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Chemical characterization, antimicrobial, antioxidant and larvicidal activities of certain fungal extracts

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ABSTRACT

Fungal extracts are considered a promising source of bioactive compounds that represent the basic core of the drug industry. This study aimed at exploring the antimicrobial, antioxidant and larvicidal activities of three ethyl acetate extracts of three fungal isolates *Fusarium oxysporum*, *Aspergillus fumigatus* and *Penicilluim griseofulvum*. These fungi were isolated from sediment samples collected in water courses of three Egyptian governorates; Giza, Qalubeya and Gharbeya, during a year from January to December 2019, molecularly identified using 18Sr RNA technique and were grown on rice medium for 14 days and extracted with ethyl acetate. GC-MS examination of the extracts led to identification of 15, 29 and 24 compounds, respectively. Hexadecanoic acid was detected as a main component in the investigated extracts. Moreover, in Folin-Ciocalteu's assay, the tested fungal extracts showed noticeable total phenolic contents (TPCs) values of 115.84, 88.24 and 73.22, respectively for *Aspergillus fumigatus*, *Penicillium griseofulvum* and *Fusarium oxysporum*. Additionally, *Aspergillus fumigatus* extract exhibited strong total antioxidant capacity (TAC) value of 409.46 mg AAE/g dry extract, followed by *Penicillium griseofulvum* and *Fusarium oxysporum* extracts with TAC values of 299.28 and 281.31 mg AAE/g dry extract, respectively. All extracts exhibited antimicrobial activities against *Staphylococcus aureus* (G+ve bacterium), *Escherichia coli* (G-ve bacterium), *Candida albicans* (yeast) and *Aspergillus niger* (Fungus). Also, the extracts showed high larvicidal activity against miracidia and cercariae of *Schistosoma mansoni*. In conclusion, *Fusarium oxysporum*, *Aspergillus fumigatus* and *Penicilluim griseofulvum* are excellent sources of bioactive compounds which have multiple biological effects.

INTRODUCTION

The phenomenon of oxidative stress caused by over-production of free radicals inside human body leads to many serious diseases such as cancer, cardiovascular, and inflammation. Moreover, the harmful effects of this phenomenon can be reduced via using naturally occurring antioxidant compounds as free radical scavengers [1-4]. Recently, pathogenic microbial strains have shown a great ability to resist antibiotics, which leads to the possibility of infection with numerous infectious diseases. Therefore, scientists turned to discovering new antimicrobial compounds from natural sources such as the secondary metabolites of fungi as an alternative to current antimicrobial agents [5-7]. Sediment fungi are a diverse group of organisms grow in extreme and unique habitats that provide them the capability to produce unique secondary metabolites which have various biological activities [8]. Fungi have appeared recently as novel antioxidants sources as a part of their bioactive secondary metabolites [9]. In addition to antioxidants, fungi exhibit various bioactivities and functions like; antifungal, antimicrobials and larvicidal activities [6,10]. *Fusarium*, *Aspergillus*, and *Penicillium* species are the most famous producers of biologically active secondary metabolites due to their ability to produce alkaloids, peptides, steroids, terpenoids,

phenols, quinines, and flavonoids, with antimicrobial and antioxidant activities [11,12]. Numerous active components have been isolated from *Aspergillus clavatus*, *A. fumigatus*, *A. giganteus*, *A. terreus*, *Penicillium expansum*, *P. urticae*, and *P. griseofulvum* [13]. Moreover, destroying the larval stages in schistosomiasis prophylaxis is very important to break the transmission cycle. The isolates of *Aspergillus, Penicillium*, *Fusarium, Rhizopus* and *Coelomycete* species were reported to have toxic effect against miracidia and cercariae of *Schistosoma mansoi* [14, 15].

Therefore, the aims of the current study were to isolate, identify molecularly the *Fusarium oxysporum*; *Aspergillus fumigatus*; *Penicilluim griseofulvum* and to evaluate the bioactive compounds produced by fungi by studying the antimicrobial, antioxidant and larvicidal activities of their extracts. GC-MS/MS investigation, determination of total phenolic content and identification of their bioactive secondary metabolites were also studied.

MATERIALS AND METHODS

Fungal isolation and purification

F. oxysporum, A. fumigatus and *P. griseofulvum* were isolated from sediment samples collected in water courses of three Egyptian governorates; Giza, Qalubeya and Gharbeya during a year from January to December 2019. 25 g of each sediment sample were transferred to 250 ml of sterilized tap water in 500 ml Erlenmeyer flasks fitted with cotton plugs for fungal isolation [16]. At room temperature and constant speed (150 rpm) for 15 minutes the flasks were shaken. Then, they were left until complete sedimentation of the soil. Serial decimal dilutions were made from the original concentration to reach dilutions 1/100 and 1/1000. 0.5ml volumes were added into Sabouraud agar media. 3 replicate plates were prepared for each concentration and were incubated for one week at 28°C (±2°C). After that, rhea growth of fungal colonies was counted and recorded in a colony forming unit per milliliter (cfu/ml). Isolated species were sent for molecular confirmation.

Molecular identification of isolated fungal strains

Molecular identification has been recognized by DNA extraction, PCR and sequencing (18SrRNA). The isolated fungi were identified using the nuclear ribosomal internal transcribed spacer (ITS) region 1, 2, along with the short structural gene (5.8 S) as they have been investigated in the supporting information of previous studies. The region ITS was selected to identify fungi because it has been recently recognized as a common marker for fungal identification [17,18]. The PCR products purification was done to remove separate PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was achieved by means of Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). to determination of sequencing products, the applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) were used. ITS1 (5'- TCC GTA GGT GAA CCT GCG G-'3) and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-'3) were the Primer sequences used for the identification in the current study.

Cultivation and extraction of fungi

The three fungal strains; *F. oxysporum, A. fumigatus* and *P. griseofulvum* were cultured in 3 Erlenmeyer flasks (1 L volume) containing 50 g rice and 50 mL distilled water for each

fungus and sterilized for 20 min at 121°C (15 lb). Each flask was inoculated with spore suspension from 1 slant (10 days old). After incubation for 15 days at 30°C, the medium was extracted with ethyl acetate several times till exhaustion and by acetone followed by ethyl acetate [6].

GC-MS analysis

According to the reported procedures [19], GC-MS investigation 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). 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 1ml/min for GC-MS detection. MS transfer line temperature and the injector was set at 280°C. The oven temperature was programmed to an initial temperature of 50°C (hold 2 min) to 150°C at an increasing rate of 7°C/min, then to 270°C at an increasing rate of 5°C/min (hold 2 min) then to 310°C 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 the irrelative retention time and mass spectra with those of the NIST, WILLY library data of the GC-MS system.

Estimation of total phenolic content (TPC)

According to the method described by [20], all determinations were carried out in triplicate and the total phenolic content was determined using Folin-Ciocalteu's reagent. The reaction mixture was composed of (100 µl) of extract, 500 µl of the Folin-Ciocalteu's reagent and 1.5 ml of sodium carbonate (20%). Then mixture was shaken and made up to 10 ml using distilled water. Gallic acid was used as standard and the mixture was allowed to stand for 2 h, then the absorbance was measured at 765 nm. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract [20].

Estimation of total antioxidant capacity (TAC)

All experiments were carried out in triplicate and phosphomolybdenum method was used for determination the antioxidant activity of each extract using ascorbic acid as standard, which based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green colored [phosphate=Mo (V)] complex at acidic pH with a maximal absorption at 695 nm. In this method, 0.5 ml of each extract (200 µg /ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). The vials containing the reaction mixture were capped and incubated in a thermal block for 90 min at 95°C. The absorbance was measured at 695 nm against a blank after the samples had cooled at room temperature. The blank consisted of all reagents and solvents without the sample, and it was incubated under the same conditions. The antioxidant activity of the sample was expressed as the number of ascorbic acid equivalent (AAE) [21].

Antimicrobial activities of the fungal extracts

Four different test microbes namely: *Staphylococcus aureus* (G+ve), *Escherichia coli* (G-ve), *Candida albicans* (yeast) and *Aspergillus niger* (fungus) were used for the investigation of the antimicrobial activity of different fungal extracts by using the agar disc diffusion method. In case of bacteria and yeast, nutrient agar plates were heavily seeded uniformly with 0.1ml of 10^5-10^6 cells/ml. Antifungal activities evaluation, potato dextrose agar plate seeded by 0.1ml the fungal inoculum. Filter paper discs (0.5cm), loaded with 1mg from each extract, were placed on the surface of inoculated plates. At low temperature (4°C) for 2-4 hours the plates were kept allowing maximum diffusion. The plates were then incubated at 37°C for 24 hours for bacteria and at 30°C for 48 hours in upright position to allow maximum growth of the organisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter (mm). The experiment was carried out more than once and mean of reading was recorded [22].

Toxicity of the fungal extracts to *Schistosoma mansoni* larval stages (Miracidia & Cercariae).

The miracidicidal and cercaricidal activities of *F. oxysporum, A. fumigatus* and *P. griseofulvum* extracts were studied on *S. mansoni* miracidia and crecariae. *S. mansoni* ova and cercariae were obtained from Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI). The concentrations used were 50, 100, 150 and 200 ppm from each extract. For 10 ml of each concentration about 100 miracidia or cercariae were introduced. Another 10 ml of dechlorinated tap water containing about 100 miracidia or cercariae was used as control. Microscopical examination of the miracidial or cercarial movement was carried out after different intervals of time either in control and fungal extracts (1/4, 1/2, 3/4,1, 2 and 3 hours). Stationary miracidia or cercariae was assumed to be dead and the total number of miracidia or cercaria was counted at the end of the experiment after adding Iodine solution [23].

Statistical analysis

All data were presented as mean ± S.D. using SPSS 13.0 program (SPSS Inc. USA).

RESULTS

Molecular identification of fungal species

The isolated fungi were molecularly identified by applying the obtained sequences from DNA of fungal isolates into BLAST search, it was found that, the fungal isolates had a similarity of 99.62, 99.48, 100% with the previously identified fungi *Fusarium proliferatum* isolate 2463 (acc. no. EU821469.1), *A. fumigatus* isolate PRN1 (acc.no. MZ328890.1) and *P. griseofulvum* strain P-1707 (acc. no. JQ316516.1), respectively. The phylogenic tree of the fungal isolates was also constructed (Figures 1, 2 & 3). Based on the above identification techniques, our local fungal isolates were identified as *F. oxysporum f. sp. cucumerinum* isolate MT1, *A. fumigatus* isolate MT21 and *P. griseofulvum* isolate MT6 with the Gene Bank accession numbers of OM722087.1, OM722121.1 and OM722119.1, respectively. The fungal strain (MM1) culture was deposited in the Microbial Chemistry Department Collection of Microorganisms.

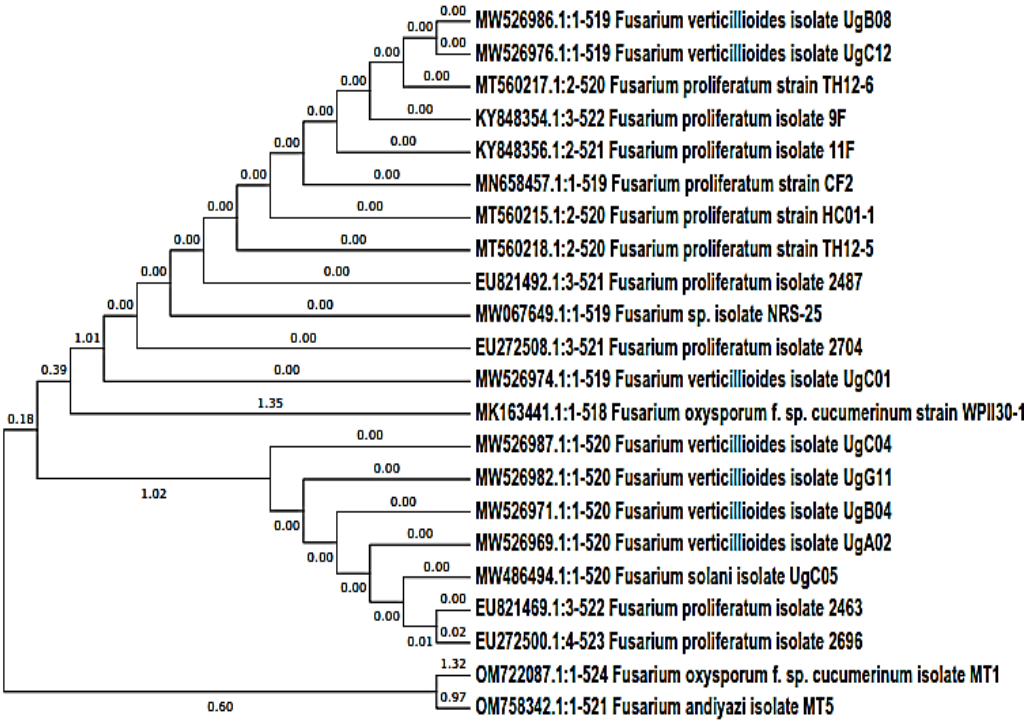

Figure 1. Phylogenetic tree of the *Fusarium oxysporum f.* sp. cucumerinum isolate MT1 strain. The phylogenetic tree has been reconstructed using MEGA7 software.

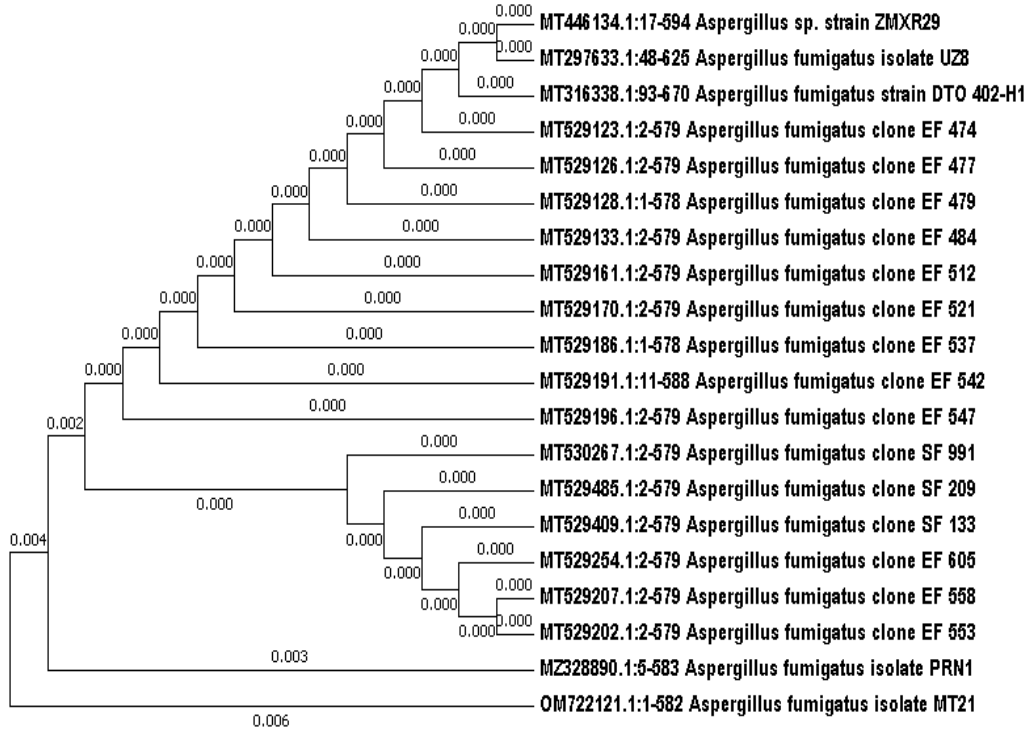

Figure 2. Phylogenetic tree of the *Aspergillus fumigatus* isolate MT21 strain. The phylogenetic tree has been reconstructed using MEGA7 software.

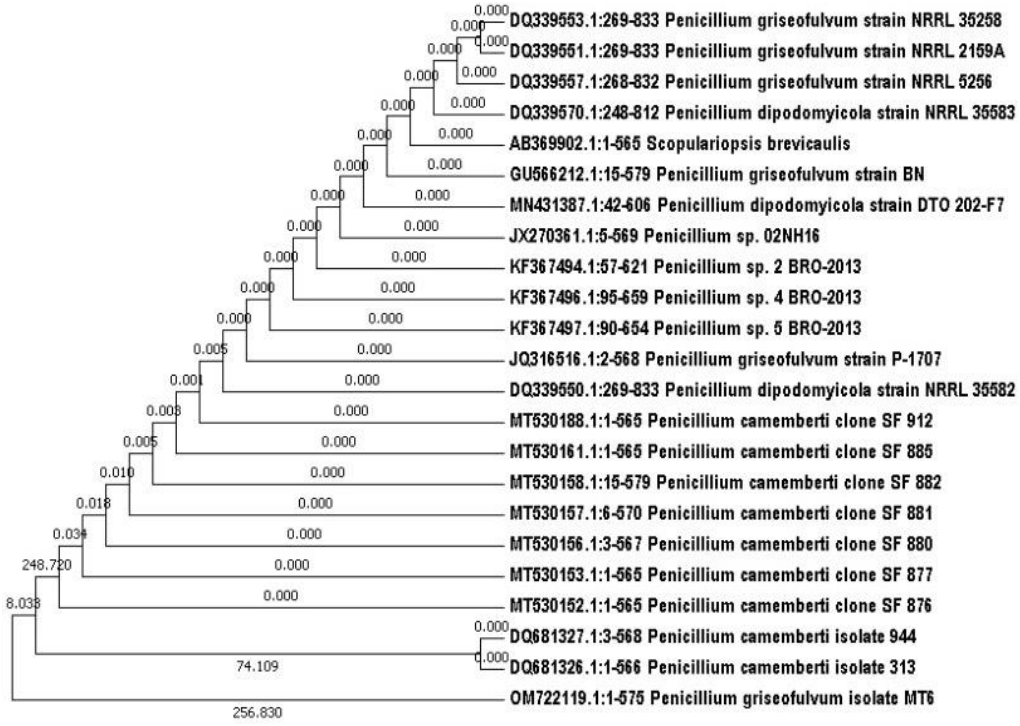

Figure 3. Phylogenetic tree of the *Penicillium griseofulvum* isolate MT6 strain. The phylogenetic tree has been reconstructed using MEGA7 software.

GC-MS investigations of the ethyl acetate extracts of tested fungal species

GC-MS examination of *F. oxysporum* f. sp. Cucumerinum extract led to identification of 15 compounds (Figure 4). The total peak areas of the identified ingredients constitutes 36.54%, the prospects of the chemical structures of the identified compounds are recorded in Table (1). The main detected compounds are Hexadecanoic acid (10.54%) and (P, P, R)-Dimethyl-5,5'-dihydroxy-1,1', 12, 12'-tetramethyl[6,6']bi(benzo[c] phenanthrenyl)-8,8' dicarboxylate (3.32%). Moreover, GC-MS/MS examination of *A. fumigatus* extract led to identification of 29 compounds (Figure 5). The total peak areas of the identified ingredients constitutes 77.24%, the prospects of the chemical structures of the identified compounds are recorded in Table (2). The main detected compounds are Hexadecanoic acid (15.77%), (R)-(-)-14-Methyl-8-hexadecyn-1-ol (15.64%), Hexadecanoic acid, methyl ester (5.78%), 9-Octadecenoic acid, methyl ester, (E) (5.25%), Hexadecanoic acid, ethyl ester (4.02%), Bicyclo[8.1.0]undecane (3.73%), 17-Pentatriacontene (2.04), and Tetradecanal (1.83%). While GC-MS/MS examination of *P. griseofulvum* extract led to identification of 24 compounds (Figure 6). The total peak areas of the identified ingredients constitute 78.32%, the prospects of the chemical structures of the identified compounds are recorded in Table (3). The main detected compounds are Hexadecanoic acid (27.59%), 9,12-Octadecadienoic acid (Z, Z) (16.65%), 9,12-Octadecadienoic acid (Z, Z), methyl ester (5.50%), and 5,6-Dihydro-4-methyl-2H-pyran-2-one (4.74%).

Table 1. Chemical composition of the ethyl acetate extract of *Fusarium oxysporum* f. sp. Cucumerinum.

No.	R_t	Area%	M.W.	M.F.	Identified compounds	Class/Category
1	26.62	1.41	161	$C_9H_7NO_2$	Phenol, 2-(5-isoxazolyl)	1-hydroxy-4-unsubstituted benzenoids
2	32.08	10.54	256	$C_{16}H_{32}O_2$	Hexadecanoic acid	Fatty acids
3	33.13	1.47	552	$C_{40}H_{56}O$	Lycoxanthin	Carotenol
4	33.54	1.58	686	$C_{41}H_{66}O_8$	(2R)-8,13-epoxy-2,2-(8',13'-epoxy-2'-bmethoxy-3'-oxolabdane-1'a,2'a-diyldioxy) 1a-hydroxylabdan-3-one	Triterpenoids
5	35.67	3.32	658	$C_{44}H_{34}O_6$	(P,P,R)-Dimethyl-5,5'-dihydroxy-1,1',12, 12'-tetramethyl[6,6']bi(benzo[c]phenanthrenyl)-8,8' dicarboxylate	Phenanthrols
6	36.46	1.39	696	$C_{40}H_{56}O_{10}$	Nephthoside-1,2',3',4'-Tetraacetate	Diterpenoids derivatives
7	37.65	2.59	694	$C_{48}H_{62}N_4$	2,7,12,17-Tetraethyl-3,5:8,10:13,15:18,20-tetra kis(2,2-dimethylpropano)porphyrin	Heterocyclic macrocycles
8	37.79	1.40	704	$C_{33}H_{36}O_{17}$	6-C-Xylosyl-8-C-glucosylapigenin permethylated derivative	Flavonoids
9	40.18	2.64	648	$C_{44}H_{56}O_4$	5,11,17,23-Tetratbutyl-25,26,27,28-tetrahydroxycalix-4-arene	Toluene derivatives
10	40.84	2.72	673	$C_{39}H_{47}NO_9$	1H-Cyclopent[c]isoxazole,1-[2,3:5,6-bis-O-(1-methylethylidene) -à-d-mannofuranosyl]hexahydro-4,5,6-tris(phenylmethoxy)-,[3aR-(3aà,4à,5á]	Heterocyclic derivatives
11	41.27	1.32	708	$C_{44}H_{36}O_9$	3,5-Diphenyl-3,5-(9,10-phenanthylene) tricyclo[5.2.1.0]decane-4-one-8-exo-9-endodicarboxylic acid diacetoxymethylester	Alkane derivatives
12	42.21	1.83	668	$C_{44}H_{48}N_2O_4$	2,13,15,26-Tetraoxa-1,14(1,3,2)-dibenzena-27(2,9)1,10-phenanthrolinabicyclo[12,12,1] heptacosaphan-7,14-diene	Heterocyclic derivatives
13	44.81	1.54	710	$C_{38}H_{50}N_2O_{11}$	Grandiflorine-1-acetate	Alkaloidderivatives
14	46.11	1.41	660	$C_{48}H_{56}N_2$	3-[9,9-Dihexyl-7-(pyridin-2-yl)fluororen-2-yl]-9-hexylcarbazole	Fluororen derivatives
15	50.75	1.38	711	$C_{43}H_{37}NO_9$	3,6-Diphenyl-3,6-(hydroxyimino)-4,5-(2,2'-diphenylene)tricyclo [6.2.1.0(2,7)]undeca-3,5-diene-9,10-(E)-dicarboxylic acid diacetoxy methylester	Alkane derivatives
		T%				
		36.54				

Rt: Retention time; M.W.: Molecular weight; M.F.: Molecular formula.

Table 2. Chemical composition of the ethyl acetate extract of *Aspergillus fumigatus*.

No.	Rt	Area%	M.W.	M.F.	Identified compounds	Class/Category
1	6.53	0.90	273	$C_{15}H_{15}NO_4$	3-[3'-(Ethoxycarbonyl)pyrrolidin-2'-ylidene]2-coumaranone	Coumaranone derivatives
2	7.96	1.05	243	$C_{17}H_{25}N$	3-Butyl-4-phenylquinuclidine	Phenylquinuclidine derivatives
3	8.37	0.89	406	$C_{31}H_{34}$	(3,6-Di-t-butyl-1-azulenyl)diphenylmethane	Azulene derivatives
4	14.74	1.02	186	$C_{11}H_{10}N_2O$	3-Methyl-2-vinyl-3H-quinazolin-4-one	Quinazolines
5	15.68	1.25	812	$C_{53}H_{64}O_7$	6-Hept-6-enyl-2-methoxy-4,2',6'-tris(4-methoxybenzyloxy)-4'-non-8-enylbiphenyl	Biphenyls and derivatives
6	15.74	1.25	690	$C_{54}H_{42}$	(E,E,E,E,E,E)-Hexakis(2-phenylethenyl)benzene	Linear diarylheptanoids
7	16.29	0.96	680	$C_{42}H_{48}O_8$	11,23-Di-tert-butyl-5,17-diethoxycarbonyl-25,26,27,28-tetrahydroxycalix[4]arene	Benzenoids derivatives
8	16.73	1.10	696	$C_{40}H_{56}O_{10}$	Nephthoside 1,2',3',4'-Tetraacetate	Diterpenoids derivatives
9	16.86	0.97	562	$C_{35}H_{46}O_6$	2-Hydroxy-3-methoxy-6,7,10,11-tetrabutyloxytriphenylene	Triphenylene derivatives
10	17.18	1.14	692	$C_{44}H_{44}N_4O_4$	N,N'-Dicyclohexyl-1,7-dipyrrolidinylperylene-3,4:9,10-tetracarboxylic acid bisimide	Perylene derivatives
11	18.92	1.62	636	$C_{36}H_{36}N_4O_7$	Methyl-13-hydroxyphaeophorbide-b	Pheophorbide derivatives
12	20.42	1.54	710	$C_{38}H_{50}N_2O_{11}$	Grandiflorine-1-acetate	Alkaloid derivatives
13	22.62	0.99	676	$C_{36}H_{60}N_4O_8$	Bis(3,6,9,12-tetraoxapentaethylene)crowno-N,N,N',N'-tetramethyl-p-phanediamine	p-phanediamine derivatives
14	27.30	1.23	658	$C_{44}H_{46}N_6$	1,2-di(3-(2-phenyl-7,7-dimethyl-4,6,7,8(5H)-tetrahydropyrrolo[3,2c] azepinyl)phenyl-diazo	Azepine derivatives
15	28.15	0.98	362	$C_{26}H_{50}$	Cyclohexane,1,1'-dodecylidenebis[4-methyl]	Cycloalkane derivatives
16	29.36	1.05	238	$C_8H_{14}O_4S_2$	Dimethyl-2-deoxyderythro-1-hexen-3-ulose-dithioacetal	Alkene derivatives
17	30.87	5.78	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	Fatty acid methyl esters
18	32.05	15.77	256	$C_{16}H_{32}O_2$	Hexadecanoic acid	Fatty acids
19	32.15	4.02	284	$C_{18}H_{36}O_2$	Hexadecanoic acid, ethyl ester	Fatty acid ethyl esters
20	34.0	3.73	152	$C_{11}H_{20}$	Bicyclo[8.1.0]undecane	Polycyclic hydrocarbons
21	34.10	5.25	296	$C_{19}H_{36}O_2$	9-Octadecenoic acid, methyl ester, (E)	Fatty acid ethyl esters
22	34.38	1.02	601	$C_{39}H_{55}NO_4$	6-Octadeca-sphingenine	Octadecanoic acid derivatives
23	35.18	15.64	252	$C_{17}H_{32}O$	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	Alkyne derivatives
24	35.49	1.83	212	$C_{14}H_{28}O$	Tetradecanal	Long-chain fatty aldehyde
25	35.63	2.04	490	$C_{35}H_{70}$	17-Pentatriacontene	Long-chain alkene
26	36.12	1.32	378	$C_{27}H_{54}$	Cyclohexane,1,3,5-trimethyl-2-octadecyl	Cycloalkanes
27	36.49	0.99	542	$C_{40}H_{62}$	Phytofluene	Carotenoids
28	38.14	0.94	708	$C_{44}H_{36}O_9$	3,5-Diphenyl-3,5(9,10-phenanthylene)tricyclo[5.2.1.0] decane-4-one-8-exo-9-endodicarboxylic acid	Alkane derivatives
29	49.98	0.97	718	$C_{40}H_{46}O_{12}$	Bicyclo[3.3.1]non-6-en-3-ol,7-methyl-,endo	Bicyclic alkene derivatives
		T%				
		77.24				

Rt: Retention time; M.W.: Molecular weight; M.F.: Molecular formula.

Table 3. Chemical composition of the ethyl acetate extract of *Penicillium griseofulvum*.

No.	Rt	Area%	M.W.	M.F.	Identified compounds	Class/Category
1	11.61	0.68	696	$C_{40}H_{56}O_{10}$	Nephthoside 1,2',3',4'-Tetraacetate	Diterpenoids derivatives
2	13.22	4.74	112	$C_6H_8O_2$	5,6-Dihydro-4-methyl-2H-pyran-2-one	Pyran derivatives
3	13.34	0.81	484	$C_{25}H_{24}O_{10}$	Tetramethyl-1,4-epoxy-1-(4'-methoxyphenyl)-1,4,5,8-tetrahydronaphthalene-2,3,6,7-tewtracarboxylate	Naphthalene derivatives
4	14.52	0.84	145	$C_7H_{15}NO_2$	3-Pentanol, 3-methyl, carbamate	Carbamate ester
5	15.59	0.69	99	$C_3H_5N_3O$	1-Methyl-3-hydroxy-V-triazole	Triazole derivatives
6	16.50	0.72	146	$C_7H_{14}O_3$	1,1-Dimethoxy-3-methylbutan-2-one	Ketone derivatives
7	17.18	1.24	98	$C_5H_6O_2$	Dihydro methyl furanone	Angelica lactone
8	17.67	1.98	596	$C_{40}H_{32}N_6$	m-Bis(3,4-diamino-2,5-diphenyl-6-pyridino)benzene	Pyridine derivatives
9	18.80	0.75	420	$C_{33}H_{24}$	2,7,12-Triethenyl-10,15-dihydro-5H-diindeno[1,2a-: 1',2'-c]fluorene	Fluorene derivatives
10	22.31	1.76	692	$C_{44}H_{44}N_4O_4$	N,N'-Dicyclohexyl-1,7-dipyrrolidinylperylene-3,4:9,10-tetracarboxylic acid bisimide	Pyrrolidine derivatives
11	23.84	1.04	378	$C_{27}H_{54}$	Cyclohexane, 1,3,5-trimethyl-2-octadecyl	Cycloalkane derivatives
12	24.32	3.15	679	$C_{42}H_{37}N_3O_6$	4,6-Dimethoxy-7-(4',6'-dimethoxyI-7'-(4",6"-dimethoxyindol-2"-yl)indol-2'-yl)-2,3-diphenylindole	Diphenylindole derivatives
13	28.13	0.77	210	$C_{15}H_{30}$	Germacrane-D	Germacrane sesquiterpenoids
14	30.86	2.03	270	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	Fatty acid methyl ester
15	30.97	1.01	676	$C_{41}H_{40}O_9$	5"-(1,1-Dimethylethyl)-2,2',2",2"',2""-pentamethoxy[1,1':3',1":3",1"' :3"'.1""-quinquephenyl]-3,3""-dicarboxylic acid	Quinquephenyl derivatives
16	32.04	27.59	256	$C_{16}H_{32}O_2$	Hexadecanoic acid	Fatty acids
17	32.14	1.54	214	$C_{13}H_{26}O_2$	Undecanoic acid, 2-methyl, methyl ester	Fatty acid methyl ester
18	33.73	0.99	256	$C_{17}H_{36}O$	1-Heptadecanol	Fatty alcohol
19	34.0	5.50	294	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z,Z), methyl ester	Fatty acid methyl ester
20	34.09	0.67	352	$C_{23}H_{44}O_2$	13-Docosenoic acid, methyl ester, (Z)	Fatty acid methyl ester
21	35.17	16.65	280	$C_{18}H_{32}O_2$	9,12-Octadecadienoic acid (Z,Z)	Fatty acids
22	35.53	1.35	310	$C_{20}H_{38}O_2$	Z-4-Octadecen-1-ol acetate	Alkene derivatives
23	37.59	0.70	184	$C_{10}H_{16}O_3$	5-Hydroxy-5-(1-hydroxy1isopropyl)-2-methyl-2-cyclohexen-1-one	Carotenoic acid derivatives
24	54.85	1.12	498	$C_{35}H_{46}O_2$	4'-Apo-á,.psi.-carotenoic acid	
		T%				
		78.32				

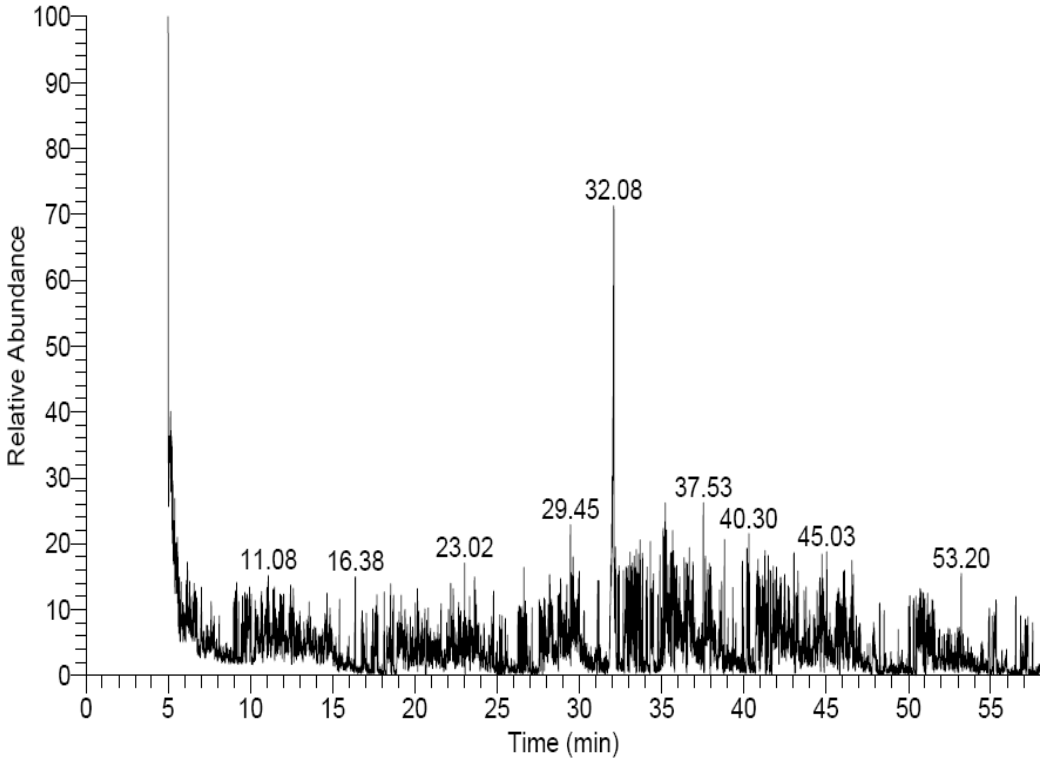

Figure 4. GC-MS chromatogram of the ethyl acetate extract of *Fusarium oxysporum* f. sp. Cucumerinum.

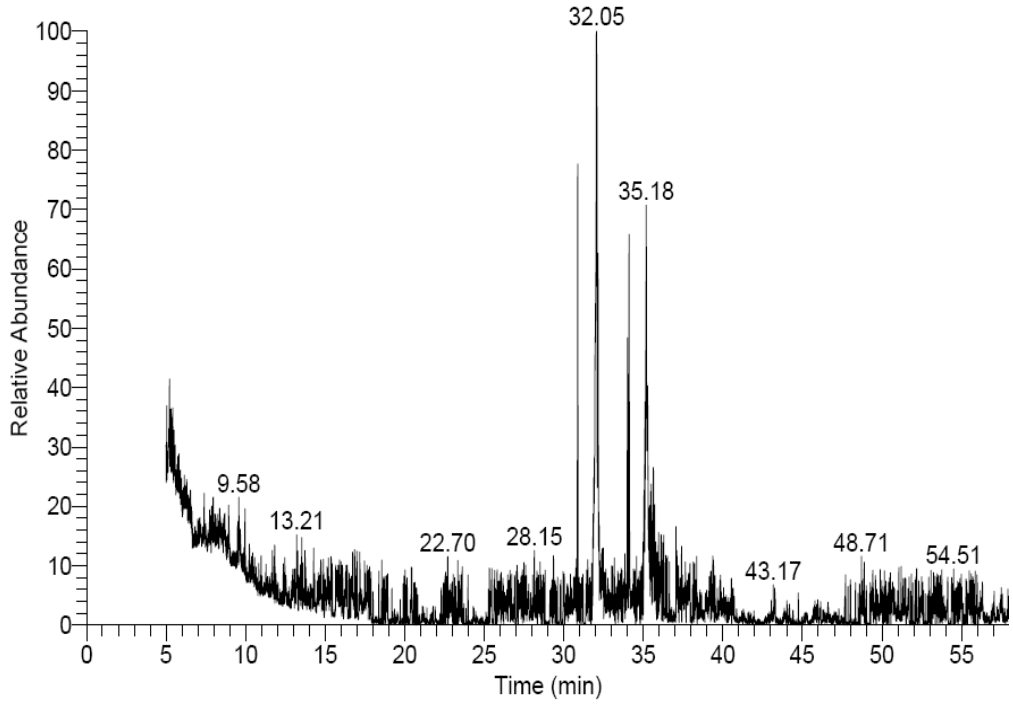

Figure 5. GC-MS chromatogram of the ethyl acetate extract of *Aspergillus fumigatus.*

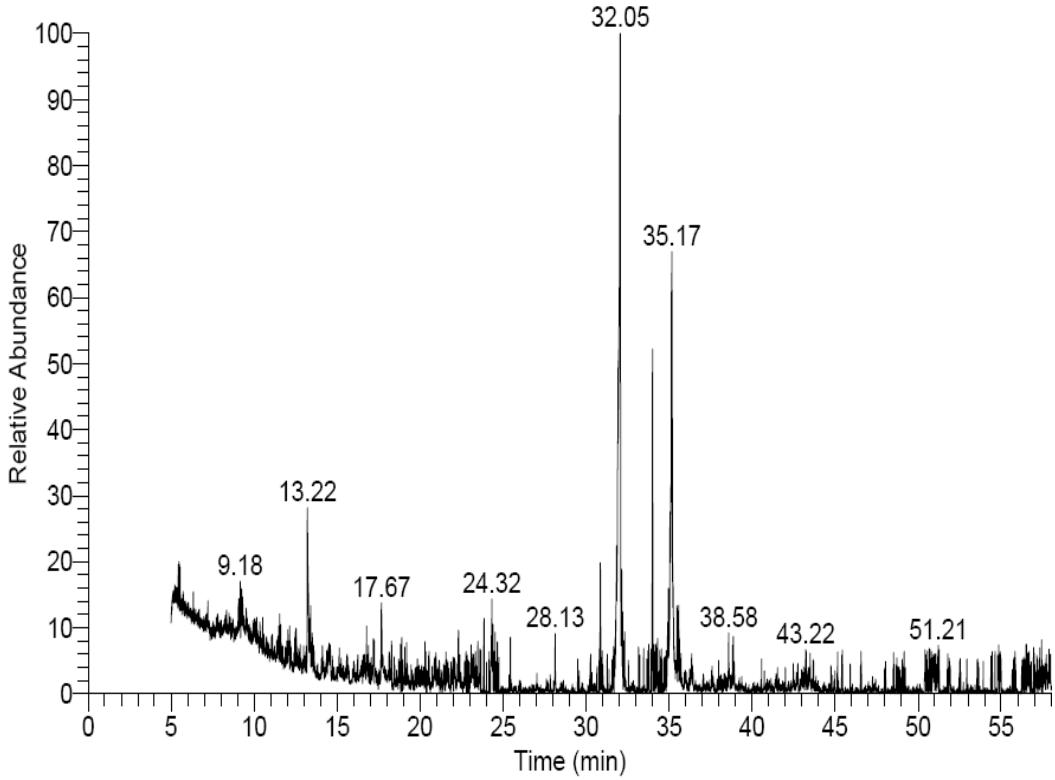

Figure 6. GC-MS chromatogram of the ethyl acetate extract of *Penicillium griseofulvum.*

Total antioxidant capacity and total phenolic content

As shown in Table 4, the ethyl acetate extract of *A. fumigatus* exhibited strong TAC value of 409.46 mg AAE/g dry extract, followed by *P. griseofulvum* and *F. oxysporum* extracts with TAC values of 299.28 and 281.31 mg AAE/g dry extract, respectively. Moreover, in Folin-Ciocalteu's assay, the tested fungal extracts showed noticeable TPC values of 115.84, 85.24 and 73.22, respectively for *A. fumigatus*, *P. griseofulvum* and *F. oxysporum* (Table 4).

Table 4. Total antioxidant capacity and total phenolic content of ethyl acetate extracts of *Fusarium oxysporum, Aspergillus fumigatus* and *Penicillium griseofulvum.*

Tested extracts	Total antioxidant capacity (mg AAE/g dry extract)[1,2]	Total phenolic content (mg GAE/g dry extract)[3]
Fusarium oxysporum	281.31 ± 3.05	73.22 ± 1.88
Aspergillus fumigatus	409.46 ± 3.25	115.84 ± 3.78
Penicillium griseofulvum	299.28 ± 5.03	85.24 ± 3.28

[1]Results are (means ± S.D.) (n = 3), [2]AAE (ascorbic acid equivalent), and [3]GAE (gallic acid equivalent).

Antimicrobial activities of the fungal extracts

Results in Table (5) and Figure (7) evaluated the antimicrobial activities of three ethyl acetate extracts of three fungal isolates (*F. oxysporum*, *A. fumigatus* and *P. griseofulvum*) grown on rice medium. It has been found that all extracts exhibited antimicrobial activities against all test microbes. Extracts of *F. oxysporum*, *A. fumigatus* and *P. griseofulvum* showed anti Gram-positive bacterial test bacterium *S. aureus* with

inhibition values of 12, 16 and 14mm, respectively. For the Gram-negative bacterial test microbe *E. coli*, extracts of *F. oxysporum*, *A. fumigatus* and *P. griseofulvum* exhibited inhibition values of 7, 13 and 11mm, respectively. Moreover, samples 1, 2 and 3 revealed anti yeast activities against *C. albicans* with inhibition values of 12, 18 and 18mm, respectively. In addition, antifungal activities were noticed for all extracts against *A. niger* with inhibition values of 13, 8 and 9mm, respectively. Purely isolated endophytic fungi were cultivated on rice as a medium at 30°C for 3-4 weeks. The secondary metabolites produced by fungi were extracted by ethyl acetate (EtOAc) as a solvent.

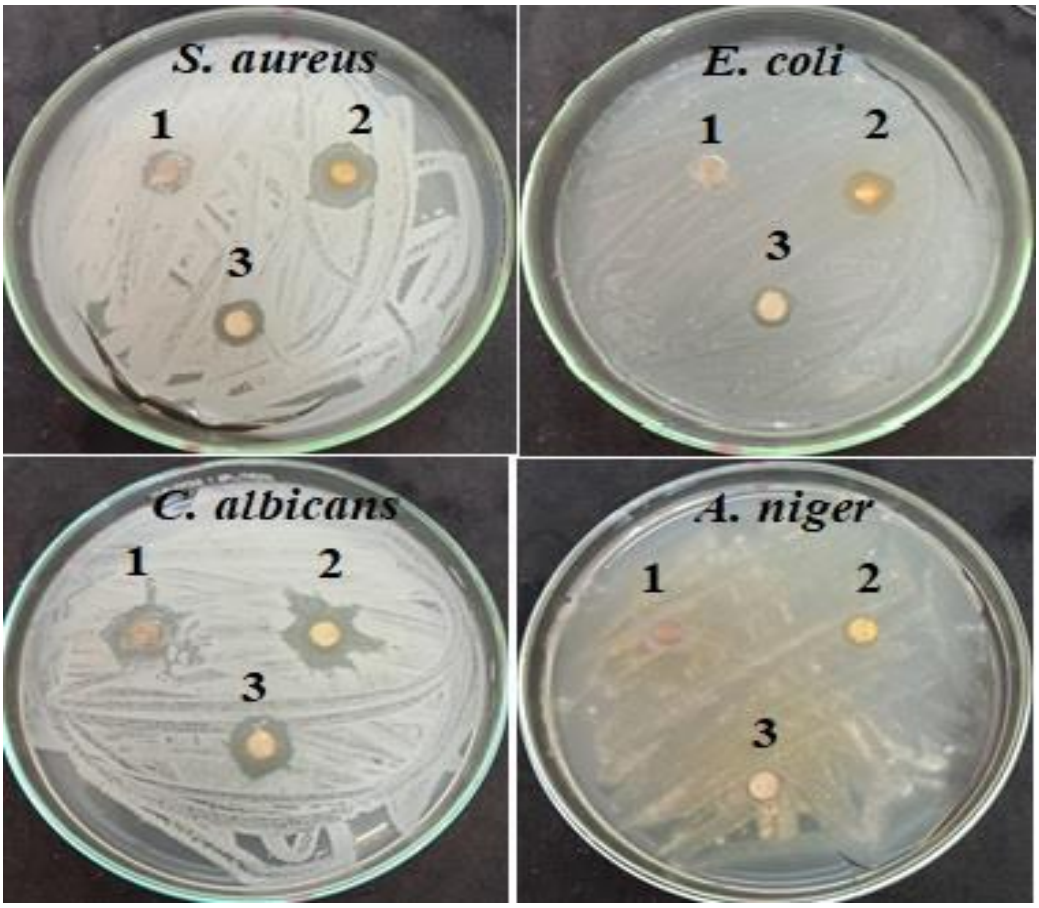

Figure 7. The antimicrobial activity of different isolated fungi grown on rice medium against different test microbes representing G+ve bacteria (*Staphylococcus aureus*), G-ve bacterium (*Escherichia coli*), Yeast (*Candida albicans*) and fungi (*Aspergillus niger*).

Table 5. Antimicrobial activity of different isolated fungi grown on rice medium against different test microbes

Fungal extract	Sample Name	Clear zone (ϕmm)			
		Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus niger*
Fusarium oxysporum	1	12	7	17	13
Aspergillus fumigatus	2	16	13	18	9
Penicilluim griseofulvum	3	14	11	18	8

Whereas G+ve bacteria (*Staphylococcus aureus*), G-ve bacterium (*Escherichia coli*), Yeast (*Candida albicans*) and fungi (*Aspergillus niger*).

Effect of fungal extracts on free larval stages of *Schistosoma mansoni* (Miracidia & Cercariae)

The data revealed that *A. fumigatus* excreted high mortalities of miracidia (Figure 8A, B and C) and cercariae (Figure 9A, B and C) followed by *P. griseofulvum* and *F. oxysporum* extracts. All fungal extracts exerted miracidial effect after 1/4 hour of their exposure to 50, 100, 150 and 200 ppm of the experimental extracts. Also, 100% of *S. mansoni* miracidia were killed after 3/4 hour of exposure to 100, 150 and 200 ppm of the tested extracts. While the mortality rate of cercariae reached 100% after 2 hours of exposure. *P. griseofulvum* and *F. oxysporum* showed cercaricidal effect after 1/2 hrs of exposure to 100 and 150 ppm, while *Aspergillus fumigatus* after 1/4 hrs of exposure to 200ppm. In general, the miracidia are more sensitive towards the toxic action of the tested agents than cercariae and the mortality percent of miracidia and cercariae is directly proportional to the time and the tested concentrations.

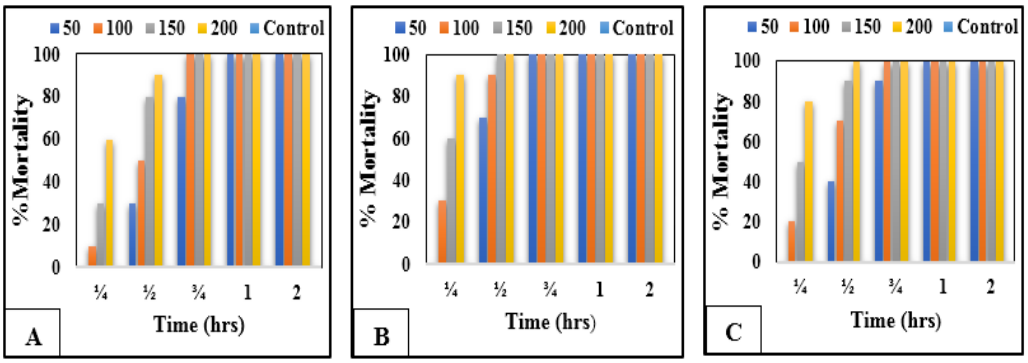

Figure 8. Miracidial activity of (A): *Fusarium oxysporum*, (B): *Aspergillus fumigatus* and(C): *Penicilluim griseofulvum* fungal extracts against *Schistosoma mansoni* miracidia post different exposure times.

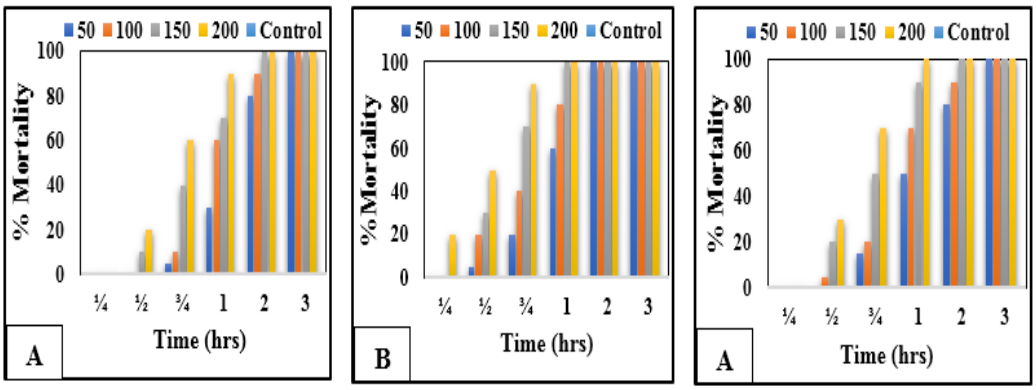

Figure 9. Cercaricidal activity of (A): *Fusarium oxysporum*, (B): *Aspergillus fumigatus* and (C): *Penicilluim griseofulvum* fungal extracts against *Schistosoma mansoni* miracidia post different exposure times.

DISCUSSION

The present study was conducted to assess the antimicrobial, antioxidant and larvicidal activities of bioactive compounds produced by *F. oxysporum*; *A. fumigatus*; *P. griseofulvum* fungal ethyl acetate extracts. The isolated fungi were molecularly identified using 18Sr RNA technique and were grown on rice medium for 14 days and extracted with ethyl acetate. Traditional fungal identification protocols have been

commonly applied and several new species till now are identified according to this method which was considered as time consuming and not accurate [24].

Moreover, GC-MS investigation, determination of total phenolic content and identification of their bioactive secondary metabolites were also studied. The identification was achieved via using computer search user-generated reference libraries, incorporating mass spectra [19, 22, 25, 26]. GC-MS investigation of *F. oxysporum* led to identification of some metabolites including methyl tetradecanone; oleic acid; eicosyl ester; methyl stearate; and bis(2-ethylhexyl) phthalate [27]. Moreover, different *Penicillium* species are characterized by the presence of biologically active compounds among them are polyketides [28-30] and alkaloids [31]. In the same context, GC-MS investigation of fermentation strain Snef1216 (*Penicillium chrysogenum*) led to identification of ten compounds like benzoic acid, 2-methoxy-, methyl ester; oxime-, methoxy-phenyl; cyclotrisiloxane, hexamethyl; undecane; benzoic acid, methyl ester; isopropyl myristate; hexadecanoic acid, methyl ester; 1,2-benzenedicarboxylic acid, butyl octyl ester; 8-octadecenoic acid, methyl ester (E) and heptadecanoic acid, 16-methyl-, methyl ester [32]. On the other side, GC-MS analysis of the ethyl acetate extract of *Aspergillus fumigatus* 269 isolated from Sungai Pinang Hot Spring, Riau, Indonesia led to identification of twenty-four components, the main identified constituents are eicosane, eicosane 2-methyl, phenol, 2,6-bis (1,1-dimethyl ethyl)-4-methyl, hexadecane 2, and 11-octadecenoic acid, methyl ester [33]. Different isolates from *A. fumigatus* isolated from fruit pulps were investigated for their chemical ingredients using GC-MS analysis led to identification of a set of fatty acids namely, 8,11-octadecadienoic acid, pentadecanoic acid, 4,7-octadecadienoic acid, hexadecenoic acid, heptadecanoic acid, cyclopropaneoctanoic acid, 5,9-octadecadienoic acid, 9,12-octadecadienoic acid and 6,8-octadienoic acid [34]. 17 compounds were detected by GC-MS analysis in the *A. fumigatus* culture filtrate including phenylethyl alcohol; 4-Hexyl-2,5-dioxo-2,5-dihydro-3-furanyl)acetic acid; phenol, 2,4-bis-(1,1-dimethylethyl); [(7-chloro-2,3-dihydro-1H-inden-4-yl)oxy](trimethyl)silane; 1-hexadecene; 4H-benzo[DE][1.6] nahthyridine,5,6-dihydro-8,9-dimethoxy; phenol,2,4-di-t-butyl-6-nitro-cis-4B,5; 1,4-benzenediol,2,6-bis(1,1-dimethylethyl)-; 6,9,12,15-docosatetraenoic acid, methyl ester; 12-hydroxy 14 methyl-oxa-cyclotetradec-6-en-2-one; 12-oxotricyclo[5.3.1.1(2,6)]dodeca-3,8-diene,11-acetoxy-4,5,9-trichloro; 2,4-oxazolidinedione, 3-(3,5-dichlorophenylo-5,5-dimethyl; (4-Methoxy-3-nitrophenyl) methano1, dimethylpentafluorophenylsilyl ether; 2h-1,4-benzodiazepin-2-one,7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy; (2e)-3-(3-chlorophenyl)-1-(4-chlorophenyl)-2-propen-1-one; 1-(3-chlor-phenyl)5-(2-methoxy-phenyl)-vinyl]-4,5-dihydro-1H-pyrazole;and picrotoxinin [35]. GC-MS analysis of *A. fumigatus* methanolic extract revealed the presence of 9-hexadecenoic acid; 2,4-dimethyl-5-methylthiopent-4-en-2-ol; E-11-hexadecenoic acid, ethyl ester; D-glucose; 6-*O*-α-D-galactopyranosyl; α-D-glucopyranoside, *O*-α-D-glucopyranosyl-(1.fwdarw.3)-β-; 6-acetyl –β-d-mannose , 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-; 5-hydroxymethylfurfural, β-D-glucopyranoside; methyl, tetraacetyl-d-xylonic nitrile; n-hexadecanoic acid; 9-octadecenoic acid , (2-phenyl-1,3-dioxolan-4-yl)methyl ester; octadecanoic acid; 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol,(3β,5Z,7E)- and pyrimidin-2-ol,4-(3,4-dimethoxyphenyl)-6-phenyl [36].

The tested fungal extracts showed high antioxidant activity and noticeable total phenolic contents (TPCs). Fungal extracts are known for their vital bioactivities [9, 37] among them are antioxidant activities [38-40]. The ethanolic extracts of *Penicillium chrysogenum* and *Penicillium fumiculosum* showed total antioxidant activity values of 3.874 and 3.171 μg AA/g, respectively. Moreover, the extracts exhibited total phenolic content values of 2.859 and 2.109 mg GAE/g, respectively [41]. Additionally, fermentation strain Snef 1216 of *P. chrysogenum* showed total phenolic content value of

135.77 mg GAE/g [32]. Furthermore, VLC fractions from the ethyl acetate extract of *Penicillium* sp. SAM16-EGY showed TAC values in the range from 212.53 to 687.56 mg AAE/g fraction [38]. In the same context, fungal extract of *A. fumigatus* displayed DPPH scavenging activity with LC_{50} value of 5µg/ mL [35].

By using the agar diffusion method [42], the antimicrobial activity of the extracts was tested against *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. For production of secondary metabolites, *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) were selected when grown in rice medium, extracted with ethyl acetate, and screened for their antimicrobial activity by agar well diffusion method. Fungal isolates were screened for antimicrobial activity against *Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium* and *Candida albicans*. The identification of the prospective endophytic fungi [43].

The data revealed that the tested fungal extracts exerted high mortality against miracidia of *S. mansoni* faster than cercariae and the mortality percent is directly proportional to the time and the tested concentrations. These observations are in agreement with the study of [44] on Kelthane that killed miracidia faster than cercariae. Also, [45] showed that miracidial mortality was greater than that of cercariae during application of Hinsan after the same time intervals. Results of the current study also revealed that *A. fumigatus* excreted high mortalities of miracidia and cercariae followed by *P. griseofulvum* and *F. oxysporum* extracts. This effect was directly proportional to the time and the concentrations tested. These findings are in accordance with those obtained by [15, 46, 47]. They reported that the mortality rate of *S. mansoni* miracidia and cercariae increased by increasing the concentration and exposure period of Abamectin, the herbicides Butachlor and Fluazifop-p-butyl, butanol extract of *Agave lophantha*, the insecticide (fenitorthion) and the fungal extract of *Aspergillus fumigatus*.

CONCLUSION

This study revealed that the *A. fumigatus* ethyl acetate extract is very promising as it showed the highest activities as antimicrobial, antioxidant and larvicidal agent followed by *P. griseofulvum* and *F. oxysporum*. Thus, it can be concluded that these fungi play an essential role in the production of several bioactive secondary metabolites which have multiple biological effects.

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AUTHOR CONTRIBUTIONS

Asmaa Abdel-Motleb, Mosad A. Ghareeb, Mohamed S. Abdel-Aziz and Maha A.M. El-Shazly: Conceived, designed the study, performed the experiments, analyzed, and interpreted the data, wrote the first draft of the manuscript. All authors revised the manuscript and formulated the final version. All authors revised the manuscript and formulated the final version. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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