fermentation

MDPI

Article

Antiviral and Antioxidant Potential of Fungal Endophytes of Egyptian Medicinal Plants

Khaled A. Selim [1,2,*], Waill A. Elkhateeb [2], Ahmed M. Tawila [3], Ahmed A. El-Beih [2,*], Tahany M. Abdel-Rahman [4], Ahmed I. El-Diwany [2] and Eman F. Ahmed [2]

1 Interfaculty Institute of Microbiology and Infection Medicine, Eberhard Karls Universität Tübingen, 72076 Tübingen, Germany
2 Chemistry of Natural and Microbial Products Department, National Research Center, Dokki, Cairo 12622, Egypt; waillahmed@yahoo.com (W.A.E.); aieldewany_1@yahoo.com (A.I.E.-D.); emanfadl.ahmed@yahoo.com (E.F.A.)
3 Division of Natural Drug Discovery, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan; pharmazone2007@yahoo.com
4 Botany Department, Faculty of Science, Cairo University, P.O. Box 12613, Giza, Egypt; prof_tahany@yahoo.com
* Correspondence: khaled.selim@uni-tuebingen.de (K.A.S.); aae2eg@yahoo.com (A.A.E.-B.)

Received: 23 April 2018; Accepted: 14 June 2018; Published: 25 June 2018

Abstract: This study aimed to explore the antioxidant potential and antiviral activity of endophytic fungi which were isolated from healthy living tissues of medicinal plants. Endophytic strains (29 different taxa) were isolated from 18 Egyptian medicinal plants collected from Saint Katherine Protectorate, Egypt. The fungal endophytes were identified based on morphological characters. All isolates were identified as ascomycetes, except two Zygomycetes strains (*Absidia corymbifera* and *Mucor fuscus*). Isolated endophytes were cultivated on potato dextrose media. The fungal metabolites were extracted by ethyl acetate and examined for their biological activities. Among 99 total extracts, only *Chaetomium globosum*, which was isolated from *Adiantum capillus*, showed a promising DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity (99% at 100 µg/mL). Fifteen extracts prohibited the reproduction of HSV-2 virus. On the other hand, the reproduction of VSV-virus was inhibited by sixteen endophytic extracts. The promising anti-(HSV-2 and VSV) extract of endophytic *Pleospora tarda* strain; that was originally isolated from the medicinal plant *Ephedra aphylla*, showed viral inhibitory activity of 40.7% and 15.2%, respectively. Two compounds, for which antiviral activates could be attributed, were isolated and identified as alternariol and alternariol-(9)-methyl ether using different NMR techniques from *P. tarda* extract. For the first time, we report here the ability of the endophytic fungus *P. tarda* to produce alternariol and alternariol-(9)-methyl ether. The results indicate that the endophytic fungi from medicinal plants are promising sources of bioactive compounds.

Keywords: medicinal plants; antioxidant; antiviral; endophytic fungi; alternariol; alternariol-(9)-methyl ether

1. Introduction

There is a global need for new antiviral compounds to solve drug resistance problems. The resistance of human disease to well-known (commercial) antibiotics is increasing rapidly nowadays, so discovering new alternative agents is indispensable required for management those maladies. Endophytes are relatively not well-studied microorganisms that recently gained importance due to their different biological activities and bioactive compounds with a high level of structural diversity [1–4]. They are considered as a rich source of novel natural metabolites for exploitation in medicine, industry and agriculture [1–5].

The medicinal plants are considered as one of the major reservoirs of endophytic bioactive metabolites for the use in the medicinal applications [2–4,6]. In the present surveys, we evaluated the antioxidant potential and the antiviral effect of the total metabolites produced by some endophytic fungi that inhabited Egyptian medicinal plants. Egyptian medicinal plants are well known for their applications in traditional medicine to cure many infectious diseases [7,8]. Saint Katherine Protectorate is an area rich with medicinal plants, but the biodiversity of microbial communities in Sinai is poorly studied. Nowadays, this area attracts more attention for studying the diversity of endophytic mycobiota from medicinal plants [8,9]. The importance of compounds with antioxidant activity is their protective effect against the oxidative stress caused by oxygen-derived free radicals [10]. The culture filtrate of *Pestalotiopsis microspora*, which was isolated from combretaceaous plant *Terminalia morobensis*, showed promising antioxidant activity [11]. Similarly, graphislactone A, a potent antioxidant agent, was identified as a phenolic metabolite from the endophytic fungus *Cephalosporium* sp., that resided in *Trachelospermum jasminoides* [12]. Moreover, many endophytes were reported as potent viral inhibitors. Recently, it was reported that an endophytic *Streptomyces* sp. strain isolated from the mangrove plant *Bruguiera gymnorrhiza*, produced xiamycin, which exhibits selective anti-HIV activity [13]. Additionally, the endophytic *Emericella* sp. (HK-ZJ) isolated from another mangrove plant *Aegiceras corniculatum*, produces several bioactive isoindolone compounds, for which two of them showed moderate activity against influenza A virus (H_1N_1) [14].

In this current report, we set to exploit the Egyptian medicinal plants in an important region of Sinai to explore the biodiversity and the bioactivity of endophytic metabolites from these medicinal plants.

2. Results and Discussion

2.1. Collected Medicinal Plants (Sampling)

The Egyptian medicinal plants were collected and identified morphologically as shown in Table 1. The healthy and disease-free samples were classified into seven families (Adiantaceae, Compositae, Ephedraceae, Euphorbiaceae, Hypericaceae, Labiatae, and Rubiaceae). Figure 1 shows the selected medicinal plants.

Table 1. Host plants used for isolation of endophytic fungi.

Plant Family	Host Medicinal Plants
Adiantaceae	*Adiantum capillus-veneris*
Asteraceae	*Achillea fragrantissima* *Artemisia herba alba* *Chiliadenus montanus* *Launaea spinosa* *Pulicaria undulate* *Tanacetum sinaicum*
Ephedraceae	*Ephedra alata* *Ephedra aphylla*
Euphorbiaceae	*Euphorbia sanctae-catherine*
Hypericaceae	*Hypericum sinaicum*
Lamiaceae	*Lavandula coronopifolia* *Phlomis aurea* *Stachys aegyptiaca* *Teucrium leucocladum* *Teucrium polium* *Thymus decussatus*
Rubiaceae	*Galium sinaicum*

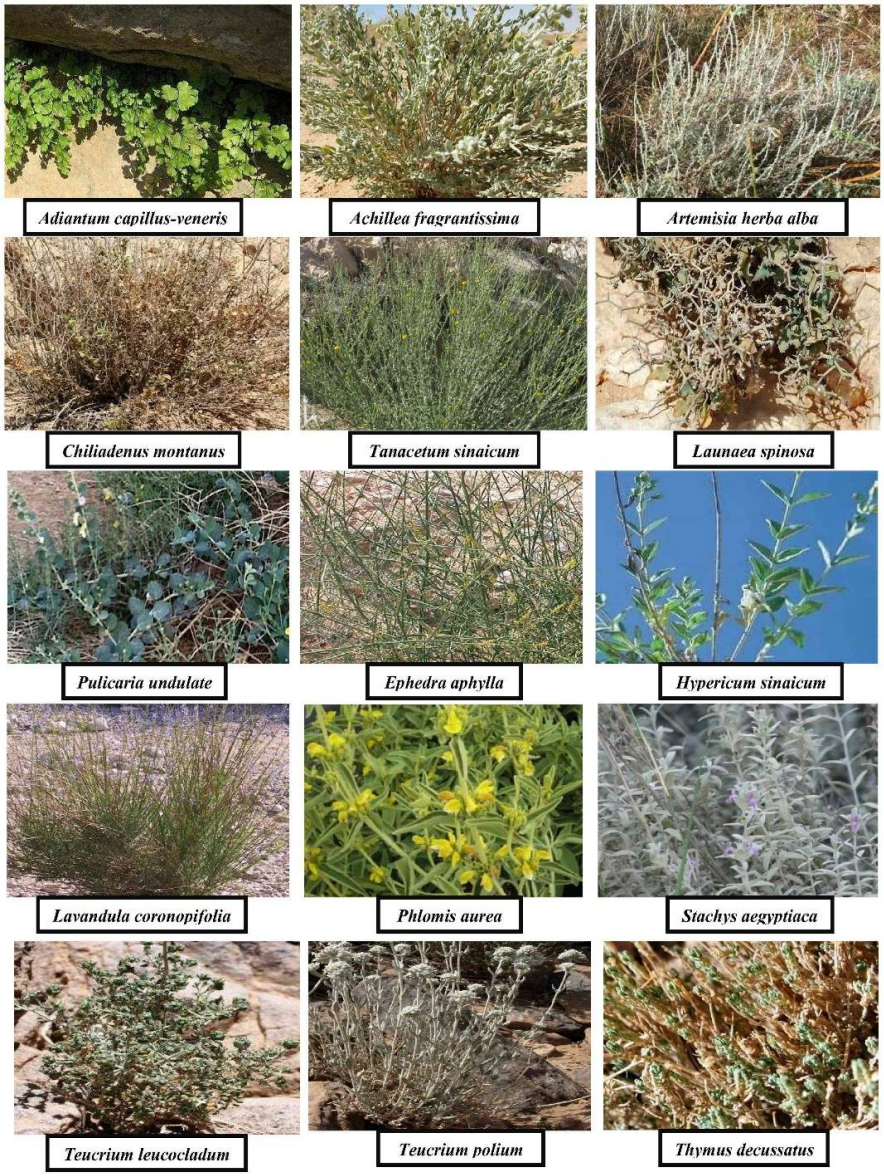

Figure 1. Some of the Saint Katherine medicinal plants which used for isolation of endophytic fungi.

2.2. Isolation and Identification of Endophytic Fungi

Plant samples were used to isolate one hundred and thirty-two endophytic strains, which were purified from the leaves and stems of 18 medicinal plants (Supplementary Tables S1 and S2). The isolated fungal endophytes were identified according to Moubasher (1993) [15]. Isolated fungal strains were cultivated on potato dextrose agar media, as presented in Figure 2.

A collection of 10 isolates from the genus *Aspergillus* was demonstrated. *Aspergillus* was represented by four species, belonging to three groups. *Aspergillus flavus* (three isolates), *Aspergillus niger* (three isolates), *Aspergillus sydowii* (two isolates), *Aspergillus versicolor* (one isolate) and one unidentified *Aspergillus* species, respectively, as in Supplementary Table S1. Furthermore, nine isolates from genus *Penicillium* were purified from the studied desert plants, *Penicillium* was represented by two species, *Penicillium chrysogenum* and *Penicillium corylophilum*, which belonged to one section: Biverticillata-Asymmetrica (two species belonged to subsection Velutina. *Penicillium chrysogenum* (three isolates), *Penicillium corylophilum* (two isolates) and unidentified *Penicillium* species (four isolates), respectively, as revealed in Supplementary Table S1.

Figure 2. Some of endophytic strains isolated from Saint Katherine medicinal plants.

The remaining isolates of different genera and species in the current study, other than *Aspergillus* and *Penicillium*, were listed in Supplementary Table S2. *Absidia corymbifora* (1 isolates), *Acremonium strictum* (two isolates), and one unidentified *Acremonium* sp., *Alternaria alternate* (one isolate), *Chaetomium globosum* (two isolates), *C. spirale* (one isolate), *Cochliobolus lunatus* (one isolate), *Fusarium oxysporum* (two isolates) and unidentified *Fusarium* sp. (one isolate), *Mucor fuscus* (one isolate), *Nigrospora sphaerica* (three isolate), *Phoma leveillei* (one isolate), *Scopulariopsis* sp. (one isolate), *Ulocladium chartarum* (three isolate), *Ulocladium atrum* (one species), *Pleospora tarda* (five isolates) and yeast (one isolate), and fungi with sterile mycelia (white, (25 isolates) and dark, (26 isolates)). All endophytic isolates belonged to ascomycetes, except two zygomycetes strains (*Absidia corymbifera* and *Mucor fuscus*). It was obvious that fungi with sterile mycelia represented 51% from the total number of isolated endophytic strains. Sterile mycelia were common endophytes, 19.69% for dark sterile mycelia, and 18.93% for white sterile mycelia, and these percentages came in accordance with many endophytic studies [16,17].

2.3. Antioxidant and Antiviral Activities

More than one hundred endophytic fungi were isolated and purified from Saint Katharine medicinal plants, and extracted with ethyl acetate. The total extracts at concentration of 50 µg/mL were investigated for their antioxidant and antiviral activities. Of all the 99 crude extracts,

only *Chaetomium globosum* isolated from the internal tissues of *Adiantum capillus-veneris* revealed a promising antioxidant activity (99%) DPPH scavenging activity at the level 100 µg/mL of total extract (Table 2). Genetic identification (18S rDNA) of the active antioxidant isolate was achieved in our previous report [10]. It showed a high similarity to *Chaetomium globosum*. The sequence of the fungus nucleotides was submitted to GenBank to estimate DNA similarities, and was assigned the accession number JN711454 [10]. In our previous study [10], we attributed the antioxidant potential of *C. globosum* to the phenolic compounds. The phenolic hydroxyl groups are considered as functional and characteristic groups responsible for the main antioxidant activity. Our results were in accordance with those of Ravindran and Naveenan (2011) [18], who investigated the bioactivity of the aqueous extracts of *C. globosum* which exhibited antioxidant activity at different concentrations using different techniques of DPPH assay, hydroxyl radical scavenging assay, and metal chelating assay. On the other hand, the moderately antioxidant property of the endophyte, *Penicillium* sp., was elucidated, which revealed (28%) DPPH scavenging activity. It is interesting that this strain was also isolated from the same plant (*Adiantum capillus-veneris*). Accordingly, we concluded that the host–microbe interaction is one of the driving forces for endophytic secondary metabolites production [3–5,8]. The previous results confirmed that *C. globosum* was considered as a promising source of bioactive metabolites which could be used in drug industry [10].

The fungal endophytic extracts which showed, in our previous report [8], promising antimicrobial activities, were selected for investigating their antiviral activities against representative DNA and RNA viruses. The results of antiviral activity were expressed in form of inhibition percent of cytopathogenic effects (% of CPE inhibition), as previously described [10]. The results of antiviral activity in the form of percent of CPE inhibition is shown in Table 2. For simplicity, the VERO cell line was treated with 50 µg/mL of selected endophytic extracts for 24 h before the viral infection, to test the potency of fungal endophytic extracts to stop reproduction of the viruses. The negative signs and zero of inhibition (% of CPE) refer to increase in virus titer in treated cells compared to control (non-treated cells); these signs mean that the viruses adhered to cells, replicated, and the extract was inactive. The positive signs indicate that the extracts are active, where they stopped the viral reproduction machinery, and decreased the viruses' titer compared to the titer of control [10,18]. Fifteen extracts inhibited viral reproduction (positive signs), and the remaining tested extracts were inactive. On the other hand, the coherence and reproduction of VSV virus was inhibited by sixteen endophytic extracts. The endophytic fungus, *Pleospora tarda*, associated with medicinal plant *Ephedra aphylla*, and sterile mycelia (w9) from host plant *Ephedra alata*, were the most potent candidates against HSV-2, with inhibitory activity of 40.7% for both. On the other hand, different endophytic metabolites interrupted the attack of VSV to Vero cells, for example, *Acremonium strictum* from *Launea spinos*, *Penicillium* sp. from *Phlomis aurea*, and *Mucor fuscus* from *Stachys aegyptiaca*. The most active strain to stop the reproduction of the VSV was *Aspergillus* sp. from *Galium sinaicum*.

The endophytic fungus *Chaetomium globosum*, which was isolated from host plant *Hypericum sinaicum*, showed a weak antioxidant and anti-VSV activities, while the same isolate from another host plant, *Adiantum capillus-veneris*, showed only promising antioxidant activity (99% of DPPH scavenging activity). The same trend was observed also in the activities of *Ulocladium chartarum*, which was isolated from *Pulicaria undulate*, and from *Hypericum sinaicum*. The first isolate exhibited a weak antioxidant activity, stopped the contact between the virus and the host cells and prevented VSV reproduction, while the second isolated strain showed a higher antioxidant, but did not influence the HSV reproduction.

Moreover, *Acremonium strictum*, which was isolated from different hosts, showed significant difference in biological activity, as shown in Table 2. From previous results, it was clear that the same isolates which inhabited different hosts exhibited different and varied biological activities. These results confirmed that in any ecosystem, the production of secondary metabolites is based on the mutualistic relationship of the endophyte and its host.

Table 2. Antioxidant and antiviral activities of different fungal endophytes extracts.

Plant Family	Host Plant	Endophyte	% of Antioxidant Activity	% of CPE Inhibition of HSV	% of CPE Inhibition of VSV	Plant Family	Host Plant	Endophyte	% of Antioxidant Activity	% of CPE Inhibition of HSV	% of CPE Inhibition of VSV
Adiantaceae	***Adiantum capillus-veneris***	*Chaetomium globosum*	99%	0%	−15.2	**Euphorbiaceae**	***Euphorbia sancte catherine***	White sterile mycelia 3	23%	ND	ND
		Penicillium sp.	28%	ND	ND			White sterile mycelia 4	9%	ND	ND
Asteraceae	***Achillea fragrantissima***	Dark sterile mycelia 1	4%	14.8%	−6.8			Dark sterile mycelia 3	8%	ND	ND
		Dark sterile mycelia 2	15%	0%	ND			*Aspergillus sydowii*	—-	ND	ND
		White sterile mycelia 1	1%	0%	0			*Acremonium strictum*	1.5%	2.2%	−6.8
		White sterile mycelia 2	6%	0%	−6.8	**Hypericaceae**	***Hypericum sinaicum***	*Ulocladium chartarum*	19%	0%	ND
		Penicillium corylophilum	—-	ND	ND			Dark sterile mycelia	14%	ND	ND
	Artimisia herba alba	*Nigrospora sphaerica*	14%	ND	ND			*Pleospora tarda*	16%	0%	8.4
		White sterile mycelia 1	15%	0%	−6.8			*Chaetomium globosum*	12%	−14.8%	6.8
		Dark sterile mycelia	14%	0%	−15.2			White sterile mycelia 1	12%	ND	ND
		White sterile mycelia 2	15%	22.2%	0			Yeast	7%	ND	ND
		Aspergillus flavus	8%	ND	ND			White sterile mycelia 2	11%	0%	8.3
	Chiliadenus montanus	Dark sterile mycelia	10%	14.8%	0	**Lamiaceae**	***Lavandula coronopifolia***	Dark sterile mycelia 1	9%	17.0%	ND
		Nigrospora sphaerica	13%	14.8%	−15.2			Dark sterile mycelia 2	21%	ND	ND
	Launea spinosa	*Acremonium strictum*	6%	14.8%	0			Dark sterile mycelia 3	5%	ND	ND
		Acremonium sp.	7%	ND	ND			*Scopulariopsis* sp.	—-	ND	ND
		Penicillium chrysogenoum	0.5%	−21.5%	−22.13		***Phlomis aurea***	Dark sterile mycelia 1	2%	ND	ND
		Aspergillus niger	0.5%	0%	−19.03			White sterile mycelia	8%	0%	ND
	Pulicaria undulate	Dark sterile mycelia 1	21%	ND	ND			Dark sterile mycelia 2	—-	−18.5%	0
		Dark sterile mycelia 2	15%	0%	8.4			*Chaetomium spirale*	2%	0%	−15.26
		Ulocladium chartarum	8%	0%	8.4			*Penicillium* sp.	—-	−18.5%	0
		Penicillium sp.	24%	0%	−22.12			*Aspergillus flavus*	5.5%	ND	ND

Table 2. *Cont.*

Plant Family	Host Plant	Endophyte	% of Antioxidant Activity	% of CPE Inhibition of HSV	% of CPE Inhibition of VSV	Plant Family	Host Plant	Endophyte	% of Antioxidant Activity	% of CPE Inhibition of HSV	% of CPE Inhibition of VSV
		Dark sterile mycelia 3	14%	26%	8.4		*Stachys aegyptiaca*	*Mucor fuscus*	—-	14.8%	0%
		Ulocladium atrum	14%	ND	ND			White sterile mycelia 1	2%	ND	ND
		White sterile mycelia	16%	ND	ND			*Pleospora tarda*	1%	ND	ND
	Tanacetum sinaicum	White sterile mycelia 1	6%	−14.8%	8.39			White sterile mycelia 2	—-	ND	ND
		White sterile mycelia 2	2%	ND	ND			*Aspergillus flavus*	0.2%	14.8%	−9.9%
		White sterile mycelia 3	1%	ND	ND		*Teucrium leucocladum*	White sterile mycelia 1	15%	18.5%	ND
		Penicillium chrysogenoum	—-	ND	ND			White sterile mycelia 2	15%	0%	0
		Penicillium sp.	6%	0%	15.2			Dark sterile mycelia 1	16%	−11.1%	−6.8
		Aspergillus sydowii	2%	ND	ND			Dark sterile mycelia 2	17%	ND	ND
Ephedraceae	*Ephedra alata*	White sterile mycelia 1	9%	40.7%	15.2			Dark sterile mycelia 3	14%	ND	ND
		White sterile mycelia 2	15%	0%	8.3			White sterile mycelia 3	15%	ND	ND
		Dark sterile mycelia 1	2%	ND	ND		*Teucrium polium*	*Alternaria alternata*	11%	0%	15.2
		Dark sterile mycelia 2	7.5%	ND	ND			*Nigrospora sphaerica*	1%	0%	−15.4
		Dark sterile mycelia 3	1%	−14.8%	0			White sterile mycelia 1	10%	0%	8.4
		White sterile mycelia 3	—-	−11.1%	−6.8			White sterile mycelia 2	18%	ND	ND
		Dark sterile mycelia 4	22%	ND	ND			*Penicillium corylophilum*	—-	0%	−15.2
		Dark sterile mycelia5	4%	ND	ND			*Penicillium chrysogenoum*	—-	ND	ND
		Unidentified bacteria	—-	ND	ND			*Aspergillus niger*	0.5%	0%	0
	Ephedra aphylla	*Pleospora tarda*	17%	40.7%	15.2%		*Thymus decussates*	Unidentified bacteria	4%	ND	ND
		Dark sterile mycelia 1	—-	0%	ND	Rubiaceae	*Galium sinaicum*	*Pleospora tarda* 1	0	0%	−15.2%
		Aspergillus versicolor	11%	ND	ND			*Fusarium oxysporum*	—-	ND	ND
		Dark sterile mycelia 2	6%	ND	ND			*Ulocladium chartarum*	—-	ND	ND
		Aspergillus niger	—-	ND	ND			*Aspergillus* sp.	—-	−3.7%	23.66%
Euphorbiaceae	*Euphorbia sancte catherine*	Dark sterile mycelia 1	21%	0%	0			*Cochliobolus lunatus*	5%	ND	ND
		White sterile mycelia 1	17%	0%	ND			*Pleospora tarda* 2	—-	NA	ND
		Phoma leveillei	19%	14.8%	15.2			*Absidia corymbifora*	—-	ND	ND
		White sterile mycelia 2	6%	ND	ND			*Fusarium* sp.	1%	11.1%	ND
		Dark sterile mycelia 2	8%	ND	ND						

ND: Not determined.

2.4. Identification of the Secondary Metabolites Compounds of Pleospora tarda

Due to the potential activity of the endophytic *Pleospora tarda* strain, isolated from the medicinal plant *Ephedra aphylla*, against HSV-2 and VSV viruses, it was selected for further chemical investigation to identify the major secondary metabolites produced by that strain. It was cultivated on potato dextrose agar (PDA) media, and the ethyl acetate extract was subjected to extensive purification using silica gel chromatography followed by purification on HPLC using C-18 column. Two compounds were obtained and identified as alternariol and alternariol-(9)-methyl ether (Figure 3).

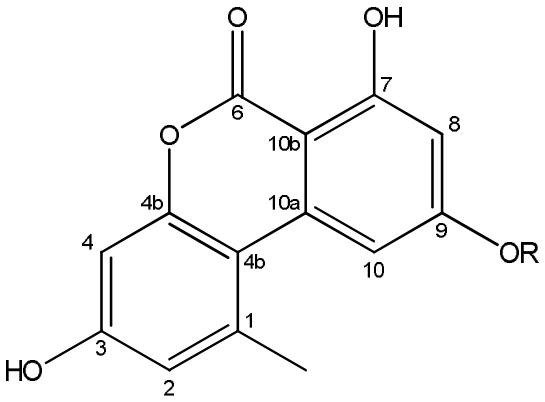

Figure 3. Compounds isolated from the endophytic fungus *P. tarda*, alternariol (compound **1**) and alternariol-(9)-methyl ether (compound **2**). 1: R = H, 2: R = CH_3.

The compound (**1**) was identified based on ^{1}H NMR and ^{13}C NMR, and 2D NMR correlation HMBC (Supplementary Figures S1–S3). ^{1}H NMR (400 MHz, (CD3)2SO): δ 7.19 (1 H, d, *J* = 2.4 Hz, H10), 6.66 (1 H, d, *J* = 2.0 Hz, H2), 6.58 (1 H, d, *J* = 2.4 Hz, H4), 6.32 (1 H, d, *J* = 2.0 Hz, H8), 2.65 (3 H, s, CH_3 at C1) ppm. ^{13}C NMR (100 MHz, (CD3)2SO): δ 166.0 (C6), 165.2 (C7), 164.5 (C9), 158.9 (C3), 153.1 (C4a), 138.8 (C10a), 138.6 (C1), 118.0 (C2), 109.4 (C4b), 104.8 (C10), 102.1 (C4), 101.4 (C8), 97.8 (C10b), 25.7 (CH_3 at C1) ppm. Based on HMBC correlation, the compound (**1**) was identified as alternariol [19].

Compound (**2**) was identified as a derivative of compound 1 with methoxy group attached to (C9) based on ^{1}H NMR (Supplementary Figure S4). ^{1}H NMR (400 MHz, (CD3)2SO): δ 7.20 (1 H, d, *J* = 2.4 Hz, H10), 6.69 (1 H, d, *J* = 2.0 Hz, H2), 6.61 (1 H, d, *J* = 2.4 Hz, H4), 6.59 (1 H, d, *J* = 2.0 Hz, H8), 3.88 (3 H, s, OCH_3 at C9), 2.71 (3 H, s, CH_3 at C1) ppm. Hence, compound (**2**) was identified as alternariol-(9)-methyl ether [19,20].

To the best of our knowledge, this is the first time the isolation of alternariol (compound **1**) and alternariol-(9)-methyl ether (compound **2**) from the endophytic fungus *Pleospora tarda* has been reported. It was demonstrated before that heptaketide compounds isolated from three endolichenic fungal strains had antiviral activity [21]. The potency of alternariol and alternariol-(9)-methyl ether to inhibit herpes simplex virus (HSV), in vitro, has been previously reported. They showed an inhibition with IC_{50} values of 13.5 and 21.3 μM with selective index of 26.5 and 17.1, respectively. Thus, we referred the antiviral activity of endophytic *P. tarda* extract to these compounds. The isolated compounds inhibited, previously, the viral replication, either by acting as a polymerase inhibitor, or via inhibiting pre-integration steps. However, it was reported previously that the alternariol and alternariol-9-methyl ether are the main mycotoxins of fungi of the genus Alternaria, which limit the usage of alternariol based compounds in pharmaceutical industry. Several research studies indicated that Alternaria mycotoxins are directly related to the increased incidence of esophageal cancer. Besides, alternariol and alternariol-9-methyl ether were able to induce gene mutations and DNA damage in animal cell lines, and to inhibit topoisomerase I and IIα [22].

From the previous, it was obvious that endophytes represented an abundant and dependable source of bioactive agents, which could be explored for exploitation in pharmaceutical applications.

In this current study, light was shed on the bioactivity of some important metabolites from endophytes which were isolated from representative medicinal plants of the Sinai region. We also elucidated the chemical purification and identification of some compounds from these active strains. These results indicated the importance of Egyptian medicinal plants as a new possible source of active compounds which can be used in pharmaceutical drug industries.

3. Conclusions

The total metabolites of a large number of endophytic fungi exhibited different bioactivities. The *Adiantum capillus-veneris* isolate, *Chaetomium globosum*, was the most potent antioxidant strain, while *Pleospora tarda*, from the medicinal plant *Ephedra aphylla*, showed promising antiviral activity. However, other isolates of the endophytic fungi stopped reproduction of HSV-2and VSV viruses in cells. The great biodiversity and promising bioactivities of endophytic isolates from medicinal plants indicated the importance and requirement for further investigations of pure compounds from the active strains. Therefore, the usage of the endophytes would be crucial approach to search for novel natural products and will help in conservation of medicinal plants and maintenance of environmental biodiversity. Interestingly, it seems that the host–endophytic interaction plays a crucial role in the direction of the endophytes secondary metabolites production.

4. Materials and Methods

4.1. Plant Material

The medicinal plants were collected from Saint Katherine Protectorate, Sinai, Egypt, in September 2010. Only apparently healthy and disease-free plants were collected, to minimize the presence of pathogenic and saprophytic microbes. The plants were stored in separate plastic bags at 4 °C in an ice box until isolation procedures were conducted [8].

4.2. Isolation of Endophytic Fungi

The endophytic fungi were isolated from fresh medicinal plants leaves by the method described by Selim and coworkers in 2011 [8].

4.3. Identification of Endophytic Fungi

Taxonomic identification of endophytic isolates was performed on the basis of morphological characters of fungal culture, colony, hyphae, and the characters of reproductive structure of endophytic fungus, according to identification keys [15].

4.4. Fermentation and Extraction of Fungal Secondary Metabolites

The isolated strains were cultivated over rich standard potato dextrose agar (PDA) medium, (peeled potato 200 g, dextrose 20 g, agar 20 g, and 1 L distilled water). The media were statically incubated at 30 °C, for 3 weeks. Then, the mycelia mat and the media were extracted with ethyl acetate by using an ultrasonic bath for 30 min. The extraction procedures were performed in triplicate. The extract was evaporated to dryness under reduced vacuum [10].

4.5. Identification of Secondary Metabolites

The total ethyl acetate extract of active *Pleospora tarda* strain grown on PDA media, was subjected to extensive purification using silica gel chromatography, followed by purification on HPLC using C-18 column. The pure compounds were identified on the basis of ^{1}H NMR, ^{13}C NMR, and 2D NMR correlation HMBC.

4.6. Antioxidant Potential of Fungal Extracts

The antioxidant activities of the extracts were assessed using free radical scavenging (FRS) model [23]. Concentrations of fungal extract in DMSO (1000 µg/mL) and DPPH (0.004%) in methanol were prepared. Different concentrations (5–25 µg) of (vitamin C and quercetin) were used as standard controls. In a 96-well plate, 20 µL of (samples or standard) solutions and 180 µL of (0.004%) DPPH were added in each well. The reaction mixture was incubated for 30 min, the assay was run in triplicate, and repeated at least once for active extracts. The absorption was measured at 540 nm by microplate reader. The percentage of scavenging activity was calculated from the following equation:

$$\% \text{ of Scavenging Activity} = [(A_{\text{Blank}} - A_{\text{Sample}})/A_{\text{blank}}] \times 100$$

where A_{Blank} is the absorbance of DPPH only, and A_{Sample} is the absorbance of reaction mixture in presence of test samples.

4.7. In Vitro Antiviral Assay of Endophytes Total Metabolites Using EPTT

The end point titration technique (EPTT) bioassay was used to evaluate the antiviral potential of the ethyl acetate extracts of endophytic fungi [24,25]. Two different viruses were chosen for this technique: herpes simplex virus type-2 (HSV-2) as a DNA virus, and vesicular stomatitis virus (VSV) as an example of an RNA virus. Both viruses were obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt. Investigation of the antiviral activity was accomplished according to the previously fully described method [10]. The antiviral activity was calculated based on the ability of the extracts to inhibit the viral cytopathogenic effects (% of CPE inhibition) [10,17,18], according to the following calculation:

$$\% \text{ of antiviral activity in form of CPE inhibition } \% = [1 - (\text{CPE test}/\text{CPE control})] \times 100$$

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/2311-5637/4/3/49/s1.

Author Contributions: K.A.S. isolated the endophytic fungi, preformed bioassays, and isolated the fungal secondary metabolites. W.A.E. identified the endophytic fungi. K.A.S., A.M.T. and A.A.E.-B. purified and elucidated the structural of fungal secondary metabolites. K.A.S., W.A.E. and E.F.A. wrote the manuscript. A.A.E.-B., T.M.A.-R. and A.I.E.-D. supervised the study.

Funding: The study was financially supported by the National Research Centre, Egypt.

Acknowledgments: The authors would like to thank the national research Centre, Egypt for financial support, and Mohamed Ismael, Katerina Peros, and Eva Bok for the continuous support and the critical reading of the manuscript. Also, we would like to express our appreciation to VACSERA, Egypt for fruitful collaboration.

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

References

1. Tan, R.; Zou, W. Endophytes: A rich source of functional metabolites. *Nat. Prod. Rep.* **2001**, *18*, 448–459. [CrossRef] [PubMed]
2. Strobel, G.; Daisy, B.; Castillo, U.; Harper, J. Natural products from endophytic microorganisms. *J. Nat. Prod.* **2004**, *67*, 257–268. [CrossRef] [PubMed]
3. Selim, K.A.; El-Beih, A.A.; AbdEl-Rahman, T.M.; El-Diwany, A.I. Biology of Endophytic Fungi. *Curr. Res. Environ. Appl. Mycol.* **2012**, *2*, 31–82. [CrossRef]
4. Selim, K.A.; Nagia, M.; El-Ghwas, D.E. Endophytic Fungi are Multifunctional Biosynthesizers: Ecological Role and Chemical Diversity. In *Endophytic Fungi: Diversity, Characterization and Biocontrol*, 1st ed.; NOVA Science Publishers: Hauppauge, NY, USA, 2016.
5. Selim, K.A.; El-Ghwas, D.E.; Selim, R.M.; Hassan, M.I.A. Microbial volatile in defense. In *Volatiles and Food Security: Role of Volatiles in Agro-Ecosystems*, 1st ed.; Springer: Singapore, 2017.
6. Bacon, C.; White, J. *Microbial Endophytes*; Marcel Dekker: New York, NY, USA, 2000.

7. Soltan, M.M.; Zaki, A.K. Antiviral screening of forty-two Egyptian medicinal plants. *J. Ethnopharmacol.* **2009**, *126*, 102–107. [CrossRef] [PubMed]
8. Selim, K.A.; El-Beih, A.A.; AbdEl-Rahman, T.M.; El-Diwany, A.I. Biodiversity and antimicrobial activity of endophytes associated with Egyptian medicinal plants. *Mycosphere* **2011**, *2*, 669–678. [CrossRef]
9. Abdel-Azeem, A.M.; Zaki, S.M.; Khalil, W.F.; Makhlouf, N.A.; Farghaly, L.M. Anti-rheumatoid Activity of Secondary Metabolites Produced by Endophytic *Chaetomium globosum*. *Front. Microbiol.* **2016**, *7*, 1477. [CrossRef] [PubMed]
10. Selim, K.A.; El-Beih, A.A.; Abdel-Rahman, T.M.; El-Diwany, A.I. Biological evaluation of endophytic fungus, *Chaetomium globosum* JN711454, as potential candidate for improving drug discovery. *Cell Biochem. Biophys.* **2014**, *68*, 67–82. [CrossRef] [PubMed]
11. Harper, J.K.E.J.; Ford, G.A.; Strobel, A.; Arif, D.M.; Grant, J.; Porco, D.P.; Tomer-Oneill, K. Pestacin: A 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron* **2003**, *59*, 2471–2476. [CrossRef]
12. Song, Y.C.; Huang, W.Y.; Sun, C.; Wang, F.W.; Tan, R.X. Characterization of graphislactone A as the antioxidant and free radical-scavenging substance from the culture of *Cephalosporium* sp. IFB-E001, an endophytic fungus in *Trachelospermum jasminoides*. *Biol. Pharm. Bull.* **2005**, *28*, 506–509. [CrossRef] [PubMed]
13. Ding, L.; Münch, J.; Goerls, M.; Fiebig, H.H.; Lin, W.H.; Hertweck, C. Xiamycin, a pentacyclic indolosesquiterpene with selective anti-HIV activity from a bacterial mangrove endophyte. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6685–668. [CrossRef] [PubMed]
14. Zhang, G.; Sun, S.; Zhu, T.; Lin, Z.; Gu, J.; Li, D.; Gu, Q. Antiviral isoindolone derivatives from an endophytic fungus *Emericella* sp. associated with *Aegiceras corniculatum*. *Phytochemistry* **2011**, *72*, 1436–1442. [CrossRef] [PubMed]
15. Moubasher, A.H. *Soil Fungi in Qatar and Other Arab Countries*; Moreno, G., Diez, J., Manjon, J.L., Eds.; University of Qatar Press: Doha, Qatar, 1993; p. 566.
16. Fröhlich, J.; Hyde, K.D.; Petrini, O. Endophytic fungi associated with palm. *Mycol. Res.* **2000**, *104*, 1202–1212. [CrossRef]
17. Promputtha, I.; Jeewon, R.; Lumyong, S.; McKenzie, E.H.C.; Hyde, K.D. Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). *Fungal Divers.* **2005**, *20*, 167–186.
18. Ravindran, C.; Naveenan, T. Adaptation of marine derived fungus *Chaetomium globosum* (NIOCC 36) to alkaline stress using antioxidant properties. *Process Biochem.* **2011**, *46*, 847–857. [CrossRef]
19. Gu, W. Bioactive metabolites from *Alternaria brassicicola* ML-P08, an endophytic fungus residing in *Malus halliana*. *World J. Microbiol. Biotechnol.* **2009**, *25*, 1677–1683. [CrossRef]
20. Lou, J.; Yu, R.; Wang, X.; Mao, Z.; Fu, L.; Liu, Y.; Zhou, L. Alternariol 9-methyl ether from the endophytic fungus Alternaria sp. Samif01 and its bioactivities. *Braz. J. Microbiol.* **2016**, *47*, 96–101. [CrossRef] [PubMed]
21. He, J.W.; Chen, G.D.; Gao, H.; Yang, F.; Li, X.X.; Peng, T.; Guo, L.D.; Yao, X.S. Heptaketides with antiviral activity from three endolichenic fungal strain *Nigrospora* sp., *Alternaria* sp. and *Phialophora* sp. *Fitoterapia* **2012**, *83*, 1087–1091. [CrossRef] [PubMed]
22. Burkhardt, B.; Pfeiffer, E.; Metzler, M. Absorption and metabolism of the mycotoxins alternariol and alternariol-9-methyl ether in Caco-2 cells in vitro. *Mycotoxin Res.* **2009**, *25*, 149–157. [CrossRef] [PubMed]
23. Selim, K.A.; El-Beih, A.A.; Abdel-Rahman, T.M.; El-Diwany, A.I. High expression level of antioxidants and pharmaceutical bioactivities of endophytic fungus *Chaetomium globosum* JN711454. *Prep. Biochem. Biotechnol.* **2016**, *46*, 131–40. [CrossRef] [PubMed]
24. Vlietinck, A.J.; Van, H.L.; Totte, J.; Lasure, A.; Berghe, D.V.; Rawangabo, P.C. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J. Ethnopharmacol.* **1995**, *46*, 31–47. [CrossRef]
25. El-Sayed, R.A. In Vitro Study on Cell–Virus Interaction under the Effect of Selected Ions Concentrations. Ph.D. Thesis, Medical Biophysics, Cairo University Egypt, Giza, Egypt, 2011.