *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus welwitschiae*, and *Corynascus sepedonium* were the most prevalent endophytic fungi from leaves and petioles of *Eichhornia crassipes* plant (water hyacinth). *Aspergillus flavus* and *Aspergillus fumigatus* extracts were the most effective as antibacterial, antiviral and cercaricidal



1-Octadecanol



(cis)-2-nonadecene



1-Heptadecanol

Figure 7. GC-MS/MS Chromatograms of the Major Identified Compounds in *Aspergillus welwitschiae* Extract.

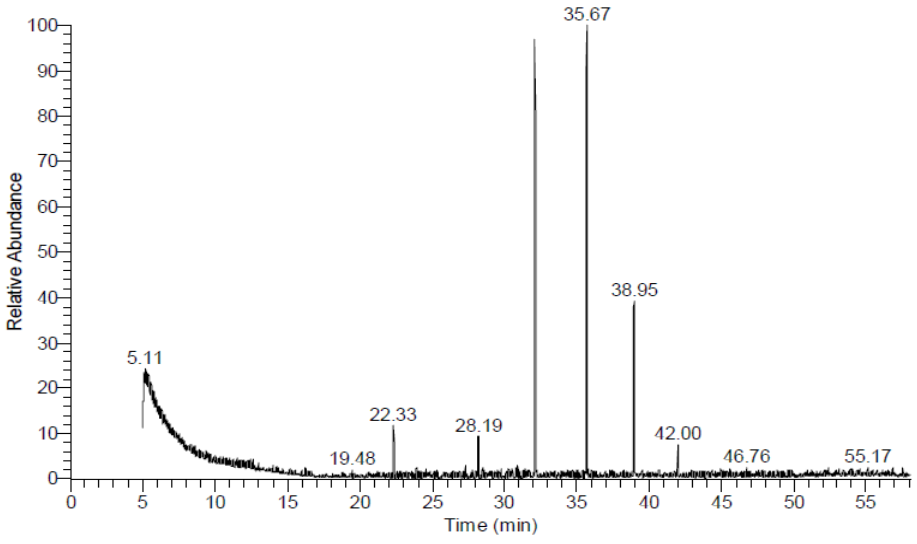


Figure 8. GC-MS/MS Chromatogram of all Identified Compounds in *Corynascus sepedonium* Extract.

agents. Among fungi, *Aspergillus* species are ubiquitous and are known for their ability to synthesize drug-lead compounds.[50] Endophytic *Aspergillus flavus* and *Aspergillus fumigatus* have been found to possess antioxidant, antifungal and antibacterial activities.[51-54] Similarly, Abdel-Wareth et al.,[55] reported on the antimicrobial effect of the acetone extract of *A. flavus* isolated from snails tissues against *Pseudomonas areuginosa* and *Candida* albicans. The current work showed that the bioactivity of *A. flavus* and *A. fumigatus* extracts might be attributed to their chemical

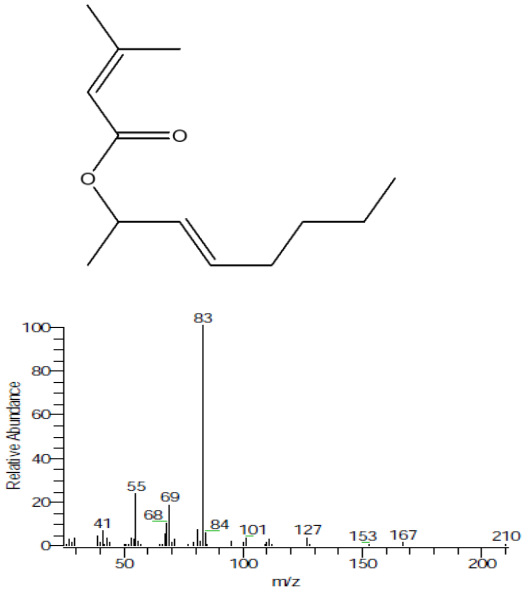
Table 9. GC-MS/MS Analysis of *Corynascus sepedonium* Extract

No.	MW	MF	Area %	RT	Compound Name
1	84	C_5H_8O	0.08	6.45	Isopropenyl Vinyl Ether
2	138	$C_9H_{14}O$	18.73	9.9	Camphenilone
3	130	$C_8H_{18}O$	2.47	10.53	1-Octanol
4	224	$C_{16}H_{32}$	0.85	13.8	1-Hexadecene
5	214	$C_{14}H_{30}O$	9.8	19.14	1-Tetradecanol
6	206	$C_{14}H_{22}O$	4.46	22.34	2-Tert Butyl-4-isopropyl-5-methylphenol
7	198	$C_{14}H_{30}$	0.53	23.3	Tetradecane<N->
8	210	$C_{13}H_{22}O_2$	19.31	23.93	3-Methyl-2-butenoicacid,oct-3-en-2-ylester
9	254	$C_{18}H_{38}$	0.1	24.05	Heptadecane,2-methyl
10	114	$C_7H_{14}O$	0.33	24.94	3-Methyl-3-hexen-2-ol
11	256	$C_{16}H_{32}O_2$	0.09	25.11	Decanoicacid,hexylester
12	152	$C_9H_{12}O_2$	0.38	25.27	2(5H)-Furanone,4-methyl-3-(2-methyl-2-propenyl)-
13	182	$C_{11}H_{18}O_2$	1.39	25.48	2,6-Dimethyl-2-vinyl
14	140	$C_9H_{16}O$	0.11	25.78	3-Nonyn-2-ol
15	298	$C_{20}H_{42}O$	0.12	25.87	1-Eicosanol
16	260	$C_{19}H_{32}$	5.09	26.46	Z,Z,Z-1,4,6,9-Nonadecatetraene
17	154	$C_{11}H_{22}$	0.13	28.08	Cyclohexane,(1,1-dimethylpropyl)
18	270	$C_{17}H_{34}O_2$	0.14	28.91	Isopropyl myristate
19	178	$C_{12}H_{18}O$	0.11	29.29	6-Dodecanone
20	324	$C_{24}H_{40}O_2$	0.16	30.04	Cyclopentanecarboxylicacid,4-pentadecylester
21	270	$C_{17}H_{34}O_2$	0.51	30.9	Hexadecanoicacid,methylester
22	118	$C_4H_6O_4$	0.15	30.64	Methyl Malonic acid
23	186	$C_{12}H_{26}O$	0.09	31.58	1-Decanol,2-ethyl-
24	278	$C_{16}H_{22}O_4$	0.47	31.72	1,2-Benzenedicarboxylic acid,dibutyl ester
25	168	$C_{12}H_{24}$	0.12	32	5-Undecene,3-methyl,(E)
26	128	$C_8H_{16}O$	14.37	32.13	2,5-Dimethylcyclohexanol
27	238	$C_{16}H_{30}O$	0.66	33.11	Cyclopropane,1-(1-hydroxy1heptyl)-2-methylene-3-pentyl
28	270	$C_{18}H_{38}O$	0.64	33.83	1-Octadecanol
29	326	$C_{22}H_{46}O$	0.28	34.02	1-Docosanol
30	284	$C_{19}H_{40}O$	9.18	35.69	n-Nonadecanol-1
31	478	$C_{34}H_{70}$	0.12	38.43	Tetratriacontane
32	242	$C_{16}H_{34}O$	3.53	38.95	1-Decanol,2-hexyl
33	390	$C_{24}H_{38}O_4$	0.89	41.49	1,2-Benzenedicarboxylicacid,bisbis(2-ethylhexyl)ester
34	242	$C_{16}H_{34}O$	0.12	41.57	Hexadecanol
35	382	$C_{26}H_{54}O$	0.55	41.99	1-Hexacosanol
36	320	$C_{23}H_{48}$	0.37	44.87	Tricosane
37	282	$C_{20}H_{42}$	0.57	45.67	Eicosane<N->
38	506	$C_{36}H_{74}$	0.15	46.22	Hexatriacontane
39	296	$C_{21}H_{44}$	0.51	48.85	Heneicosane<N->
40	310	$C_{22}H_{46}$	0.13	51.92	Docosane<N->
Oxygenated compounds			89.12%		
Deoxygenated compounds			8.67%		
Isoprenes	36.47%				
Monoterpenes	35.94%				
Sesquiterpenes	21.38%				
Diterpenes	3.03%				
Sesterterpenoids	0.55%				
Triterpene	0.12%				
Sesquarterterpene	0.15%				
Others	0.15%				
Total identified compounds			97.79%		

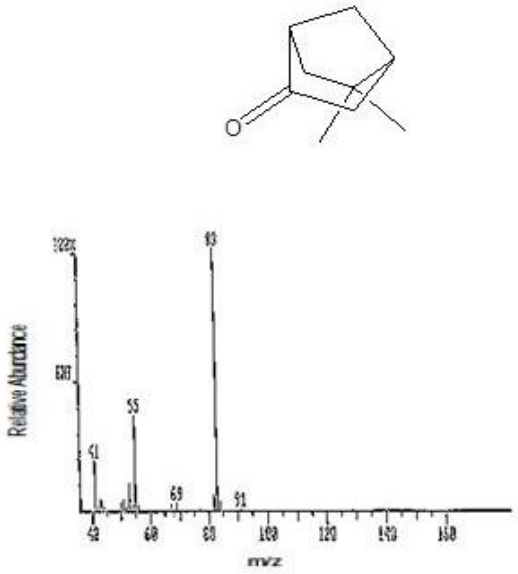
MW: Molecular weight; MF: Molecular formula; RT: Retention time.

constituents. The major compounds identified in *Aspergillus flavus* extract belong to different chemical classes including a hydrocarbon, an alkene and long chain fatty alcohols. These chemical classes were found to have pronounced biological activities like antimicrobial,[56-59] antioxidant, anticancer,[60] antihyperglycemic, insecticidal,[61,62] and cytotoxic properties.[63] In addition, the monoterpenes and sesquiterpenes identified in *A. fumigatus* extract were previously reported as compounds having antimicrobial,[56-59] antioxidant, anticancer,[60] and anti-inflammatory activities.[64] Recently, Abdel-Motleb et al.,[65] demonstrated that ethyl acetate extract of *A. fumigatus* isolated from a freshwater environment had larvicidal activity on both miracidia and cercariae of *Schistosoma mansoni*. To the best of our knowledge, this is the first study to evaluate the cercaricidal and antiviral activities of *A. flavus* and *A. welwitschiae* extracts.

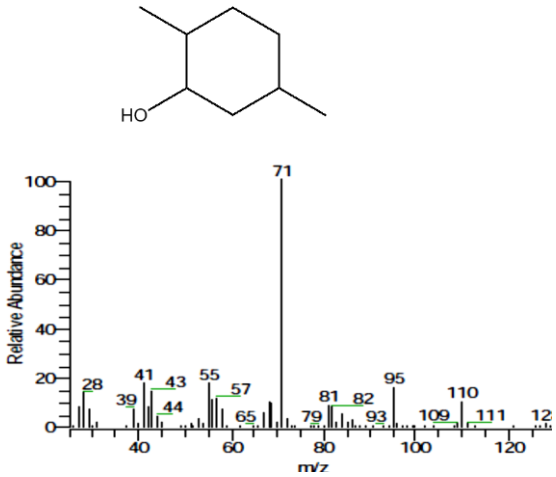
Moreover, we observed that *A. welwitschiae* extract did not only show a pronounced antibacterial effect, but also was the only extract that showed antifungal effect on *Aspergillus fumigatus*. Maliehe et al.,[66] isolated the endophytic *Aspergillus welwitschiae* from *Aloe ferox*. *A. welwitschiae* is a filamentous fungus that belongs to the Nigri group, known to be a saprotroph that decomposes plant material.[67] It produces secondary metabolites with anti-virulence[68] and antibacterial activities,[69] as it successfully inhibited *Salmonella enterica*, *Proteus* sp.,[70] *Staphylococcus aureus* and *E. coli*.[66] Also, the biological activity of *A. welwitschiae* extract can be explained in the light of its GC-MS/MS analysis which revealed the presence of various compounds, as the identified compounds belong to long-chain fatty alcohols, which were



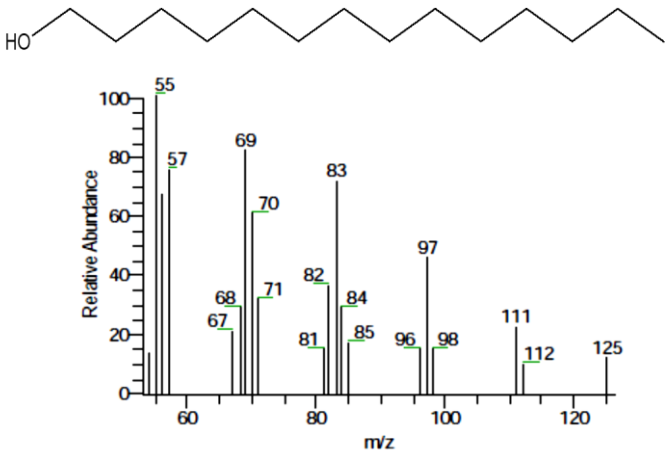
3-Methyl-2-butenoicacid, oct-3-en-2-ylester



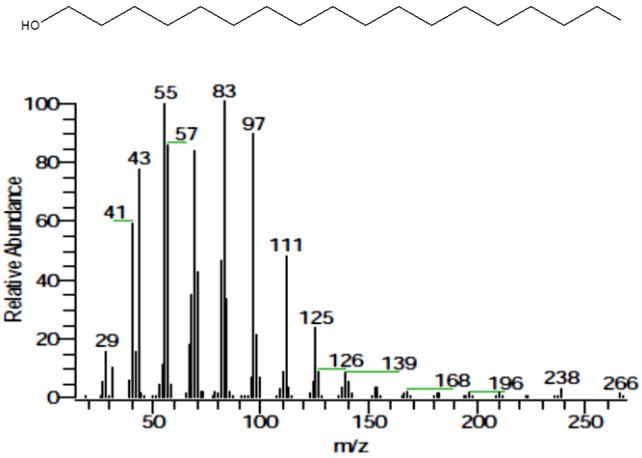
Camphenilone



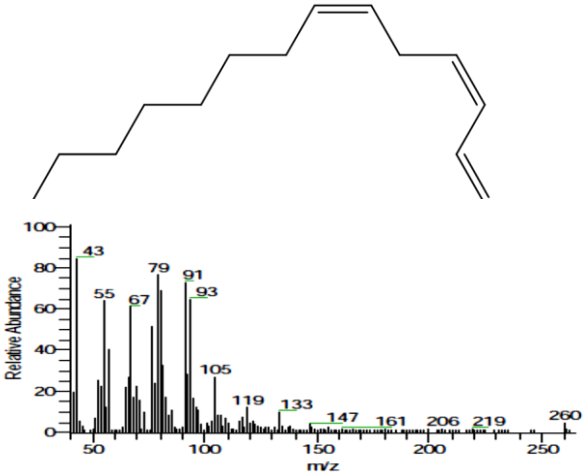
2,5-Dimethylcyclohexanol



1-Tetradecanol



n-Nonadecanol-1



Z,Z,Z-1,4,6,9-Nonadecatetraene

Figure 9. GC-MS/MS Chromatograms of the Major Identified Compounds in *Corynascus sepedonium* Extract.

shown to possess antimicrobial,[71] antifungal, antimalarial, and antioxidant activities.[72]

Regarding *Corynascus sepedonium*, it is characterized by reticulate peridial cells, broadly fusiform ascospores with two terminal germ pores, and an asexual state of echinulate conidia.[73-75] *C. sepedonium* is the teleomorph of *Myceliophthora sepedonium*. It is usually isolated from soil.[76-78] The major identified compounds in *Corynascus sepedonium* belong to various chemical classes such as cyclic terpenes, ketones, straight-chain saturated fatty alcohol, and alkenes.[79-81] It was demonstrated that these chemical classes had good antimicrobial[82] and cytotoxic effects.[63,57] To the best of our knowledge, this is the first study to isolate *Corynascus sepedonium* from *Eichhornia crassipes*, and to evaluate its biological activity as antibacterial, antiviral and cercaricidal agent.

Conclusion

The present study indicated that the fungal spp. that reside in *Eichhornia crassipes* tissues possess enormous biological activities that are more or less resemble those of their host plant. In addition, we shed the light on *Aspergillus welwitschiae* and *Corynascus sepedonium* for the first time as endophytes and candidates that can be exploited for antimicrobial, antiviral and cercaricidal purposes.

Authors' Contributions

MTAA contributed to methodology, resources, visualization, investigation, writing-original draft, review and editing; EAMA contributed to methodology, visualization, investigation, writing original draft; MAE contributed to methodology, visualization, investigation, writing original draft.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

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