chemistry

MDPI

Article

LC-ESI-MS and GC-MS Profiling, Chemical Composition, and Cytotoxic Activity of Endophytic Fungus *Pleosporales* sp. Derived from *Artemisia annua*

Mamdouh Nabil Samy [1,2,†], Eman Zekry Attia [1,†], Basma Ali Khalifa [3], Usama Ramadan Abdelmohsen [1,4] and Samir Anis Ross [2,5,*]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia 61519, Egypt; mamdouh.eskandr@mu.edu.eg (M.N.S.); eman_zekry@mu.edu.eg (E.Z.A.); usama.ramadan@mu.edu.eg (U.R.A.)
[2] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA
[3] Department of Botany and Microbiology, Faculty of Science, Minia University, Minia 61519, Egypt; basma-ali@mu.edu.eg
[4] Department of Pharmacognosy, Faculty of Pharmacy, Deraya University, New Minia 61111, Egypt
[5] Division of Pharmacognosy, Department of BioMolecular Sciences, School of Pharmacy, University of Mississippi, University, MS 38677, USA
* Correspondence: sross@olemiss.edu
† These authors contributed equally to this work.

Abstract: The chemical profiling of ethyl acetate extract of the endophytic fungus *Pleosporales* sp. using liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) revealed the presence of 12 metabolites of different chemical classes such as steroids, α-pyrones, asterric acid derivatives, and quinones. Additionally, the gas chromatography–mass spectrometry (GC-MS) profiling of the ethyl acetate (EtOAc) and methanol extracts exhibited the presence of fatty acids and their esters, in which methyl palmitate (18.72%, and 25.48%, respectively) and methyl linoleate (11.92% and 23.39%, respectively) were found in both extracts. On the other hand, palmitic acid (12.60%), methyl oleate (26.90%), oleic acid (4.01%) and linoleic acid (3.25%) were present only in methanol extract. Furthermore, ethyl palmitate (12.60%), 13-octadecenoic acid (19.36%), and ethyl linoleate (3.25%) occurred in EtOAc extract. A phytochemical investigation of both extracts led to the isolation of fatty acids such as palmitic acid (**18**), oleic acid (**20**), and linoleic acid (**21**) and their esters including methyl palmitate (**13**), methyl stearate (**22**), methyl linoleate (**16**), methyl 3-hydroxy-5-methylhexanoate (**23**), and monomethyl azelate (**27**), in addition to monoacyl derivatives of glycerol such as 3,3-dihydroxypropyl hexadecanoate (**24**), 2,3-dihydroxypropyl elaidate (**25**), and 1-linoleoyl-sn-glycerol (**26**). The structures of the isolated compounds were identified by different spectroscopic analyses including ^{1}H- and ^{13}C-NMR and GC-MS. The EtOAc extract exhibited a cytotoxic effect against MCF-7 and HepG-2 cell lines, with IC_{50} values of 4.12 ± 0.10 and 10.05 ± 0.05 µg/mL, respectively.

Keywords: *Pleosporales* sp.; LC-ESI-MS; GC-MS; fatty acids; esters of fatty acids; glycerol derivatives; cytotoxicity

Citation: Samy, M.N.; Attia, E.Z.; Khalifa, B.A.; Abdelmohsen, U.R.; Ross, S.A. LC-ESI-MS and GC-MS Profiling, Chemical Composition, and Cytotoxic Activity of Endophytic Fungus *Pleosporales* sp. Derived from *Artemisia annua*. *Chemistry* **2024**, *6*, 1336–1346. https://doi.org/10.3390/chemistry6060078

Academic Editor: George Grant

Received: 23 September 2024
Revised: 23 October 2024
Accepted: 24 October 2024
Published: 26 October 2024

1. Introduction

Natural products are chemical substances produced by living organisms. Plants, animals, marine macroorganisms (sponge, corals, and algae), and microorganisms (bacteria, actinomycetes, and fungi) are among the most important producers of natural products [1]. The role of natural products in the discovery of new therapeutic agents can be proved by examining the number and sources of bioactive compounds [2].

Endophytes primarily consist of fungi and bacteria, inhabiting the living tissues of healthy plants and marine organisms. Both chemists and biologists are paying close attention to them since they have been identified as a valuable source of secondary metabolites

that are both biologically and chemically varied [3–5]. Endophytes have long been recognized as prospective pharmaceutical leading sources, with numerous endophytic fungi producing novel bioactive compounds such as antibacterial, anticancer, and antiviral drugs [1].

Different types of metabolites have been produced by the fungus *Pleosporales* sp. such as azaphilones, polyketides, phenalenones, drimane sesquiterpenoids, diketopiperazine alkaloids and steroids, with various biological activities such as cytotoxic, antibacterial, antifungal, anti-inflammatory activities [6–16]. It was found that dipleosporalone A, an azaphilone dimer isolated from *Pleosporales* sp. CF09-1, displayed potent cytotoxicity towards certain cell lines equal to or stronger than the effects of the control anticancer drug cisplatin; therefore, this could be a promising lead for the development of antitumor agents [6]. An azaphilone derivative, pleosporalone B, exhibited potent antifungal activities against the fungi *Alternaria brassicicola* and *Fusarium oxysporum*, which were stronger than the positive control ketoconazole; hence, it has potential as a leading drug for antifungal agents [7]. Dimeric benzophenones, dipleosporones, isolated from the fungus *Pleosporales* sp. YY-4 exhibited anti-inflammatory potential, being more potent than the positive control, dexamethasone; consequently, they could be promising metabolites for developing anti-inflammatory agents [17].

The endophytic fungus *Pleosporales* sp. AAnEF1 isolated from a healthy plant, *Artemisia annua*, collected from Minia University was cultured on MPDB to produce metabolic compounds. This study aims to isolate and characterize the secondary metabolites of *Pleosporales* sp. and evaluate their cytotoxic activity against cancer cell lines.

2. Materials and Methods

2.1. Plant Material

The fresh leaves and stems of *Artemisia annua* were gathered from a cultivated field close to Minia University, 28°7′24.3″ N, 30°44′27.1″ E. The plant was deposited as a voucher specimen in the herbarium of the Faculty of Science, Minia University (No.: AA-112).

2.2. Isolation of Endophytic Fungi

The obtained fresh plant material was rinsed with running tap water to remove the attached soil particles. They were then sterilized with ethanol for 1 min, followed by 3% sodium hypochlorite solution for 1 min, and finally, a series of washing processes with sterile distilled water took place. After that, each plant organ (e.g., leaves and stems) was cut into smaller pieces and placed on malt extract agar plates (LOBACHEMIE, Mumbai, India) supplemented with ampicillin (0.5 mg/mL) to inhibit the growth of associated bacterial endophytes. Finally, the plates were incubated at 28 ± 2 °C. The colonies that formed underwent multiple sub-cultures to obtain pure fungal isolates, which were stored at 4 °C (voucher specimen codes: AAF-101 to AAF-111).

2.3. Molecular Identification and Phylogenetic Analysis

The genomic DNA materials of the isolated fungal strain were extracted, and then the fungal internal transcribed spacer (ITS) region was amplified and sequenced using the universal primers ITS1 and ITS4 to taxonomically define the strain. In order to find the nearest related species with extremely similar sequences to the amplified ones, the high-quality sequences were compared to those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information (NCBI). Lastly, MEGA7 software was used to complete the phylogenetic analysis and multiple sequence alignment [18,19].

2.4. Fermentation in Liquid Medium

The isolated fungal strain *Pleosporales* sp. AAnEF1 was cultivated in an Erlenmeyer flask (2 L), containing 400 mL of modified potato dextrose broth (MPDB), along with its main components: 0.5 g of peptone, 0.8 g of yeast extract, 3 mg of $(NH_4)_2SO_4$, 2 g of KH_2PO_4, and 0.5 g of $MgSO_4$. The flask was incubated statically at 20 ± 2 °C for 30 days.

Subsequently, the fermentation process was ended by adding 100 mL of EtOAc to the flask. The fermented broth was then centrifuged at 6000 rpm for 15 min at 4 °C before being filtered to remove the wet mycelia. The obtained filtrate (300 mL) was subjected three times to successive liquid–liquid extraction with 250 mL of EtOAc and methanol. The obtained EtOAc and methanol solutions were then concentrated under reduced pressure to give semisolid brown residues.

2.5. LC–HR–ESI-MS

The recovered ethyl acetate fungal extract was subjected to liquid chromatography–high resolution-electrospray ionization–mass spectrometry (LC-HR-ESI-MS) metabolomics analyses on an Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled with a Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, MA, USA). The separation was performed on a BEH C18 column (2.1 × 100 mm, 1.7 µm particle size; Waters, Milford, MA, USA) with a guard column (2.1 × 5 mm, 1.7 µm particle size) and a linear binary solvent gradient of 0–100% eluent B for 6 min at a flow rate of 0.3 mL min^{-1}, using 0.1% formic acid in water (*v/v*) as solvent A and acetonitrile as solvent B. The injection volume was 2 µL and the column temperature was 40 °C. The metabolites were then detected by mass spectrometry using electrospray ionization (ESI). The mass range for time of flight–mass spectrometry (TOF–MS) was set from *m/z* (mass-to-charge ratio) 50–1200. In MZmine 2.12, the raw data were analyzed to identify the metabolites. After processing, the data set was subjected to peak identification and molecular formula prediction. The corresponding fungal extract's positive and negative ionization mode data sets were dereplicated against those in the DNP database (Dictionary of Natural Products) [20].

2.6. GC-MS Analysis

An Agilent 7890B gas chromatographic (GC) instrument that was equipped with a RS185 PAL3 autosampler, connected to an Agilent 7250 Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) mass spectrometer (Agilent, Santa Clara, CA, USA) was used to analyze the sample. A 5% phenyl methyl siloxane (Agilent J&W HP-5MS) capillary column (30 m × 0.25 mm i.d.) was used. The carrier gas was helium at a constant flow rate of 1 mL/min. The analysis was carried out in a programmed temperature using a flame ionization detector (FID). The oven temperature was programmed as follows: heated to 50 °C for one min., then it was raised to 280 °C at a rate of 5 °C/min., and finally, it was held at 280 °C for ten min. The inlet was set to operate in split mode at 280 °C, with a split ratio of 50:1. The temperature of the transfer tube from the GC to the Q-ToF was maintained at 280 °C.

The Q-ToF mass spectrometer utilized a high-emission low-energy electron ionization source (EI^+) with 70 eV electron energy and 25.0 µA emission current. Throughout the experiment, the temperatures of the source, quadrupole, and transfer line were 230, 150, and 280 °C, respectively. The MS scan was from 40 to 450 *m/z* at a rate of 5 Hz. Agilent MassHunter software (version B7.06.274) was used to acquire the data. Tentative compound identification was performed using the NIST database (version 2.3).

2.7. Extraction and Isolation of Secondary Metabolites

The EtOAc extract (1.73 g) was fractionated through silica gel CC (77 × 2 cm, 70 g), using dichloromethane–methanol (DCM-MeOH) gradient solutions to gradually increase the polarity by 2% by MeOH until DCM-MeOH in a ratio of 80:20 was attained, and finally, the column washed by MeOH (500 mL of each polarity). The effluents were fractionally collected and concentrated under reduced pressure, yielding 23 subtractions. Subfraction E-9 (45.6 mg) gave a mixture of methyl palmitate (**13**), methyl stearate (**22**), and methyl linoleate (**16**) (38.0 mg). Subfraction E-11 (86.3 mg) was purified through sephadex LH-20 CC using 600 mL of MeOH, yielding eight subfractions. Fraction E-11-2 produced a mixture

of methyl palmitate (**13**) and methyl linoleate (**16**) (26.6 mg). Fraction E-11-4 produced a mixture of methyl 3-hydroxy-5-methylhexanoate (**23**) and palmitic acid (**18**) (28.6 mg).

The MeOH extract (1.74 g) was fractionated through silica gel CC (77 × 2 cm, 70 g), using DCM-MeOH gradient mixtures solutions to gradually increase the polarity by 2% by MeOH until DCM-MeOH in a ratio of 80:20 was attained, and finally, the column washed by MeOH (500 mL of each polarity). The effluents were fractionally collected and concentrated under reduced pressure, producing 23 subtractions. Subfraction M-10 (23.5 mg) produced palmitic acid (**18**) (18.2 mg). Subfraction M-11 (55.7 mg) was purified by sephadex LH-20 CC using 600 mL of MeOH, yielding a mixture of palmitic acid (**18**), 3,3-dihydroxypropyl hexadecanoate (**24**), 2,3-dihydroxypropyl elaidate (**25**) and 1-linoleoyl-sn-glycerol (**26**) (16.0 mg). Subfraction M-12 (70.3 mg) was purified through sephadex LH-20 CC using 800 mL of MeOH, yielding a mixture of methyl 3-hydroxy-5-methylhexanoate (**23**) and methyl palmitate (**13**), monomethyl azelate (**27**), palmitic acid (**18**), oleic acid (**20**), and linoleic acid (**21**) (11.0 mg).

2.8. MTT Cytotoxicity Assay

The cytotoxicity of the EtOAc and MeOH extracts was tested against HepG-2 (human hepatocellular carcinoma) and MCF-7 (human breast cancer) cell lines. Cell lines were purchased from vacciera, Cairo, Egypt. Cells were cultured using Dulbecco's Modified Eagle's Medium (DMEM)–high-glucose medium (Gibco, Carlsbad, CA, USA) and incubated at 37 °C with 5% CO_2. When cells reached 100% confluency, they were trypsinized and cultured in a b96-well plate at a density of 10,000 cell/well using DMEM–high-glucose with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). Cells were incubated overnight. The next day, the extracts were dissolved in 100 µL of dimethyl sulfoxide (DMSO) at concentrations of 20, 30, 40, 50, and 60 µg/mL, and then 1 µL was added to 100 µL media in each well and incubated for 48 h. After that, 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (5 mg/mL) powder (Sigam-Aldrich, Darmstadt, Germany) dissolved in 10% fetal bovine serum (FBS) was added per well and incubated for 2 h at 37 °C in a CO_2 incubator. After incubation, the medium was discarded and 200 µL of DMSO was used as the baseline for the relative cytotoxicity calculation to dissolve the formazan crystals. Doxorubicin (Sigam-Aldrich, Darmstadt, Germany) was used as the positive control. Then, absorbance was read in a spectrophotometer at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (BioTek Epoch 2, Santa Clara, CA, USA). This method is based on the ability of live but not dead cells to reduce a tetrazolium dye to a purple formazan product. All experiments were repeated three times [21].

3. Results and Discussion

3.1. LC-ESI-MS Profiling of Pleosporales sp.

The chemical profiling of the EtOAc extract was performed using LC-ESI-MS metabolomics tools (Supplementary Figures S1 and S2). Twelve compounds were tentatively identified (Table 1 and Figure 1). The DNP database was used to dereplicate the positive and negative ionization mode data sets from *Pleosporales* sp. AAnEF1 extract. The mass ion peak at *m/z* 223.0607 $[M + H]^+$, which corresponded to the molecular formula $C_{11}H_{10}O_5$, was identified as 3,4-dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-methyl carboxylate (**1**), which was earlier obtained from *Alternaria tenuissima* [22]. Furthermore, the mass ion peak at *m/z* 249.1492 $[M - H]^-$ for the predicted molecular formula $C_{15}H_{22}O$ was identified as (1′*S*, 2*Z*)-3-methyl- 5-(2,6,6-trimethyl-4-oxocyclohex-2-enyl)pent-2-enoicacid (**2**), which was formerly characterized from *Pleosporales* sp. SK7 and exhibited antibacterial activity against Gram-positive and Gram-negative bacterial strains including *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella* ATCC 14028 [23].

Table 1. LC-ESI-MS results of the EtOAc extract after dereplication of compounds by metabolomics analysis in *Pleosporales* sp. AAnEF1.

No.	Compound Name	Mode	*m*/*z*	R_t	Molecular Weight	Exact Mass	Delta M	Molecular Formula
1	3,4-Dihydro-8-hydroxy-1-oxo-1*H*-2-benzopyran-3-methyl carboxylate	$[M + H]^+$	223.0607	6.46	222.0534	222.0528	0.0006	$C_{11}H_{10}O_5$
2	(1′*S*, 2*Z*)-3-methyl-5-(2,6,6-trimethyl-4-oxocyclohex-2-enyl)pent-2-enoicacid	$[M - H]^-$	249.1492	11.83	250.1727	250.1569	0.0158	$C_{15}H_{22}O$
3	Desmethylaltenusin	$[M + H]^+$	277.0714	4.51	276.0641	276.0634	0.0007	$C_{14}H_{12}O_6$
4	Pleosporone	$[M - H]^-$	289.1067	6.09	290.1140	290.0790	0.0350	$C_{15}H_{14}O_6$
5	Altersolanol G	$[M + H]^+$	335.1130	4.05	334.1058	334.1053	0.0005	$C_{17}H_{18}O_7$
6	Asterric acid	$[M - H]^-$	347.07601	4.38	348.0832	348.0845	0.0013	$C_{17}H_{16}O_8$
7	10,11-Dideoxy, 6,19-dihydro alternaric acid	$[M - H]^-$	379.2131	11.88	380.2204	380.2199	0.0005	$C_{21}H_{32}O_6$
8	Ergosta-4,6,8(14),22-tetraen-3-one	$[M + H]^+$	393.3153	15.30	392.3080	392.3079	0.0001	$C_{28}H_{40}O$
9	Methyl 3-chloroasterric acid	$[M - H]^-$	395.15207	11.96	396.1593	396.0612	0.0981	$C_{18}H_{17}ClO_8$
10	20-Hydroxyergosta-4,6,8(14),22-tetraen-3-one	$[M + H]^+$	409.3096	12.59	408.3024	408.3028	0.0004	$C_{28}H_{40}O_2$
11	Herbarulide	$[M + H]^+$	425.3049	10.61	424.2976	424.2977	0.0001	$C_{28}H_{40}O_3$
12	Alternapyrone	$[M + H]^+$	429.3363	13.40	428.3290	428.3290	0.0000	$C_{28}H_{44}O_3$

R_t: retention time in min.

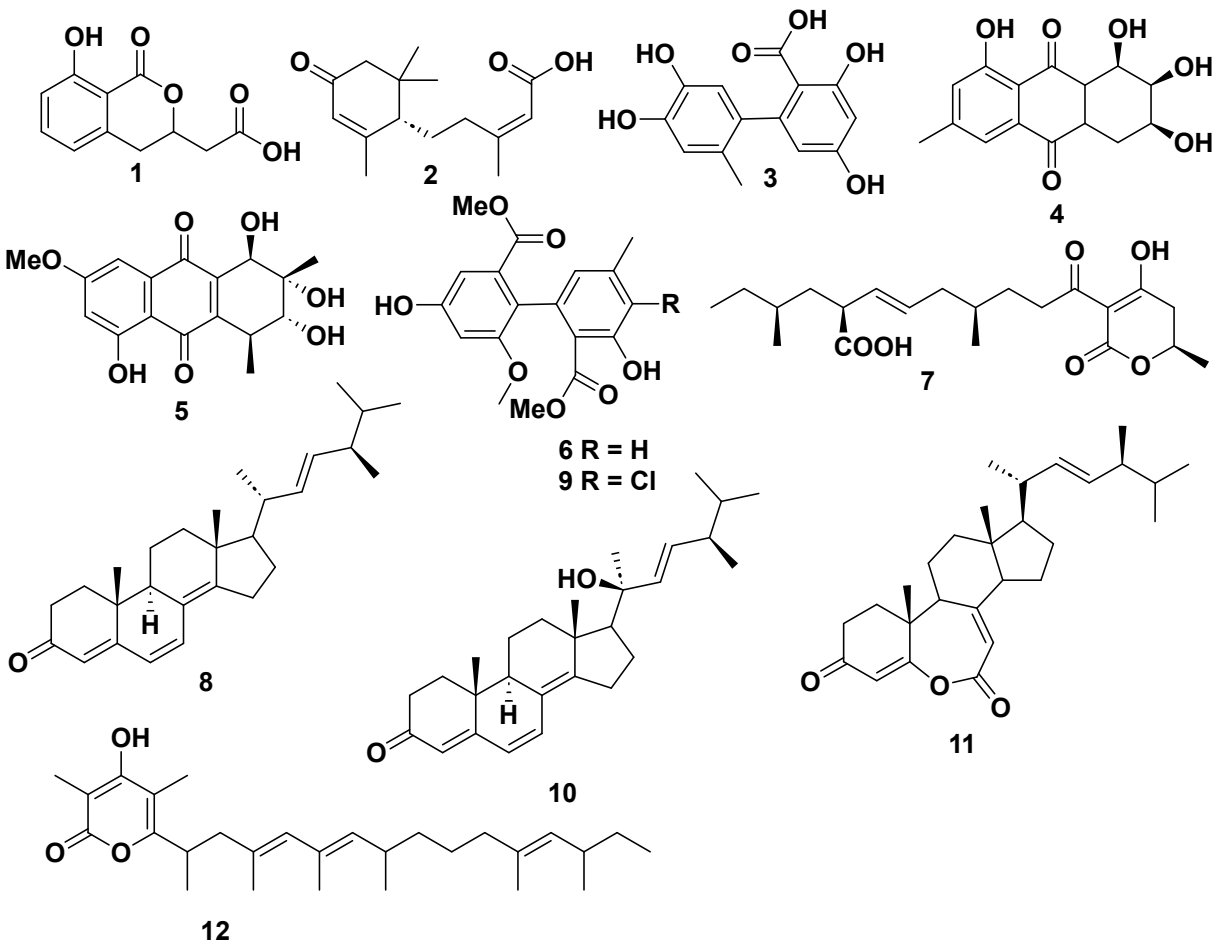

Figure 1. Structures of the identified metabolites (**1**–**12**) in the EtOAc extract of *Pleosporales* sp. by LC-ESI-MS.

Additionally, the mass ion peak at *m/z* 277.0714 $[M + H]^+$ in accordance with the molecular formula $C_{14}H_{12}O_6$ was recognized as desmethylaltenusin (**3**), which was previously isolated from *Alternaria* sp. II2L4 and showed cytotoxicity activity against L5178Y mouse lymphoma cells using a microculture tetrazolium (MTT) assay with EC_{50} values of 6.8 µg/mL [24]. Conversely, at *m/z* 289.1067 $[M-H]^-$, the suggested molecular formula $C_{15}H_{14}O_6$ was identified as pleosporone (**4**). This compound was previously isolated from *Pleosporalean ascomycete* and exhibited antibacterial activity, with MICs ranging from 1 to 64 µg/mL. It displayed the maximum sensitivity against *Streptococcus pneumoniae* and *Haemophilus influenzae*, with MICs of 4 and 1 µg/mL [25]. Another metabolite with the molecular formula $C_{17}H_{18}O_7$ indicated by the mass ion peak at *m/z* 335.1130 $[M + H]^+$ was characterized as altersolanol G (**5**), which was previously described from *Alternaria solani* and showed antibacterial activity against *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli*, and *P. aeruginosa* [26]. Additionally, that at *m/z* 347.07601 $[M - H]^-$ corresponding to the molecular formula $C_{17}H_{16}O_8$ was suggested to be asterric acid (**6**), which was previously isolated from *Pleosporales* sp. SK7. This compound showed antibacterial activity against Gram-positive and Gram-negative bacterial strains (*S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *Salmonella* ATCC 14028) and antioxidant potential with DPPH radical scavenging activity [23].

Furthermore, the mass ion peak at *m/z* 379.2131 $[M - H]^-$, indicating the predicted molecular formula $C_{21}H_{32}O_6$, was distinguished as 10,11-dideoxy, 6,19-dihydro alternaric acid (**7**), which was isolated before from *Alternaria solani*. This compound has antifungal properties [27].

On the other hand, the mass ion peak at *m/z* 393.3153 corresponding to the molecular formula $C_{28}H_{40}O$ $[M + H]^+$ was identified as ergosta-4,6,8(14),22-tetraen-3-one (**8**). This compound was previously isolated from *Alternaria alternate* [28]. In addition, the metabolite, namely methyl 3-chloroasterric acid (**9**), with the molecular formula $C_{18}H_{17}ClO_8$, was also dereplicated from the mass ion peak at *m/z* 397.8 $[M + H]^+$. This compound was previously reported to derive from *Pleosporales* sp. SK7, and it showed cytotoxicity properties against the MDA-MB-435 cell line with an IC_{50} of 25.96 ± 0.32 Mm [23]. Moreover, the predicted MF $C_{28}H_{40}O_2$ at m/z 409.3096 $[M + H]^+$ was distinguished as 20-hydroxyergosta-4,6,8(14),22-tetraen-3-one (**10**), formerly reported to derive from *Alternaria solani* [29]. Likewise, the mass ion peak at *m/z* 425.3049 $[M + H]^+$ indicating the molecular formula $C_{28}H_{41}O_3$ was identified as herbarulide (**11**), previously isolated from *Pleospora herbarum*, and it was reported as a antimicrobial agent [30].

Finally, the mass ion peak at *m/z* 429.3363 $[M + H]^+$, in agreement with the molecular formula $C_{28}H_45O_3$, was dereplicated as alternapyrone (**12**), which was characterized from *Alternaria solani* [31].

The recorded antimicrobial activities of *Pleosporales* sp. might be consolidated with such a metabolomics analysis that revealed the presence of various metabolites, namely, (1′*S*, 2*Z*)-3-methyl-5-(2,6,6-trimethyl-4-oxocyclohex-2-enyl)pent-2-enoicacid (**2**), pleosporone (**4**), altersolanol G (**5**), 10,11-dideoxy, 6,19-dihydro alternaric acid (**7**), herbarulide (**11**), and alternapyrone (**12**) [23,25–27,30,31], and the recorded cytotoxic activities of *Pleosporales* sp. might be consolidated with such a metabolomics analysis that revealed the presence of various metabolites, namely, desmethylaltenusin (**3**), asterric acid (**6**), and methyl 3-chloroasterric acid (**9**); thus, this suggests the involvement of such compounds in the antimicrobial capacity and anticancer potency of *Pleosporales* sp [23,24].

3.2. GC-MS Profiling of Pleosporales sp.

The secondary metabolites produced by the endophytic fungus *Pleosporales* sp. AAnEF1 isolated from a healthy plant, *Artemisia annua*, were identifying by using GC-MS. Their chemical composition with their retention time (R_t), molecular formula, molecular weight, and peak area %, which is calculated from the area under the peak by integration, are illustrated in Tables 2 and 3 and Figure 2 (Supplementary Figures S3 and S4). The calculated retention indices (RIs) were compared with those reported in the literature (the

National Institute of Standard and Technology (NIST)). The GC-MS of the endophytic fungus *Pleosporales* sp. revealed the presence of five compounds in ethyl acetate extract, of which 13-octadecenoic acid (19.36%), methyl palmitate (18.72%), ethyl palmitate (12.60%), and methyl linoleate (11.92%) were the major compounds, and six compounds in methanol extract, of which methyl oleate (26.90%), methyl palmitate (25.48%), and methyl linoleate (23.39%) were the major compounds.

Table 2. GC-MS profile of EtOAc extract of *Pleosporales* sp. AAnEF1.

No.	Compound Name	R_t	Area %	Molecular Weight	Molecular Formula	Retention Index	Retention Index Standard
13	Methyl palmitate	30.29	18.72	270.5	$C_{17}H_{34}O_2$	1649	1922
14	Ethyl palmitate	31.51	12.60	284.5	$C_{18}H_{36}O_2$	1790	1996
15	13-Octadecenoic acid, methyl ester	34.01	19.36	296.5	$C_{19}H_{36}O_2$	1747	2098
16	Methyl linoleate	34.29	11.92	294.5	$C_{19}H_{34}O_2$	1755	2097
17	Ethyl linoleate	35.35	3.25	270.5	$C_{20}H_{36}O_2$	1786	2173

R_t: retention time in min.

Table 3. GC-MS profile of methanol extract of *Pleosporales* sp. AAnEF1.

No.	Compound Name	R_t	Area %	Molecular Weight	Molecular Formula	Retention Index	Retention Index Standard
13	Methyl palmitate	30.26	25.48	270.5	$C_{17}H_{34}O_2$	1648	1922
18	Palmitic acid	31.48	12.60	256.42	$C_{16}H_{32}O_2$	1677	1970
19	Methyl oleate	33.99	26.90	296.5	$C_{19}H_{36}O_2$	1746	2062
16	Methyl linoleate	34.28	23.39	294.5	$C_{19}H_{34}O_2$	1754	2097
20	Oleic acid	35.19	4.01	282.5	$C_{18}H_{34}O_2$	1781	2120
21	Linoleic acid	35.52	3.25	280.4	$C_{18}H_{32}O_2$	1791	2104

R_t: retention time in min.

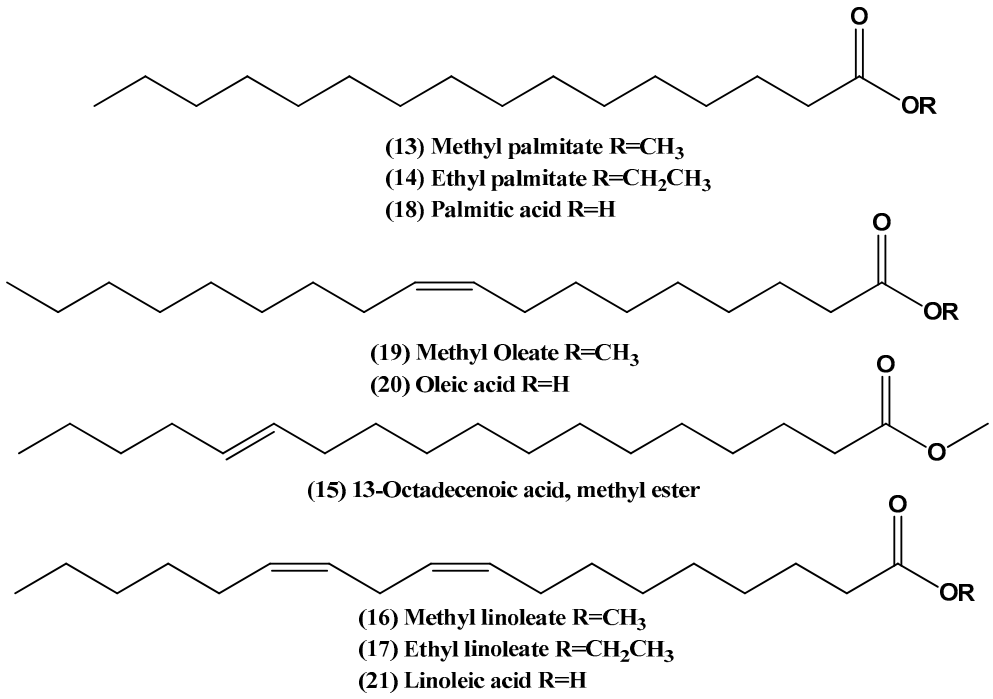

Figure 2. Structures of the identified compounds (**13**–**21**) in EtOAc and MeOH extracts from *Pleosporales* sp. discerned by GC-MS.

The GC-MS profiling of the ethyl acetate and methanol extracts of the endophytic fungus *Pleosporales* sp. indicated the presence of fatty acids and their esters, in which

methyl palmitate (18.72% and 25.48%, respectively) and methyl linoleate (11.92% and 23.39%, respectively) were found in both extracts. On the other hand, ethyl palmitate (12.60%), 13-octadecenoic acid (19.36%), and ethyl linoleate (3.25%) occurred in EtOAc extract. Additionally, palmitic acid (12.60%), methyl oleate (26.90%), oleic acid (4.01%), and linoleic acid (3.25%) were present only in MeOH extract.

3.3. Isolation of Secondary Metabolites

A chemical investigation of both extracts of *Pleosporales* sp. using silica gel and sephadex LH-20 column chromatography led to the isolation of fatty acids such as palmitic acid (**18**), oleic acid (**20**), and linoleic acid (**21**) and their esters including methyl palmitate (**13**), methyl stearate (**22**), methyl linoleate (**16**), methyl 3-hydroxy-5-methylhexanoate (**23**), and monomethyl azelate (**27**), in addition to monoacyl derivatives of glycerol such as 3,3-dihydroxypropyl hexadecanoate (**24**), 2,3-dihydroxypropyl elaidate (**25**), and 1-linoleoyl-sn-glycerol (**26**). The structures of the isolated compounds were identified by different spectroscopic analyses including ^{1}H- and ^{13}C-NMR and GC-MS (Figure 3) (Supplementary Figures S5–S38). The isolated compounds were obtained for the first time from *Pleosporales* sp.

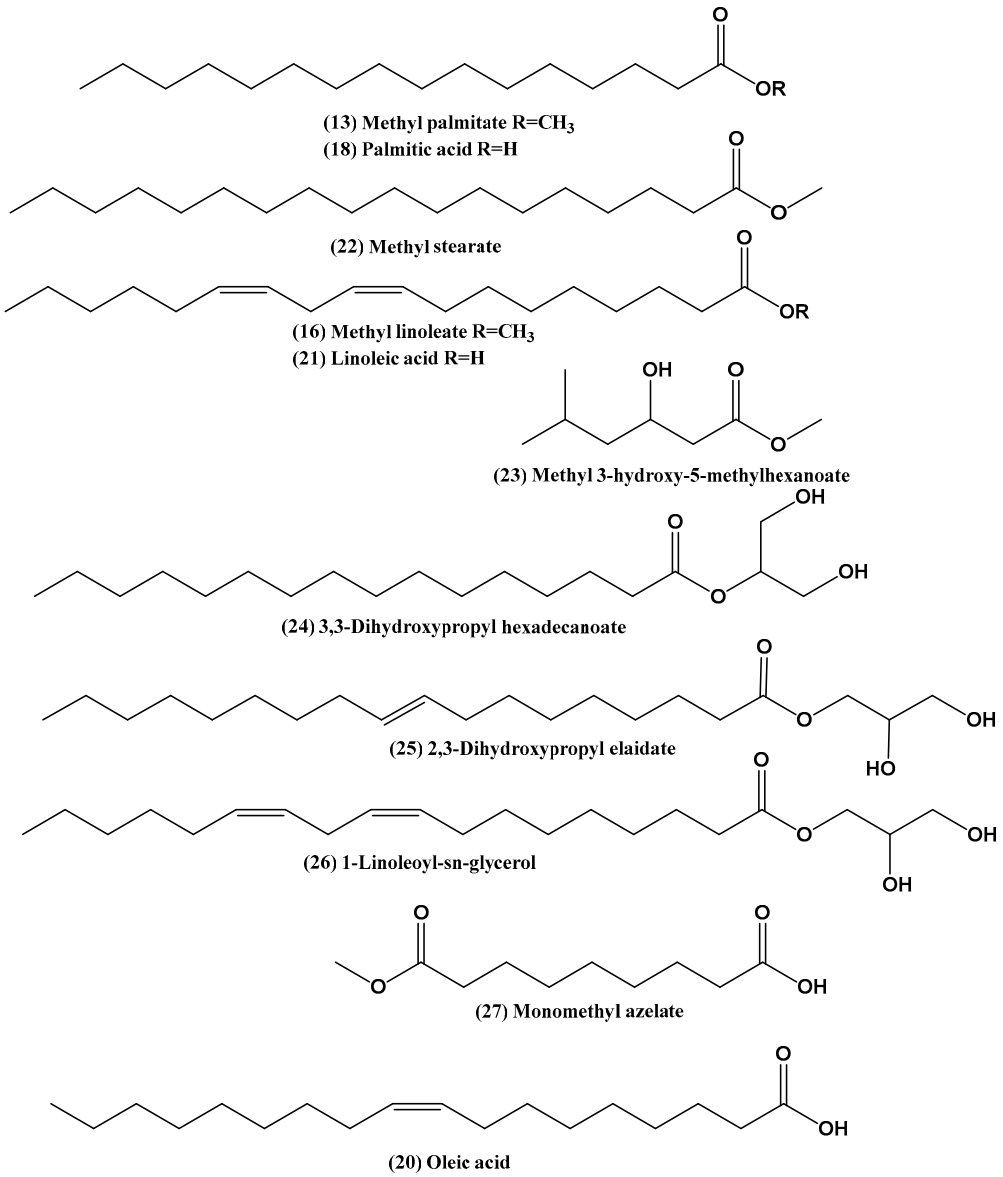

Figure 3. Structures of the isolated compounds (**13**, **16**, **18**, **20**–**27**) from *Pleosporales* sp.

3.4. Cytotoxic Activity

The cytotoxic activity of the EtOAc extract of *Pleosporales* sp. was evaluated using an MTT assay against MCF-7 (a human breast cancer cell line) and HepG-2 (human hepatocellular carcinoma). It showed a remarkable growth-inhibitory effect against MCF-7 and HepG-2 cell lines, with IC_{50} values of 4.12 ± 0.1 and 10.05 ± 0.05 µg/mL, respectively, comparable with those of the standard doxorubicin (IC_{50} = 1.72 ± 0.03 and 1.32 ± 0.06 µg/mL, respectively). On the other hand the MeOH extract showed negligible cytotoxic activity against the tested cell lines.

Previously, the cytotoxic effects of the isolated compounds from *Pleosporales* sp. have been reported in the literature. Two new azaphilone dimers, dipleosporalones A and B, obtained from marine-derived *Pleosporales* sp. CF09-1, were potent against MDA-MB-231, HeLa, MGC-803, MCF-7, and A549 cell lines, where dipleosporalone A displayed potent cytotoxic activity toward the MGC-803 cell line, with an IC_{50} value of 1.3 µM, stronger than that of cisplatin (IC_{50} = 1.5 µM) [6]. A new polyketide, globosuxanthone F, produced by *Pleosporales* sp. NBUF144, demonstrated cytotoxicity against CCRF-CEM human acute lymphatic leukemia cells, with an IC_{50} value of 0.46 µM [14]. Pleosporalin F, a new heptaketide isolated from *Pleosporales* sp. F46, demonstrated considerable cytotoxicity against MDA-MB-231, with an IC_{50} of 22.4 ± 1.1 µM [13].

The identified secondary metabolites in the EtOAc extract of *Pleosporales* sp. by LC-ESI-MS possessed cytotoxic activities, where desmethylaltenusin (**3**), which was previously isolated from *Alternaria* sp. II2L4, showed cytotoxicity activity against L5178Y mouse lymphoma cells using an MTT assay with EC_{50} values of 6.8 µg/mL [24], and methyl 3-chloroasterric acid (**9**), which was previously reported from *Pleosporales* sp. SK7, showed cytotoxicity properties against the MDA-MB-435 cell line, with an IC_{50} of 25.96 ± 0.32 Mm [23]. On the other hand, the saturated fatty acids such as stearic acid and palmitic acid and monounsaturated fatty acids, like oleic acid, are unable to induce a cytotoxic effect unlike the polyunsaturated fatty acids [32]. Moreover, the consumption of polyunsaturated fatty acids can delay the growth of tumors by activating apoptotic processes in tumor cells [33,34] or by preventing angiogenesis [35,36]. These data may explain the stronger cytotoxic effect of the EtOAc extract of *Pleosporales* sp, which contains cytotoxic metabolites such as desmethylaltenusin (**3**) and methyl 3-chloroasterric acid (**9**).

4. Conclusions

The chemical investigation of the different extracts of *Pleosporales* sp. via LC-ESI-MS and GC-MS suggested the presence of various secondary metabolites produced by this fungus. Additionally, fatty acids and their esters and monoacyl derivatives of glycerol were isolated. The EtOAc acetate had a remarkable growth-inhibitory effect against MCF-7 and HepG-2 cell lines.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/chemistry6060078/s1, Figures S1 and S2: LC-MS base peak chromatograms of the ethyl acetate extract of the MPDB culture filtrate of *Pleosporales* sp; Figures S3 and S4: GC chromatogram of EtOAc and methanol extracts; Figures S5–S38: The ^{1}H NMR, DEPTQ NMR, GC, EI-MS of the isolated compounds from *Pleosporales* sp.

Author Contributions: Conceptualization, M.N.S. and E.Z.A.; methodology, M.N.S. and E.Z.A.; software, U.R.A.; investigation, M.N.S., E.Z.A. and B.A.K.; writing—original draft preparation, M.N.S. and E.Z.A.; writing—review and editing, U.R.A. and S.A.R.; supervision, S.A.R.; project administration, S.A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are contained within the article.

Acknowledgments: The authors are grateful to the Egyptian Government and the National Center for Natural Products Research, the School of Pharmacy, the University of Mississippi, the USA, for their financial support.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Selim, K.A.; El-Beih, A.A.; AbdEl-Rahman, T.M.; El-Diwany, A.I. Biology of Endophytic Fungi. *Curr. Res. Environm. Appl. Mycol.* **2012**, *2*, 31–82. [CrossRef]
2. Bérdy, J. Bioactive Microbial Metabolites. *J. Antibiot* **2005**, *58*, 1–26. [CrossRef] [PubMed]
3. Gao, H.; Li, G.; Lou, H.-X. Structural Diversity and Biological Activities of Novel Secondary Metabolites from Endophytes. *Molecules* **2018**, *23*, 646. [CrossRef] [PubMed]
4. Li, G.; Lou, H.-X. Strategies to Diversify Natural Products for Drug Discovery. *Med. Res. Rev.* **2018**, *38*, 1255–1294. [CrossRef]
5. Zhang, H.W.; Song, Y.C.; Tan, R.X. Biology and chemistry of endophytes. *Nat. Prod. Rep.* **2006**, *23*, 753–771. [CrossRef] [PubMed]
6. Cao, F.; Meng, Z.H.; Wang, P.; Luo, D.Q.; Zhu, H.J. Dipleosporalones A and B, dimeric azaphilones from a marine-derived *Pleosporales* sp. fungus. *J. Nat. Prod.* **2020**, *83*, 1283–1287. [CrossRef]
7. Cao, F.; Meng, Z.H.; Mu, X.; Yue, Y.F.; Zhu, H.J. Absolute configuration of bioactive azaphilones from the marine-derived fungus *Pleosporales* sp. CF09-1. *J. Nat. Prod.* **2019**, *82*, 386–392. [CrossRef]
8. Cao, F.; Zhao, D.; Chen, X.-Y.; Liang, X.D.; Li, W.; Zhu, H.J. Antifungal drimane sesquiterpenoids from a marine-derived *Pleosporales* sp. fungus. *Chem. Nat. Compd.* **2017**, *53*, 1189–1191. [CrossRef]
9. Zhang, L.-H.; Bai, J.; Yan, D.-J.; Wang, Y.-N.; Zhang, Y.-L.; Li, L.; Liu, B.-Y.; Hu, Y.-C. Pleosporalesones A–B, two unique polyketides isolated from *Pleosporales* sp. *Tetrahedron Lett.* **2019**, *60*, 375–377. [CrossRef]
10. Chen, C.J.; Zhou, Y.Q.; Liu, X.X.; Zhang, W.J.; Hu, S.S.; Lin, L.P.; Huo, G.M.; Jiao, R.H.; Tan, R.X.; Ge, H.M. Antimicrobial and anti-inflammatory compounds from a marine fungus *Pleosporales* sp. *Tetrahedron Lett.* **2015**, *56*, 6183–6189. [CrossRef]
11. Dong, L.; Kim, H.J.; Cao, T.Q.; Liu, Z.; Lee, H.; Ko, W.; Kim, Y.C.; Sohn, J.H.; Kim, T.K.; Yim, J.H.; et al. Anti-Inflammatory effects of metabolites from antarctic fungal strain *Pleosporales* sp. SF-7343 in HaCaT human keratinocytes. *Int. J. Mol. Sci.* **2021**, *22*, 9674. [CrossRef] [PubMed]
12. Dong, L.; Cao, T.Q.; Liu, Z.; Tuan, N.Q.; Kim, Y.C.; Sohn, J.H.; Yim, J.H.; Lee, D.S.; Oh, H. Anti-Inflammatory effects exerted by 14-methoxyalternate C from antarctic fungal strain *Pleosporales* sp. SF-7343 via the regulation of NF-κB and JAK2/STAT3 in HaCaT human keratinocytes. *Int. J. Mol. Sci.* **2022**, *23*, 14642. [CrossRef] [PubMed]
13. Li, G.; Xu, K.; Chen, W.Q.; Guo, Z.H.; Liu, Y.T.; Qiao, Y.N.; Sun, Y.; Sun, G.; Peng, X.P.; Lou, H.X. Heptaketides from the endophytic fungus *Pleosporales* sp. F46 and their antifungal and cytotoxic activities. *RSC Adv.* **2019**, *9*, 12913–12920. [CrossRef] [PubMed]
14. Zhou, J.; Zhang, H.; Ye, J.; Wu, X.; Wang, W.; Lin, H.; Yan, X.; Lazaro, J.E.H.; Wang, T.; Naman, C.B.; et al. Cytotoxic polyketide metabolites from a marine mesophotic zone Chalinidae sponge-associated fungus *Pleosporales* sp. NBUF144. *Mar. Drugs* **2021**, *19*, 186. [CrossRef] [PubMed]
15. Han, Y.; Sun, C.; Li, C.; Zhang, G.; Zhu, T.; Li, D.; Che, Q. Antibacterial phenalenone derivatives from marine-derived fungus *Pleosporales* sp. HDN1811400. *Tetrahedron Lett.* **2021**, *68*, 152938. [CrossRef]
16. Xu, Y.; Zhu, A.; Cao, F.; Liu, Y.-F. Diketopiperazine alkaloids and steroids from a marine-derived *Pleosporales* sp. fungus. *Chem. Nat. Compd.* **2018**, *54*, 818–820. [CrossRef]
17. Zeng, H.T.; Yu, Y.H.; Zeng, X.; Li, M.M.; Li, X.; Xu, S.S.; Tu, Z.C.; Yuan, T. Anti-inflammatory dimeric benzophenones from an endophytic *Pleosporales* species. *J. Nat. Prod.* **2022**, *85*, 162–168. [CrossRef]
18. Alhadrami, H.A.; Sayed, A.M.; El-Gendy, A.O.; Shamikh, Y.I.; Gaber, Y.; Bakeer, W.; Sheirf, N.H.; Attia, E.Z.; Shaban, G.M.; Khalifa, B.A.; et al. A metabolomic approach to target antimalarial metabolites in the *Artemisia annua* fungal endophytes. *Sci. Rep.* **2021**, *11*, 2770. [CrossRef]
19. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]
20. Abdelaleem, E.R.; Samy, M.N.; Ali, T.F.S.; Mustafa, M.; Ibrahim, M.A.A.; Bringmann, G.; Ahmed, S.A.; Abdelmohsen, U.R.; Desoukey, S.Y. NS3 helicase inhibitory potential of the marine sponge *Spongia irregularis*. *RSC Adv.* **2022**, *12*, 2992–3002. [CrossRef]
21. Attia, E.Z.; Khalifa, B.A.; Shaban, G.M.; Amin, M.N.; Akil, L.; Khadra, I.; Al Karmalawy, A.A.; Alnajjar, R.; Zaki, M.Y.W.; Aly, O.M.; et al. Abdelmohsen, U.R. Potential topoisomerases inhibitors from *Aspergillus terreus* using virtual screening. *S. Afr. J. Bot.* **2022**, *149*, 632–645. [CrossRef]
22. Noor, A.O.; Almasri, D.M.; Bagalagel, A.A.; Abdallah, H.M.; Mohamed, S.G.A.; Mohamed, G.A.; Ibrahim, S.R.M. Naturally occurring isocoumarins derivatives from endophytic fungi: Sources, isolation, structural characterization, biosynthesis, and biological activities. *Molecules* **2020**, *25*, 395. [CrossRef] [PubMed]
23. Wen, S.; Fan, W.; Guo, H.; Huang, C.; Yan, Z.; Long, Y. Two new secondary metabolites from the mangrove endophytic fungus *Pleosporales* sp. SK7. *Nat. Prod. Res.* **2020**, *34*, 2919–2925. [CrossRef] [PubMed]
24. Aly, A.H.; Edrada-Ebel, R.; Indriani, I.D.; Wray, V.; Müller, W.E.; Totzke, F.; Lin, W. Cytotoxic metabolites from the fungal endophyte *Alternaria* sp. and their subsequent detection in its host plant *Polygonum senegalense*. *J. Nat. Prod.* **2008**, *71*, 972–980. [CrossRef] [PubMed]
25. Zhang, C.; Ondeyka, J.G.; Zink, D.L.; Basilio, A.; Vicente, F.; Collado, J.; Platas, G.; Huber, J.; Dorso, K.; Motyl, M.; et al. Isolation, structure and antibacterial activity of pleosporone from a *Pleosporalean ascomycete* discovered by using antisense strategy. *Bioorg. Med. Chem.* **2009**, *17*, 2162–2166. [CrossRef] [PubMed]

26. Okamura, N.; Haraguchi, H.; Hashimoto, K.; Yagi, A. Altersolanol-related antimicrobial compounds from a strain of *Alternaria solani*. *Phytochemistry* **1993**, *34*, 1005–1009. [CrossRef]
27. Brian, P.W.; Curtis, P.J.; Grove, J.; Hemming, H.G.; Unwin, C.H.; Wright, J.M. Alternaric Acid, a Biologically Active Metabolic Product of the Fungus *Alternaria solani*. *Nature* **1949**, *164*, 534. [CrossRef]
28. Seitz, L.M.; Paukstelis, J.V. Metabolites of *Alternaria alternata*: Ergosterol and ergosta-4, 6, 8 (14), 22-tetraen-3-one. *J. Agric. Food Chem.* **1977**, *25*, 838–841. [CrossRef]
29. Ai, H.-L.; Zhang, L.-M.; Chen, Y.-P.; Zi, S.-H.; Xiang, H.; Zhao, D.-K.; Shen, Y. Two new compounds from an endophytic fungus *Alternaria solani*. *J. Asian Nat. Prod. Res.* **2012**, *14*, 1144–1148. [CrossRef]
30. Krohn, K.; Biele, C.; Aust, H.J.; Draeger, S.; Schulz, B. Herbarulide, a ketodivinyllactone steroid with an unprecedented homo-6-oxaergostane skeleton from the endophytic fungus *Pleospora herbarum*. *J. Nat. Prod.* **1999**, *62*, 629–630. [CrossRef]
31. Fujii, I.; Yoshida, N.; Shimomaki, S.; Oikawa, H.; Ebizuka, Y. An iterative type I polyketide synthase PKSN catalyzes synthesis of the decaketide alternapyrone with regio-specific octa-methylation. *Chem. Biol.* **2005**, *12*, 1301–1309. [CrossRef] [PubMed]
32. Heidary Jamebozorgi, F.; Yousefzadi, M.; Firuzi, O.; Nazemi, M.; Jassbi, A.R. In vitro anti-proliferative activities of the sterols and fatty acids isolated from the Persian Gulf sponge; *Axinella sinoxea*. *DARU J. Pharm. Sci.* **2019**, *27*, 121–135. [CrossRef] [PubMed]
33. Chamras, H.; Ardashian, A.; Heber, D.; Glaspy, J.A. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. *J. Nutr. Biochem.* **2002**, *13*, 711–716. [CrossRef]
34. Serini, S.; Piccioni, E.; Merendino, N.; Calviello, G. Dietary polyunsaturated fatty acids as inducers of apoptosis: Implications for cancer. *Apoptosis* **2009**, *14*, 135–152. [CrossRef] [PubMed]
35. Wen, B.; Deutsch, E.; Opolon, P.; Auperin, A.; Frascogna, V.; Connault, E.; Bourhis, J. n-3 Polyunsaturated fatty acids decrease mucosal/epidermal reactions and enhance antitumour effect of ionising radiation with inhibition of tumour angiogenesis. *Br. J. Cancer* **2003**, *89*, 1102–1107. [CrossRef]
36. Spencer, L.; Mann, C.; Metcalfe, M.; Webb, M.; Pollard, C.; Spencer, D.; Berry, D.; Steward, W.; Dennison, A. The effect of omega-3 FAs on tumour angiogenesis and their therapeutic potential. *Eur. J. Cancer* **2009**, *45*, 2077–2086. [CrossRef]