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Chemical Profile Of Endophyte Culture Extracts Isolated From Two Arenga Species, And Evaluation Of Their Antimicrobial And Antioxidant Activities

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Abstract

Fungi are considered a vital source of chemical entities possessing a wide range of biological potentials. The aim of this work is investigating the fungal endophyte associated with *Arenga pinnata* and *Arenga engleri* to be isolated, purified, cultured, extracted with acetone & ethyl acetate, and the obtained extracts were investigated for their total phenolic content and total antioxidant capacity. Based on the results two endophytic fungi (*Stemphylium simmonsii* and *Aspergillus terreus*) were selected for more indepth chemical profiling, radical scavenging and antimicrobial investigation. *S. simmonsii* and *A. terreus* culture ethyl acetate extracts were investigated for their antioxidant activity using DPPH assay for estimation of the diffusion inhibition zones and minimum inhibitory concentrations to assess their antimicrobial potential against *S. aureus* (Gram+ve), *E. Coli* (Gram-ve) and *C. albicans* (fungi). The chemical profile was conducted using LC-MS for tentative identification of the produced secondary metabolites. Fourteen and eighteen secondary metabolites were identified from *S. simmonsii* and *A. terreus* culture ethyl acetate extracts respectively. Anthracenes were the major metabolites of *S. simmonsii* whereas fatty acyls were the major metabolites of *A. terreus* culture ethyl acetate extract. *S. simmonsii* ethyl acetate extract was active against both Gram +ve and Gram -ve bacteria with inhibition zone 36 and 35 mm and MIC 4.88 and 4.85 µg/ml respectively. *A. terreus* ethyl acetate extract showed higher potential against Gram -ve bacteria with inhibition zone 33 mm and MIC 4.88 µg/ml. Further investigation and bio-guided fractionation are recommended to be carried out on both extracts to isolate the most active fractions and pure compounds for a better understanding of the results obtained.

Keywords: Endophyte; LC-MS; Stemphylium, Aspergillus, total phenolic; antimicrobial, antioxidant

1. Introduction

Endophytic fungi are living microorganisms that are hosted partially or totally inside the plant tissues over their life span [1]. During their symbiotic life cycle with the host, endophytic fungi through their metabolism and defensive mechanisms produce a wide range of low-molecular-mass secondary metabolites (SMs) with diverse chemical structures. [2,3]. The produced SMs roles are mainly concerned with the organism's survival under different environmental parameters, and in the case of plant pathogenic fungi, many of these metabolites are factors that have toxic effects on plant cells and, others are produced as potential mycotoxins. So, through symbiotic or pathogenic relationships of endophyte to the host plant, SMs produced represent a research focal point for natural product researchers to discover new biological active agents. Until now, humans have always been dealing with the uprising situations of spreading microbial infections. since kingdom fungi is known for being a valuable source of different bioactive secondary metabolites that have a powerful therapeutic potential that started from the discovery of penicillin in 1928 isolated from *Penicillium notatum* [4]. One of the challenges during the management of bacterial infections is the continuous development of bacterial resistance to current antibacterial compounds [5]. So, the urge to discover novel antibacterial agents with higher potential and new mechanistic antibacterial action increased and became an important research point especially from natural sources including terrestrial, plant and marine environments [6]. Among these sources, fungi is well known to produce several classes of secondary metabolites during their lifespan and most importantly for their colony protection the produced metabolites possess powerful antibacterial effect to prevent invasion [4].

The fungal genus Stemphylium comprises several pathogenic species to Fabaceae members and other plant species including garlic, onion, parsley, tomato and beet [7]. Chemically, *Stemphylium* species have been reported to synthesis a variety of SMs, that act as phytotoxins during plant pathogenicity [8]. Several reports have revealed that Stemphylium SMs have several biological potentials, including cytotoxic and antibacterial effects [9-11]. The marine and mangrove derived fungus Stemphylium is another good source for biologically active anthraquinones such as altersolanol and macrosporin derivatives, ampelanol, alterporriol, and auxarthrol C which showed antibacterial activity against wide range of Gram +ve and Gram -ve bacterial strains [12].

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Among the discovered fungi, genus Aspergillus is the most diverse fungal species identified with huge diversity in the produced and identified secondary metabolites. The chemical diversity of this genus is because of Aspergillus are fast growing and salt tolerant species that can grow easily in wide range of culture media [13]. Species of genus Aspergillus have been extensively reported as source of several chemical classes with significant biological potential, most importantly with antibacterial activity [4,14,15]. According to previous reports, a growing number of Aspergillus species, have been studied and proven to produce novel antibacterial bioactive SMs, such as asperchondols A and B, aspergivones A and B and aspochalasin B and D [16-18]. Another reports have highlighted the antibacterial efficacy of ethyl acetate extracts of different Aspergillus species [19,20].
The aim of this work is to isolate and purify fungal endophytes associated with plant samples from two *Arenga* species, extract the culture media of the grown endophytes using two solvents (acetone and ethyl acetate), and then evaluate the total phenolic content, total antioxidant activity, and antimicrobial efficacy of the obtained extracts. The most active strains will be selected for LC-MS chemical profiling of the produced metabolites, and the activity will be correlated with the produced secondary metabolites (SMs).

2. Methods and Materials

General Experimental Material

Solvents for endophyte extraction were obtained from El Nasr Chemicals Co, Egypt. HPLC grade solvents were obtained from Merck, Germany. **DNA** extraction by ABT DNA mini extraction kit (Applied Biotechnology Co. Ltd, Egypt)**,**2X Red master Mix (Applied Biotechnology Co., Egypt), Oligonucleotide (AlphaDNA Co, Canada). Agarose gel (1%), Tris acetate EDTA buffer (TAE buffer) 50 X, Ethidium bromide (Bioshop company), Molecular Marker (DNA ladder). Fungal growth media, potato dextrose agar (PDA: potato infusion, dextrose and agar) and bacterial growth media, nutrient agar medium with the following ingredients (g/l): peptone (5), meat extract (3) and agar (15-20) and pH of 7.0 obtained from Merck, Germany. *Staphylococcus aureus* ATCC 6538 (Gram +ve), *Escherichia coli* ATCC 25922 (Gram -ve), and *Candida albicans* ATCC 10231 (yeast) obtained from Microbial Chemistry Laboratory, National Research Center, Egypt.

Plant samples

Arenga pinnata (Wurmb) Merr and *Arenga engleri* Becc. leaves were collected from Zohrea Garden, EL-Giza, Egypt in May 2023 and authenticated taxonomically by Dr. Therese (Plant specialist) at Mazhar Garden, El-Giza, Egypt. A voucher sample of 03*Aen*/2024 and 34 38*Api*/2024 for *A. engleri* and *A. pinnata*, respectively were archived at the Pharmacognosy Department Herbarium (Faculty of Pharmacy, Helwan University), Cairo, Egypt.

Fungal isolation and extraction

Small pieces of *A.pinnata* and *A. engleri* leaves were cleaned with sterile distilled water followed by disinfection of the surface by 70% aqueous methanol (three times, 2 min), then washed with sterilized distilled water and aseptically dried [21,22]. The outer surface removed using a sterile blade, and the inner part was cut into small parts, and then carefully placed onto agar plates of PDA [23]. In order to favor the fungal growth, an antibacterial (neomycin 50 mg/L) was added to arrest the bacterial growth [24] and the plates incubated at 27°C for 3-6 weeks. The resultant colonies were transported based on the morphological differences to new PDA media then the solation and purification of the pure strains was performed by repeated sub-culturing (Supplementary figure 1). Pure fungal isolates were fermented using two 1 L Erlenmeyer flasks. The culture media used contains rice (100 g) and distilled water (100 mL), sterilized for 20 min at 121°C (15lb). The prepared culture flasks were seeded with spore suspension from 10 days old slant. After 14 days of incubation (30°C), the culture media were extracted several times with ethyl acetate till exhaustion that monitored with TLC. Ethyl acetate was evaporated by rotary evaporation to afford twelve ethyl acetate extracts. This step was then repeated using acetone as an extraction solvent to afford twelve acetone extracts.

Fungal identification

DNA extraction and polymerase chain reaction

Pure isolated colonies are collected using a sterilized toothpick and suspended in sterile saline (0.5 ml) centrifuged for 10 min at 10,000 rpm. The supernatant removed and, the produced pellet is resuspended in InstaGene Matrix (Bio-Rad, USA) (0.5ml) and incubated for 30 min (56°C), then the mixture heated for 10 min at 100°C to make the produced supernatant ready for PCR. 1 µl of template DNA added in 20 µL of PCR reaction mixture. 27F/1492R primers for bacteria utilized and then conduct thirty-five amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. The DNA fragments are amplified at about 1,400 bp in the case of bacteria. A positive control of genomic DNA of *Candida sp.* and a negative control in the PCR was included. Remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore) [25]. To confirm the targeted PCR amplification, five µl of the PCR product was electrophoresed along with 100bp DNA molecular weight 1% agarose gel containing ethidium bromide (at the rate of 0.5µg/ml) at constant 80V for 30min in 1X TAE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by Samsung Note4 smart phone.

Sequencing of the PCR product:

The amplified PCR products were submitted to Solgent Co Ltd (South Korea) for gel purification and sequencing. The resulted sequences were trimmed and assembled in Geneious software (Biomatters). Consequently, the trimmed sequences were identified by searching in basic local alignment search tool (BLAST) in GenBank. They used ITs 2 and 3 at PCR and ITs 1 and 4 at sequencing (Table 1), using *Candida* sp. as control.

Table 1. Internal transcribed spacer region (ITS) sequencing

Name	Primer sequence
TS1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC

Total phenolic assay

The total phenolic content was measured using different concentrations of twelve ethyl acetate extracts, and twelve acetone extracts as well as the two methanolic plant extracts by Folin-Ciocalteu reagent (FCR) assay [26]. A mixture of sample (50µl), water (950µl), FCR (500µl), 20% sodium carbonate solution (2.5 ml) was prepared. The mixture was incubated at room temperature for 40 min and the absorbance (725 nm) was recorded. A standard calibration curve of was prepared using gallic acid as a reference as the total phenolic contents were presented as gallic acid equivalent (GAE) (mg/g). A control solution was tested using distilled water instead of the sample and the absorbance was calculated based on the control results. The results were estimated from the gallic acid standard calibration curve based on the following equation: **y=0.00x-0.0139**

LC-MS metabolite profiling

The LC-MS profiling was conducted through chromatographic separation using 6530 Q-TOF lc/ms (agilent technologies) supplied with an autosampler (G7129A), and column comp (G7116A), quaternary pump (G7104C), at faculty of pharmacy-Fayoum university, Egypt. Zorbax RP-18 column (dimensions: 150 mm × 3 mm, dp = 2.7µm) from Agilent technologies, injection volume (1 µl) and flow rate (3ml/min). The mass spectrum was acquired using ESI in (-) ionization modes with a capillary voltage of 4000 v. The mass spectra m/z range was adjusted to 50 to 3000 m/z. The gas temperature and drying gas flow were 250^0C and 8 l·/min, respectively. The collision energy was 10v and skimmer and fragmentation voltages (65 and 130 v), respectively. the nebulization pressure (58psi).

Estimation of antioxidant potential

Total antioxidant capacity

The total antioxidant capacity (TAC) of each fungal extract and plant extracts was evaluated using phosphomolybdenum method [27]. A mixture of 100 µL extract solution was combined with 900 µL of phosphomolybdenum reagent solution. Deionized water (100 µL) was used to prepare tested blank instead of extracts. For 90 min, the mixtures were kept in a boiling water bath (95°C). After cooling at room temperature, the absorbance was measured (695nm) using Shimadzu UV1024-PC spectrophotometer. A standard calibration curve has been constructed using ascorbic acid (0.2-1mg/ml) to determine the total antioxidant as ascorbic acid equivalent. The total antioxidant activity was calculated by the following equation: **y=0.0036-0.2129**

Determination of antioxidant activity by DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was adopted to evaluate the free radical scavenging capacity of the ethyl acetate and acetone extracts of endophytic isolates W5 and W7 following the methods reported by Wu et al., 2019 [28]. Briefly, 50µL of each extract with the following concentrations (1000-500-250-125-62.5 and 31.25 µg/mL) was mixed with 1950 µL DPPH solution (100µM). The mixture was shaken and kept for 30min in dark 25^0C[29]. Absorbance was measured at 517 nm. The DPPH scavenging activity was estimated according to the following equation:

DPPH scavenging activity % = A_0-A_E/A_0×100

Where A_0 and A_E are the absorbance of the control and extract, respectively, then the IC_{50} for each extract was calculated.

Estimation of antimicrobial potential

Antimicrobial activity by agar well diffusion method

Ethyl acetate and acetone extracts of twelve endophytic isolates were tested against *S. aureus* (Gram+ve), *E. Coli* (Gram-ve) and *C. albicans* (fungi)starting by dissolving 10mg in DMSO (2ml) then the antimicrobial activity was measured using the agar well diffusion technique. In the nutrient agar medium plates, the test microbes were seeded 0.1ml of 107-108 cells/ml. A hole (1cm diameter) was created in the agar media by a sterile cork borer. After that a specific amount of tested sample (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours for bacteria and at 30ºC for 48 hours in an 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 the reading was recorded [30,31].

Determination of minimum inhibitory concentrations (MIC's)

The ethyl acetate and acetone extracts of two endophytic W_5 and W_7 were evaluated for their MIC. A sterile labeled 96well microplates were prepared for testing. 50µL of tested extracts in DMSO (5mg/mL stock concentration for extracts) was inoculated into the first row of the 96wel plate. 50 µL broth medium was added to all other wells and serial dilutions were performed. 10µL of resazurin indicator solution was mixed with the content of each well. Bacterial suspension (5 x106 cfu/mL) with volume of 10µL was inoculated to each well. The plates were prepared (n=2) and incubated for 18–24 h at temperature of 37°C. Visual examination was adopted for the color change where any color changes from purple to pink or colorless were recorded as positive results. MIC was calculated as the lowest concentration at which color change happened [32,33].

Determination of minimum bactericidal concentrations (MBC's)

From the inhibition zone of MIC plates, streaks were swapped from two plates having the lowest concentrations of the ethyl acetate and acetone extracts of two endophytic W_5 and W_7 which exhibit invisible growth. The swaps were sub-cultured onto sterilized nutrient agar plates. The plates were kept inside the incubator for 24 h at 35°C then visually inspected for any bacterial growth in comparison to sample concentration. MBC was calculated as the tested concentration of extract that did not show any bacterial growth [32,33].

3. Results

Fungal isolation and Identification of the isolates

Culturing of the sterilized pieces of *A. pinnata* leaf on solid PDA media afforded twelve different endophytic fungi (W_1-W_{12}) according to mycelia, colony, and morphological features. The fungal isolates W_5 & W_7 were molecularly identified by 18SrRNA protocol. W_5 fungus was isolated from *A. engleri* samples and W_7 fungus was isolated from *A. pinnata* samples. Nucleotide sequences of 644 and 607bp of the whole 18S rRNA gene of W_5 & W_7 (respectively) were determined in both strands. Blast search revealed that Isolate W_5 exhibited 100% similarity to *Stemphylium simmonsii* culture WAC: 3063 (acc. no. MN401378.1) whereas W_7 exhibited 100% similarity to *Aspergillus terreus strain* OUC-MDZ5136 (acc. no. MK351266.1). The base sequences of the identified microorganisms and the AB1 for the isolated microbes are presented in the supplementary file (Supplementary figure 2-3). The phylogenetic trees of these fungi were also constructed (Figure 1 and 2) of W_5 & W_7 respectively. These microbes were identified as *Stemphylium simmonsii* isolate W_5 and *Aspergillus terreus* isolate W_7 with the GeneBank accession numbers PP372679 and P359608 for W_5 and W_7, respectively. The traditional fungal identification is also done by cultivation and microscopic examination including mycelial color, size and shape as well as conidial shape and the morphology of conidiophores ([34,35]. These methods need highly experienced taxonomists and also time consumed [36]. But molecular methods are considered as effective, fast and easy tools for fungal identification [37]. Endophytic fungi represented a great part of undiscovered fungi. They are considered as a prolific source for novel, potential and active metabolites. The main role of bioactive compounds produced by endophytic fungi is to help plants to be protected from extremal biotic and abiotic stress and thus helping the plants to survive [38]. Alternaria, Penicillium and Talaromyces spp. were among the isolated and molecularly identified endophytic fungi based on morphological features [39-41].

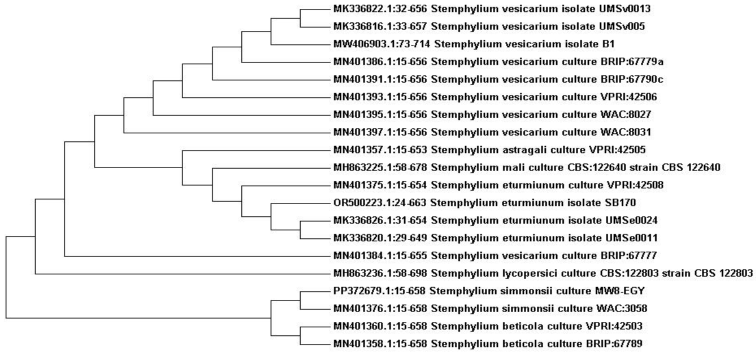

Fig. 1. Phylogenetic trees showing relationship of strain W_5 with other related fungal species retrieved from GenBank based on their sequence homologies of 18srDNA

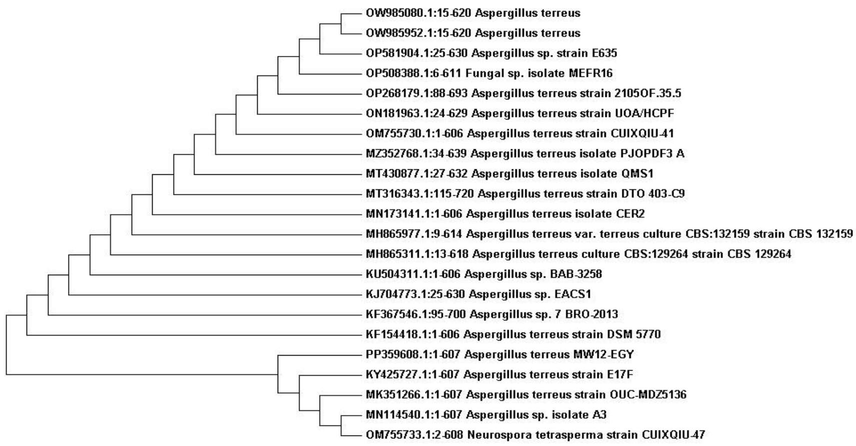

Fig. 2. Phylogenetic trees showing relationship of strain W_7 with other related fungal species retrieved from GenBank based on their sequence homologies of 18srDNA

Estimation of the total phenolic contents of fungal isolate extracts

The rice culture of the twelve fungal isolates were extracted separately with ethyl acetate and acetone to afford 24 extracts that were subjected to estimation of their phenolic content. The results revealed that the ethyl acetate extract of fungal isolates ($W_{5\&7}$) showed the highest phenolic content 1259, 1270 GAE/g dry weight respectively, and for the acetone extract, the fungal isolate (W_7) showed also the highest phenolic content with estimated value of 1311.8 GAE/g dry weight but the acetone extract of fungal isolate (W_7) showed low total phenolic content with estimated value of 193.58 GAE/g dry weight (Table 2, Figure 3).

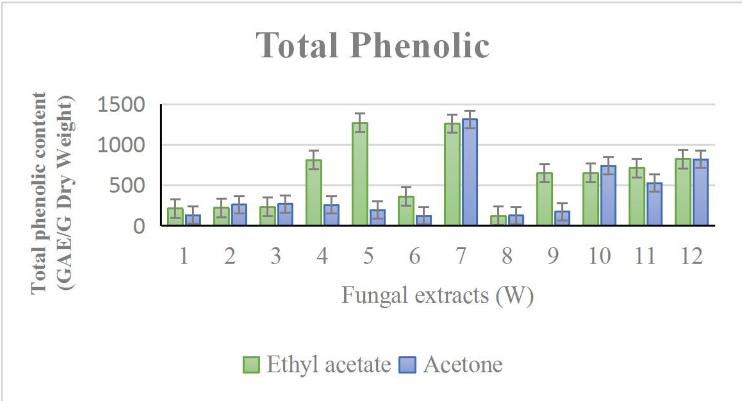

Fig. 3. Total phenolic content of ethyl acetate and acetone extracts from fungal endophytes grown on rice medium

Table 2. Total phenolic contents of ethyl acetate & acetone extracts from fungal endophytes grown on rice medium

Fungi serial number (W)	Total phenolic content (GAE/g dry weight) Ethyl acetate extracts	Total phenolic content (GAE/g dry weight) acetone extracts
1	212.67 ± 3.785	128.12 ± 5.914
2	220.21 ± 9.745	259.94 ± 8.447
3	233.88 ± 8.592	268.12 ± 9.635
4	811.46 ± 13.757	255.39 ± 5.48
5	1270.2 ± 12.106	193.58 ± 5.48
6	358.73 ± 10.909	120.55 ± 0.909
7	1259.9 ± 14.78	1311.8 ± 8.249
8	122.67 ± 1.389	125.09 ± 4.545
9	649.64 ± 6.364	171.15 ± 4.296
10	652.37 ± 5.53	738.73 ± 3.278
11	711.76 ± 6.943	526.61 ± 5.845
12	822.97 ± 3.193	816.61 ± 3.785

GAE: gallic acid equivalent, n=3

LC-MS tentative chemical profile of ethyl acetate extract of *S. simmonsii*

The obtained ethyl acetate extract of the rice media of fungal isolate W_5 (*S. simmonsii*) and W_7 (*A. terreus*) were subjected to LC-MS analysis to tentatively determine the chemical profile of the produced secondary metabolites. The obtained chromatogram was processed (Figure 4 & Figure 5), and the data were compared to the literature and available chemical library including PubChem, and reaxys through comparison of molecular weight and retention time in order to tentatively identify fourteen secondary metabolites from S. *simmonsii* extract (Table 3, Figure 6) and eighteen secondary metabolites from *A. terreus* extract (Table 4, Figure 7).

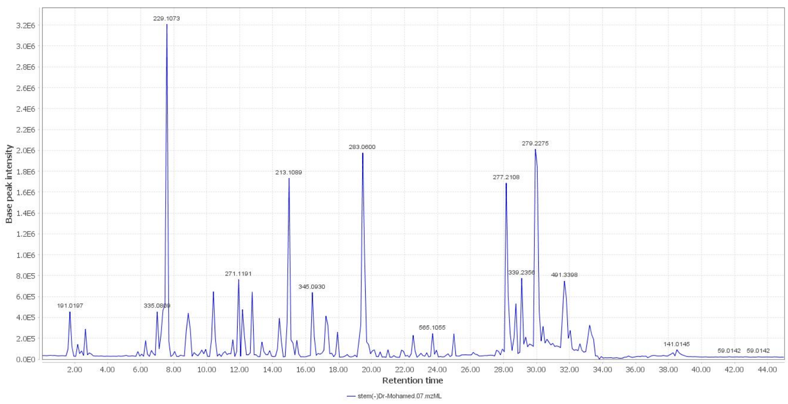

Fig. 4. LC-MS profile (Total Ion Chromatogram acquired in negative ion mode of *S. simmonsii* ethyl acetate extract

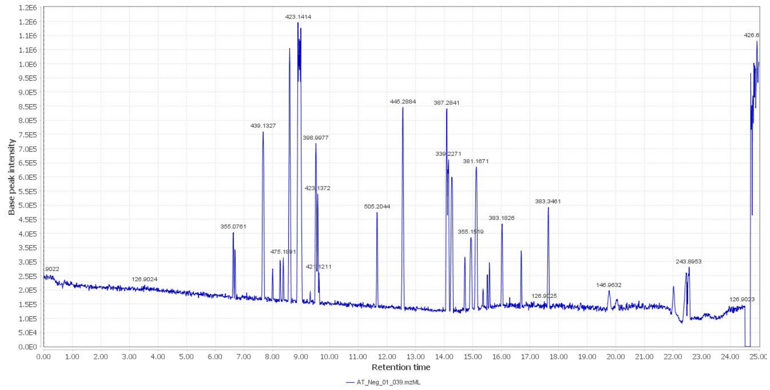

Fig. 5. LC-MS profile (Total Ion Chromatogram acquired in negative ion mode of *A. terreus* ethyl acetate extract

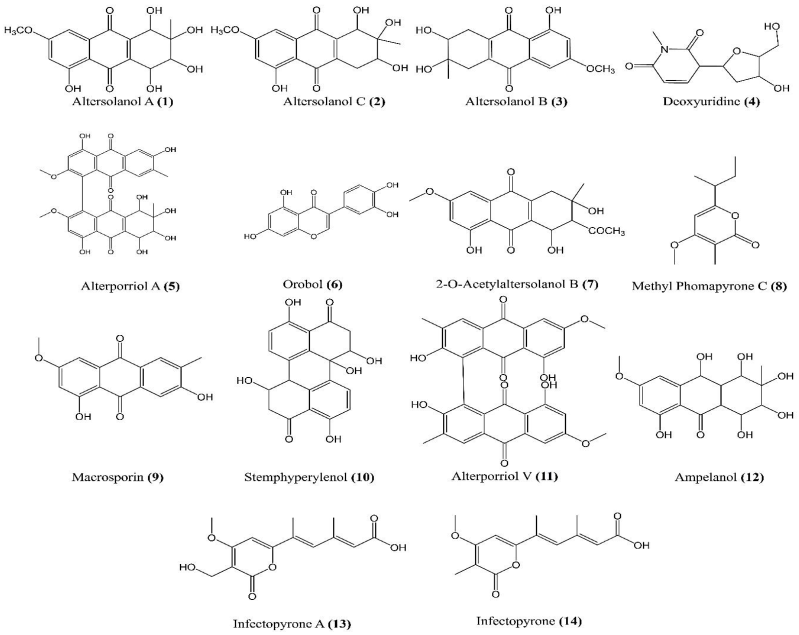

Fig. 6: LC- MS identified phytochemicals (1-14) for crude *S. simmonsii* ethyl acetate extract

Table 3. LC-MS tentative profile of the secondary metabolite's identification for *S. simmonsii* ethyl acetate extract.

	Compound	Rt	m/z $[M-1]^-$	Chemical class	References
1.	Altersolanol A (Stemphylin)	7.00	335.0809	Anthracenes	[42,43]
2.	Altersolanol C (Dactylariol)	8.88	319.0779	Anthracenes	[44] [42]
3.	Altersolanol B (Dactylarin)	12.17	303.0854	Anthracenes	[43,45]
4.	Deoxyuridine	14.41	227.1246	Pyrimidine	[46]
5.	Alterporriol A or B	14.51	617.1238	Anthracenes	[9]
6.	Orobol	15.46	285.0395	Isoflavonoid	[7]
7.	2-*O*-Acetylaltersolanol B	16.40	345.0930	Anthracenes	[11]
8.	Methyl phomapyrone C	17.23	195.9960	Pyran derivative	[46,47]
9.	Macrosporin	19.46	283.0600	Anthracenes	[43]
10.	Stemphyperylenol	22.52	367.1905	Phenanthrene derivatives	[48]
11.	Alterporriol V	23.69	565.1055	Anthracenes	[11]
12.	Ampelanol	29.10	339.2356	Anthracenes	[43]
13.	Infectopyrones A	29.92	279.2275	Fatty acyls	[11]
14.	Infectopyrone	30.98	263.0930	Fatty acyls	[11]

*Rt: retention time

Table 4. LC-MS tentative profile of the secondary metabolite's identification for *A. terreus* ethyl acetate extract.

	Compound	Rt	m/z $[M-1]^-$	Chemical class	References
15.	Butyrolactone II	6.64	355.0761	Fatty acyls	[14]
16.	Aspulvinone E	6.69	295.0562	Phenolic derivative	[49,50]
17.	Butyrolactone III	7.68	439.1327	Fatty acyls	[51,52]
18.	Asterric acid	8.01	347.0709,	Benzene and substituted derivatives	[50]
19.	*Epi*-aszonalenin A	8.27	414.9916	Indoles and derivatives	[53]
20.	Asperterpene B	8.38	475.1891	Steroids and steroid	[54]
21.	Terretonin M	8.60	445.1795	derivatives	[55]
22.	Butyrolactone I	9.58	423.1237	Fatty acyls	[56]
23.	Dihydrogeodin	9.53	398.9977	Benzene and substituted derivatives	[57]
24.	Terretonin G	11.65	505.2044	Steroids and steroid derivatives	[15]
25.	Serantrypinone	14.09	387.2814	Pyridopyrimidines	[58]
26.	3α-Hydroxy-3,5-dihydromonacolin L acid	14.16	339.2271	Hydroxy acids and derivatives	[59]
27.	Asperteretone A	14.26	379.1515	Linear 1,3-diarylpropanoids	[60]
28.	Isobutyrolactone V	15.12	381.1671	Phenolic derivative	
29.	Butyrolactone VI	15.36	457.2178	Fatty acyls	[61]
30.	Asperteretone E	15.51	393.2725	Phenolic derivative	[62]
31.	Asperteretal C	16.02	383.1626	Lignans, neolignans and related compounds	[63]
32.	3-Hydroxy-4-(4-hydroxyphenyl)-5-methoxycarbonyl-5-(4-hydroxy-3-formylbenzyl)-2,5-dihydro-2-furanone	17.64	383.3461	Organooxygen compounds	[51]

*Rt: retention time

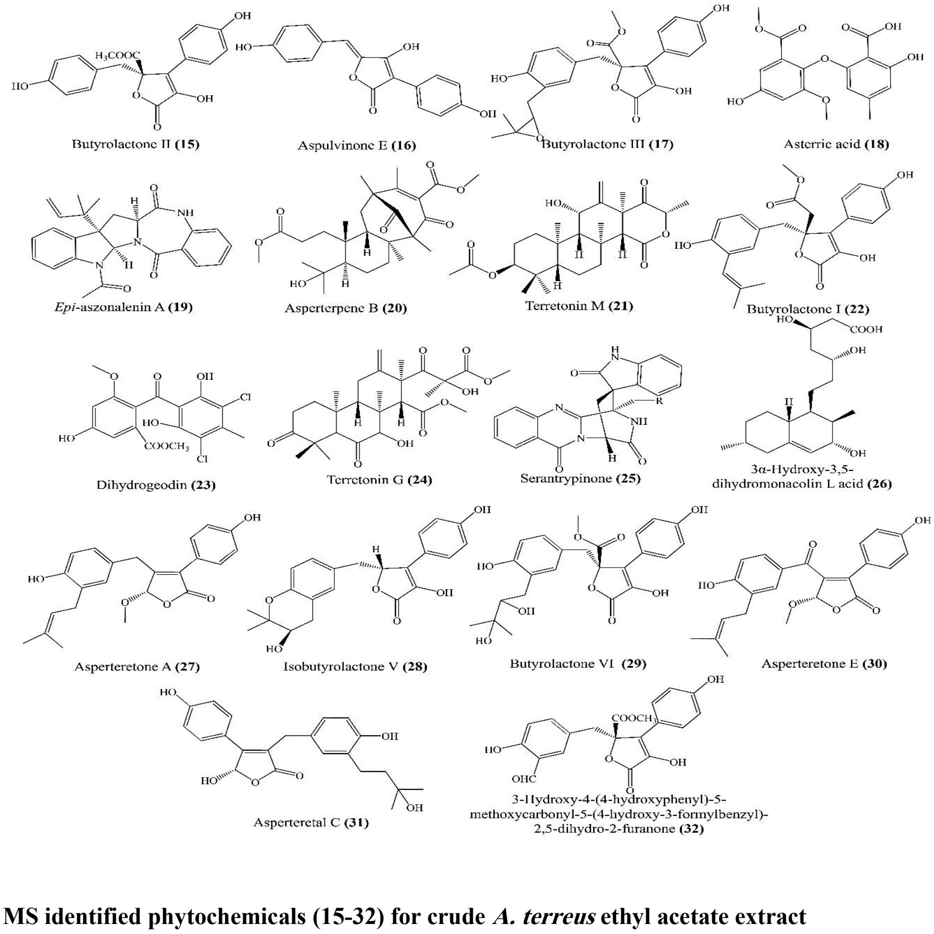

Fig. 7: LC- MS identified phytochemicals (15-32) for crude *A. terreus* ethyl acetate extract

Determination of total antioxidant capacity (TCA) of endophytic isolates extracts

The total antioxidant activities were measured for ethyl acetate and acetone extracts of twelve isolated fungal endophytes (W1-12) grown on rice medium. For ethyl acetate extraction solvent, fungal isolate $W_{7, 5, 6, 4 \& 1}$ showed the higher TCA (1699, 1587.49, 1304.71, 1236.56 and 1157.68 AAE/g dry extract, respectively). For acetone as extraction solvent, fungal isolate W_7 showed the highest TCA (1621.95 and 1520.8 AAE/ g dry extract, respectively) **(Table 5)**.

Table 5. Total antioxidant contents of ethyl acetate & acetone extract from fungal endophytes grown on rice medium

Sample Code (W)	Et acetate extract (AAE/g dry weight)	Acetone extract (AAE/g dry weight)
1	1157.68±2.851	395.45±3.792
2	538.05±2.581	644.71±5.389
3	366.01±3.244	469.25±6.810
4	1236.56±5.041	432.40±5.010
5	1587.49±2.796	500.55±7.584
6	1304.71±13.314	294.71±3.220
7	1698.97±1.925	1621.95±7.398
8	459.81±4.470	241.10±2.698
9	759.34±13.136	269.44±3.255
10	849.62±5.162	757.12±1.951
11	934.62±2.566	575.36±1.821
12	908.79±4.516	854.34±2.085

* AAE/g dry weight: ascorbic acid equivalent

Determination of antioxidant activity by DPPH assay

Anti-DPPH assay was carried out for ethyl acetate and acetone extracts of fungal isolates W_5 & W_7 compared to ascorbic acid as positive control. The calculated IC_{50} is displayed in **Table 6**. All extracts exhibited very strong anti-DPPH activity that was even higher than ascorbic acid. Ethyl acetates of W_5 & W_7 showed higher scavenging activity of the free radical DPPH more than acetone extracts with IC_{50}=34.984, 58.259 µg/ml respectively.

Table 6. DPPH scavenging activities of ethyl acetate & acetone extract W_5 and W_7

Sample code	Ethyl acetate extract (IC_{50} µg/ml)	Acetone extract (IC_{50} µg/ml)
W_5	58.259 ± 1.899	137.41 ± 1.438
W_7	34.984 ± 1.198	89.50 ±0.996
Ascorbic acid	111.828 ± 0.343	

IC_{50}: Half inhibitory concentration

Antimicrobial activity by agar well diffusion method

The antimicrobial activities of the twelve fungal isolates ethyl acetate and acetone extracts were tested against Gram +ve *S. aureus*, Gram -ve *E. coli* and *C. albicans* fungi. As shown in **Table 7**, the results showed that the ethyl acetate extracts of fungal isolates $W_{4, 5, 6 \& 7}$ exhibited the highest inhibition zone against Gram +ve *S. aureus* with inhibition values of 38, 36, 31 and 30 mm, respectively. Whereas the rest of the ethyl acetate extracts showed moderate activities. In case of Gram -ve *E. coli*, fungal isolates $W_{4, 5, 6, 7 \& 12}$ ethyl acetate extracts had significant activity with inhibition zones of 38, 35, 35, 33, and 30 mm, respectively. Whereas the rest of the ethyl acetate extracts showed moderate activities. Regarding their antifungal potential of the obtained ethyl acetate extracts, their effect was tested against *C. albicans* and the results showed that fungal isolates $W_{4, 6, 5 \& 9}$ ethyl acetate extracts exerted higher potential with inhibition values of 39, 34, 32 and 30 mm, respectively. In conclusion the ethyl acetate of fungal isolates $W_{4, 5, 6 \& 7}$ possess significant antimicrobial among the tested ethyl acetate extracts (Supplementary figure 4&5).

As shown in **Table 8**, the results showed that the acetone extracts of fungal isolates $W_{11, 3 \& 4}$ showed higher activities against *S. aureus* with inhibition values of 50, 45 and 34 mm, respectively. Acetone extracts of fungal isolates $W_{11, 3, 4 \& 1}$ showed the highest inhibition zones against *E. coli*, extracts with inhibition values of 49, 40, 35 and 34 mm, respectively. In case of *C. albicans*, acetone extracts of fungal isolates $W_{11, 3 \& 4}$ were the extracts with the higher antifungal potential with inhibition values of 48, 40 and 32 mm, respectively. In conclusion the acetone extract of fungal isolates $W_{3, 4 \& 11}$ possess the most significant antimicrobial among the tested acetone extracts.

Table 7. Antimicrobial activities of ethylacetate extracts of twelve endophytic isolates (clear zone in mm diameter)

Microbe	Mean diameter of inhibition zone (mm) ethyl acetate extracts											
	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8	W_9	W_{10}	W_{11}	W_{12}
S. aureus	25	26	20	38	36	31	30	23	27	22	25	26
E. Coli	27	24	21	38	35	35	33	24	27	23	25	30
C. albicans	25	24	18	39	32	34	29	22	30	23	27	28

Table 8: Antimicrobial activities of acetone extracts of twelve endophytic isolates (clear zone in mm diameter)

Microbe	Mean diameter of inhibition zone (mm) acetone extract											
	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8	W_9	W_{10}	W_{11}	W_{12}
S. aureus	29	28	45	34	13	23	24	28	29	15	50	21
E. Coli	34	27	40	35	16	10	17	24	19	NA	49	24
C. albicans	25	27	40	32	13	23	20	28	24	11	48	21

Determination of minimum inhibitory concentrations (MIC's) and minimum bactericidal concentrations (MBC's)

MIC and MBC of ethyl acetate and acetone extracts of endophytes $W_{5\&7}$ were estimated. As represented in Table 9**,** ethyl acetate extract of endophyte W_5 had strong antimicrobial activity with low MIC (4.88 µg/ml) against both of *S. aureus* and *E. coli* which is approximately double that of the positive control (levofloxacin=2.44 µg/ml) and had MBC against *S. aureus* equal to that of levofloxacin. While ethyl acetate extract of endophyte W_7 exhibits strong antimicrobial activity with low MIC of 4.88 µg/ml that double the value of that of levofloxacin against *E. coli* only and show MBC equal to levofloxacin's (9.77 µg/ml**)**. Acetone extracts of endophytes $W_{5\&7}$ showed high MIC's and MBC's.

Table 9. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of ethyl acetate and acetone extracts of endophytes $W_{5\ \&\ 7}$

Extract	MIC (µg/ml)			MBC (µg/ml)		
	S. aureus	*E. coli*	*C. albicans*	*S. aureus*	*E. coli*	*C. albicans*
W_5 (Et. Acetate)	4.88	4.88	78.25	9.77	19.54	156.25
W_7 (Et. Acetate)	19.53	4.88	39.06	39.06	9.77	78.25
W_5 (Acetone)	39.09	78.125	78.125	78.125	312.5	156.25
W_7 (Acetone)	78.125	156.25	156.25	312.5	625	312.5
Levofloxacin	2.44	2.88	4.88	9.77	9.77	4.88

4. Discussion

Culturing of different samples of *Arenga* leaves collected from Egyptian garden after surface sterilization using PDA solid media plate, afforded twelve different endophytic fungi (W_1-W_{12}) based on differences in their morphological features. Each fungus was cultured in rice media and secondary metabolites extracted using two extraction solvents (ethyl acetate and acetone) affording twenty-four extracts that monitored with TLC. Screening assays for phenolic content and total antioxidant capacity were performed for all twenty-four extracts obtained. Results of these screening assays showed that ethyl acetate and acetone extracts of fungal isolates $W_{5\text{-}7}$ showed the highest phenolic contents and TCA which was encouraging to investigate the fungal identity chemical profile, antioxidant and antimicrobial potential for the extracts of fungal isolates $W_{5\text{-}7}$ (Table 2&5). Phenotypic and 18S-rDNA sequence analyses, morphological and chemotaxonomic investigations were used for taxonomic identification. The phylogeny of fungal isolates $W_{5\text{-}7}$ was inferred by sequence comparison of its amplified internal transcribed spacer (ITS) with published sequences obtained from GenBank. The most related sequences were aligned, and a neighbor-joining tree was calculated. The isolated endophytic fungus was identified as *S. simmonsii* (W_5) and *A. terreus* (W_7). The ethyl acetate extract chemical profile of the rice culture of $W_{5\text{-}7}$ was analyzed using LC-MS and the tentative identification for the metabolites using negative mode allowed a comparison of the mass spectra and retention times of the extract secondary metabolites with mass spectra from a data library. The chromatogram of *S. simmonsii* (W_5) ethyl acetate extract is shown in Figure 4 and fourteen compounds were tentatively identified (Table 3), including anthracenes more commonly anthraquinones, as well as fatty acyls and other polyphenols. According to our results *S. simmonsii* (W_5) ethyl acetate extract showed radicle scavenging activity with IC_{50} 58.259 ± 1.899 µg/ml (Table 6) which was more effective than ascorbic acid and the proposed justification of this finding is the significant synergism between the identified anthracenes and polyphenols. The anthraquinones and polyphenolics possess a well-studied antioxidant capacity. Li et al., 2017 and coauthors have reported the antioxidant potential of the anthraquinone and polyphenols extracted from *S. lycopersici* where the report focused on the potential of *Stemphylium* produced colored pigments [64]. According to previous reports, the antioxidant potential of anthraquinones were estimated using several assays. The basic chemical skeleton of anthraquinone play a role as electron acceptor, and the hydroxyl group substitution pattern in the aromatic region accompanied with hydroxyl methylation are considered a multifunctional antioxidant beside the significant scavenging effect [65]. On the other hand, *S. simmonsii* (W_5) ethyl acetate extract in a well diffusion method showed significant inhibition zone against Gram +ve bacteria *S. aureus* and Gram -ve bacteria *E. coli* with acceptable MIC in comparison to levofloxacin (Table 9). Altersolanol A and other derivatives, one of the tentatively identified metabolites of *S. simmonsii* (W_5) ethyl acetate extract was reported for its potent antimicrobial activity and our results was coincided with [10,11]. Alterporriol derivatives, the anthraquinone dimers were also reported for their significant antimicrobial effect against wide range of microbes with MIC range 2.5-10 µM. Macrosporin were also reported for their antimicrobial potential against *S. aureus* and *E. coli* with reported MIC of 4.6, and 9.2 µM respectively [11]. The chromatogram of *A. terreus* (W_7) ethyl acetate extract is shown in Figure 5 and eighteen secondary metabolites were tentatively identified (Table 4), including Fatty acyls, as well as fatty acyls, steroids and steroid derivatives and other benzene and substituted derivatives. According to our results, *A. terreus* (W_7) ethyl acetate extract showed radicle scavenging activity with IC_{50} 34.984 ± 1.198 µg/ml (Table 6) which was more effective than ascorbic acid and the proposed justification of this finding is also the higher phenolic content and the significant synergism between the identified major fatty acyls and other metabolites which is coincided with previous investigations. The radicle scavenging potential of butyrolactone I was investigated and the results showed that it has closer activity to quercetin with calculated IC_{50} was 21.68 µg/mL [66]. Aspulvinone E is a phenolic derivative that previously reported for its stability under different temperature and pH, and showed notable antioxidant potential [67]. Regarding the antimicrobial activity results, *A. terreus* (W_7) ethyl acetate extract in a well diffusion method showed significant inhibition zone against Gram +ve bacteria *S. aureus* and Gram -ve bacteria *E. coli* with acceptable MIC in comparison to levofloxacin (Table 8&9). Our results were supported with several previous reports of the potent antimicrobial effect of *A. terreus* extracts where it proves that this fungus can produce a variety of secondary metabolites that could be potential antibiotics [19,68,69]. Butyrolactones was proved to exhibit bactericidal effect against methicillin resistant ***S. aureus*** (MRSA) and also tested for inhibiting the production of staphylococcal protease and showed more activity than vancomycin [70]. Terretonin G also was reported for it activity against ***S. aureus*** with calculated MIC of 8 µg/mL [71].

This work reported the potential of *S. simmonsii* and *A. terreus* endophyte as a source of antioxidant and antimicrobial extracts and revealed the chemical profile of the obtained active extract suggesting that rice culture is an available, affordable and reproducible culture media to culture tested strains and ethyl acetate is the solvent of choice to obtain biologically active extracts. According to our literature survey, *S. simmonsii* has very limited reports regarding chemical profiling and biological evaluation. This is the first report of total phenolic content, antioxidant, antimicrobial potentials and LC-MS chemical profile of *S. simmonsii* fungal extract. *Aspergillus terreus* is one of the most common fungi attached to the living organisms and has been reported and isolated endophyte several time. So, it was common to be among the isolated fungi in our original work. Adding the obtained chemical and biological results and comparing them with the previous reports of *Aspergillus terreus* help us to validate our adopted methods and results as our obtained results were coincided with literature.

Research limitations & recommendations for future directions

- In this study, we extracted from the solid culture (rice media) of *S. simmonsii* and *A. terreus* endophyte. Still, we did not include the extracted compounds from liquid culture due to the facility's limitations in our lab.
- To add value to our work, we may test the extracts against highly resistant strains. However, these assays were unavailable when the research study was done.
- The study did not include an *in vivo* evaluation of the safety of the extract due to the limited quantity at the time. So, it is recommended for future work to maximize the extraction part to produce enough extract for *in vivo* evaluation and isolation of pure compounds.

5. Conclusion

The development of treatments derived from natural products remains a key focus in the search for novel antimicrobial agents. Since the discovery of penicillin in 1928, there has been a growing interest in identifying natural sources capable of producing less resistance-prone antimicrobial compounds. Fungi have shown great potential due to their ability to generate a wide variety of bioactive chemical entities, making them a valuable resource for new medicinal agents. Our findings demonstrated the antioxidant activity of ethyl acetate extracts from *S. simmonsii* and *A. terreus*, which is attributed to their phenolic content. In addition, the ethyl acetate extract of *S. simmonsii* showed notable antimicrobial activity against both Gram-positive and Gram-negative bacteria with inhibition zone 36 and 35 mm and MIC 4.88 and 4.85 µg/ml respectively. Similarly, the extract of *A. terreus* exhibited strong antimicrobial potential against Gram-negative bacteria with inhibition zone 33 mm and MIC 4.88 µg/ml. These biological activities were supported by the chemical profiling obtained through LC-MS analysis, which identified fourteen and eighteen tentative secondary metabolites in the extracts of *S. simmonsii* and *A. terreus*, respectively. Future research should focus on bio-guided fractionation of *S. simmonsii* active extracts to isolate and identify the specific compounds responsible for the observed activities since limited studies are available for this identified strain which higher potentials of isolating novel active chemical entities.

6. Conflicts of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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9. Data availability statement

The original contributions presented in the research are included in the manuscript / supp. material/referenced in the article and further queries can be engaged to the corresponding authors.

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