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Contributed Paper

In vitro Antioxidant and Antitumor Activities of an Endophytic Fungus *Phomopsis liquidambari* QH4 from *Artemisia annua*

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Abstract

Endophytic fungi from medicinal plants are prolific producers of bioactive secondary metabolites. The present study aimed to evaluate the antioxidant and antitumor activities of mycelia of an endophytic *Phomopsis liquidambari* (strain QH4), from *Artemisia annua*. The antioxidant activities of QH4 extracts extracted from methanol, acetone, ethyl acetate, chloroform and hexane were determined via five different antioxidant models namely free radical scavenging capacity using 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$), 2,2-dipheny l-1-picrylhydrazyl (DPPH) and hydroxyl radicals, reducing power assay and ferrous ion chelating ability. The antitumor activity was determined by MTT assay. The methanol extract (200 μg/ml and 4.0mg/ml) exhibited strong antioxidant capacity in each antioxidant model systems, while the ethyl acetate extract (20 μg/ml) was active against HL-60 leukemia, MCF-7 breast and COLO205 colon cell lines with a growth inhibition of 71.99 %, 53.66 % and 92.70 %, respectively. Total phenolic contents (TPC) of the extracts with different solvents were determined using the Folin-Ciocalteu method. The TPC was highest in methanol extract (60.07 ± 0.31 mg gallic acid equivalent/g dry weight of the mycelia) and lowest in hexane extract (6.54 ± 0.29 mg GAE/g DW of the mycelia). The correlation coefficients from regression analysis showed various relationships between TPC in the extracts and antioxidant activities tested via different models including $ABTS^+$ ($R^2 = 0.9651$), DPPH ($R^2 = 0.8490$), hydroxyl radical scavenging ability ($R^2 = 0.9438$), reducing power assay ($R^2 = 0.8266$) and ferrous ion chelating ability ($R^2 = 0.4836$). The endophytic *Phomopsis liquidambari* QH4 may have the potential to become a source of natural antioxidant and/or antitumor agents.

Keywords: endophytic fungus, *Phomopsis liquidambari*, *Artemisia annua*, antioxidant activity, antitumor activity

1. INTRODUCTION

Endophytic fungi, which inhabit normal tissues of hosts without causing apparent symptoms of pathogenesis [1], exist in association with the plant kingdom and are viewed as a rich source of bioactive natural products [2]. These endophytes also have the capacity to produce diverse classes of secondary metabolites with a wide range of biological activities such as antioxidant agent Chlorogenic acid [3], antimicrobial agent hypericin [4], acetylcholinesterase inhibitor huperzine A [5] and antitumor agents taxol [6]. Bioprospecting endophytes thus offer tremendous promise to discover natural products with therapeutic value [7], which have attracted increasing attention among microbiologists, ecologists, agronomists, chemists and pharmacologists.

The herb *Artemisia annua* L. is a member of the family Asteraceae that has been used in traditional Chinese medicine (TCM) for the treatments of fever and malaria since ancient times [8]. To date, artemisinin and its derivatives have provided the basis for the most effective treatments for malaria [9]. Besides malaria, artemisinin and its derivatives have also shown to be effective against a number of viruses, prokaryotic and eukaryotic organisms, and useful in the treatment of ovarian, oral and prostate cancer [10]. Several studies have been performed to explore the endophytes of *A. annua* as new resources, including fungi, actinomycetes and bacteria. Zhang *et al.* [11] evaluated the antimicrobial activities of extracts from 11 endophytic fungi associated with *A. annua*, and concluded that these endophytes could be applied as new sources of antibiotics in agriculture and/or pharmaceutical industries. Wang *et al.* [12] reported that an endophytic fungus with its elicitor from *A. annua* was able to stimulate artemisinin production in hairy roots. Lu *et al.* [13] isolated three new antimicrobial metabolites produced by endophytic fungus *Colletotrichum* sp. from *A. annua*. So far, however, the endophytic fungus *Phomopsis liquidambari* associated with *A. annua* has not been reported previously. Therefore, the aim of this study was to evaluate antioxidant and antitumor activities of the endophytic fungus *Phomopsis liquidambari* QH4 from *A. annua* via different methods, and to determine the relationships between the Total phenolic contents (TPC) of the extracts and their antioxidant activity.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Endophytic Fungi

The endophytic fungi were isolated from fresh leaves of an apparently healthy *A. annua*, collected in July 2012, in the suburb of Guiyang city, Guizhou province, China. The general isolation procedures followed the method described by preceding researchers [3]. Specifically, the leaves of *A. annua* were washed softly with running tap water, then sterilized with 75 % ethanol for 1 min and 2.5 % sodium hypochlorite for 15 min, and again 75 % ethanol for 1 min, followed by rinsing in sterile water for three times. The sterilized leaves tissue were then cut into segments (5×5 mm) and six of them placed on a plate containing potato dextrose agar (PDA) supplemented with penicillin (100 μg/ml) and streptomycin sulphate (100 μg/ml) to prevent bacterial growth, and incubated at 28 °C. After 1 week, the emerging hyphae from segments were transferred onto new PDA Petri dish for purification. The isolated endophytic fungi was maintained on a PDA slant and subcultured once a month. Slants were incubated at 28 °C for 5 days and subsequently stored at 4 °C.

Total genomic DNA of the endophytic fungi was extracted directly from actively growing mycelium scraped from PDA plates,

using the Fungal gDNA Kit GD2416 (Biomiga, CA, USA), according to manufacturers' protocol. The ITS region of nuclear ribosomal RNA genes was amplified with primer pairs ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC). The PCR amplification reactions were performed in a Bio-RAD PTC-200 thermocycler. The PCR reaction mixture (25 μl) contained 12.5 μl of PCR master mix, 9.5μl of molecular biology grade water, 1 μl of each primer and 100 ng of template DNA. The PCR procedure for ITS was as follows: initial denaturation 95 °C for 2 min, followed by 35 cycles at 94 °C for 1 min; annealing 51 °C for 1 min; primer extension 72 °C for 1 min; and a final extension of 72 °C for 1 min. PCR amplification products were visualized using 1 % agarose gel. Sequencing was performed with an ABI PRISM 3730 DNA autosequencer using either dRhodamine terminator or Big Dye Terminator (Applied Biosystems Inc., Foster 19 City, California). Sequences were aligned with additional sequences from GenBank using BioEdit [14] and ClustalX [15]. Alignment was manually adjusted to allow maximum alignment and minimize gaps. Maximum parsimony was applied to the ITS dataset. All characters were weighted and gaps were treated as missing data. Maximum parsimony analysis (PAUP* version 4.0b10) was used [16-17].

2.2 Fermentation and Sampling

The endophytic *P. liquidambari* QH4 was grown under static conditions at 28 °C for 30 days in 80 × 1000 ml conical flasks containing Potato dextrose broth (300 ml/flask). The mycelia were air-dried in a ventilated oven at 35 °C to constant weight and powdered by an electric blender (Yinhe, China). About 1 g of the powdered mycelia was extracted with hexane, dichloromethane, ethyl acetate, acetone and methanol respectively, in a ratio of 1:50 (w/v) for 2h with the aid of ultrasonic waves. Five extract solutions were concentrated by rotary vacuum evaporator (Yarong, China), respectively. All of these extracts in different solvents obtained were weighed and stored at -20 °C until they were used.

2.3 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$) Radical Scavenging Activity

The $ABTS^+$ radicals scavenging activity was measured using the improved method given by Huang *et al.* [3]. The ABTS radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature in darkness for 16 hours. The $ABTS^+$ solution was diluted with 80 % ethanol to an absorbance of 0.700 + 0.005 at 734 nm. Each different solvent extracts of *P. liquidambari* QH4, 0.1 ml, at various concentrations (100-800 μg/ml) in 80 % ethanol was respectively added to 3.9 ml of freshly $ABTS^+$ solution. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance was recorded at 734 nm. Different concentrations (2.5-20 μg/ml) of trolox standard solution in 80 % ethanol were prepared and assayed under the same conditions. Results were expressed in terms of trolox equivalents antioxidant capacity (TEAC) (mg trolox/g dry weight of the mycelia).

2.4 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

The scavenging effects of extracts for DPPH radicals were monitored according to the method described by Duan *et al.* [18]. Each test extract (0.1 ml) at various concentrations (50-400 μg/ml) in 80 % ethanol was mixed with 2.9 ml DPPH solution (120 μM) in methanol and incubated in darkness at 37 °C for 30 min. The absorbance was recorded at 517 nm. Inhibition of free radical by DPPH in percentage (I %) was calculated with the

following equation: I %= $[(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract), and A_{sample} is the absorbance of the test extract. The values of inhibition were calculated for the various concentrations of each extract from the mycelia of *Phomopsis liquidambari* QH4. Butylated hydroxytoluene (BHT) was used as positive control, and all tests were carried out in triplicate.

2.5 Hydroxyl Radical Scavenging Activity

The scavenging ability of the five sample extracts on hydroxyl radicals was determined according to the method described by Smirnoff and Cumbes *et al.* [19] with some modifications. Briefly, individual test extracts (1 ml) at different concentrations (3.0-5.0 mg/ml) in 80 % ethanol was added to the reagent containing 1 ml 1.5 mM $FeSO_4$, 0.7 ml 6 mM H_2O_2 and 0.3 mL 20 mM sodium salicylate. After incubation for 1h at 37 °C, absorbance of the reaction mixture was read at 562 nm. The scavenging ability on hydroxyl radicals in percentage (I %) was calculated using the following equation: I %= $[(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract), and A_{sample} is the absorbance of the test extract. BHT was used as positive control, and all tests were carried out in triplicate.

2.6 Reducing Power Assay

The reducing power assay was conducted as previously described by Oyaizu *et al.* [20]. In brief, each test extract (1 ml) at different concentrations (50-400 μg/ml) in 80 % ethanol was first mixed with 2.5 ml 0.2 M phosphate buffer, pH 6.6, and 2.5 ml 1 % potassium ferricyanide. After incubation at 50 °C for 20 min, 2.5 ml 10 % trichloroacetic acid was added to the mixture followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 ml of the upper layer of the mixture was added to 2.5 ml distilled water and 0.5 ml 0.1 % ferric chloride, and the absorbance of the resulting solution was read at 700 nm against a blank. BHT was used as positive control. The higher absorbance indicated greater reducing power.

2.7 Ferrous Ion Chelating Assay

The chelating ability of the five test extracts was determined by the method previously reported by Singh and Rajini [21]. Individual test extracts (1 ml) at different concentrations (3.0-5.0 mg/ml) in 80 % ethanol were mixed with an equal volume of 0.1 mM $FeSO_4$ and 0.25 mM ferrozine. After incubation for 10 min, absorbance of the mixture was measured at 562 nm. The chelating ability on ferrous ion in percentage (I %) was calculated using the following equation: I %= $[(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract), and A_{sample} is the absorbance of the test extract. EDTA disodium salt (EDTA-2Na) was used as the positive control.

2.8 Determination of Total Phenolic Contents

The amount of total phenol was determined using the Folin-Ciocalteu reagent [22]. One ml each of test extract at different concentrations (50-400 μg/ml) in 80 % ethanol was dissolved in 1.5 ml distilled water and 0.5 ml Folin-Ciocalteu's reagent. After 1 min, 1 ml 20 % sodium carbonate solution was added. The final mixture was shaken three times and incubated for 2 h in the dark at 25 °C. The absorbance of the mixture was measured at 760 nm. A standard curve was first plotted using gallic acid as a standard. All test extracts were carried out in triplicate and the total phenolic contents (TPC) were expressed as gallic acid equivalents (mg GAE/g dry weight of the mycelia).

2.9 Antitumor Activity Assay

The determination of antitumor activity was carried out based on the MTT assay, which was first described by Mosmann to quantitate mitochondrial dehydrogenase activity [23]. The human tumor cell lines [breast (MCF-7), colon (COLO205) and leukemia (HL-60)] obtained from the Cell Line Bank of the Chinese Academy of Science were used in the present study. The cell lines were grown in RPMI-1640 supplemented with 10 % fetal bovine serum under a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C. An aliquot (200 μl) of these cell suspensions at a density of 3×10^4 cell/ml was plated in 96-well microtiter plates and incubated for 24 h at the above conditions. Then 2 μl of the various extracts dimethyl sulfoxide (DMSO) solutions at different concentrations (1.25-200 μg/ml) was added to each well and further incubated for 72 h in the same conditions. MTT solution (20 μl of 5 mg/ml in IPMI-1640 medium) was added to each well and incubated for 4 h. After addition of 100 μL DMSO and incubation for 1h, the cells were lysed to liberate the formed formazan crystals. Absorbance was then determined on a Spectra Max Plus plate reader at 570 nm. The growth inhibition rate in percentage (I %) was calculated using the following equation: $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test extract and using "RPMI-1640 supplemented with 10 % fetal bovine serum" instead of "the test extract"), and A_{sample} is the absorbance of the test extract. Adriamycin was used as the positive control.

2.10 Statistical Analysis

Values were expressed as means ± standard deviation (SD). Analysis of variance was conducted and differences between variables were tested for significance by one-way ANOVA with Tukey test using the SAS 8.0 program. Differences at $P < 0.05$ were considered statistically significant. Correlation coefficients (R^2) were calculated using Microsoft Excel 2003.

3. Results

3.1 Identification of Endophytic Fungus

In order to identify the fungus isolated from the leaves of *Artemisia annua*, its internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) were sequenced and identified by BLAST searches with the nucleotide database in GenBank, which showed that the sequences similar to our sequence were *Phomopsis*. We therefore chose sequences of *Phomopsis* and that of *Valsa mali* (GU174589) as an outgroup for phylogenetic analysis. The ITS dataset included sequences from 10 fungal specimens. Parsimony analysis yielded one tree (TL=169, CI=0.834, RI=0.622, RC=0.519, HI=0.166) that was shown in Figure 1, indicating that this isolate QH4 is a *Phomopsis* sp. closely related to these taxa. Isolate QH4 clustered together with *Phomopsis liquidambari* (AY601919) supported by a high bootstrap value (99 %), which was consistent with the ITS analysis.

3.2 Antioxidant Activity

The antioxidant activities of methanol, acetone, ethyl acetate, chloroform and hexane extracts of QH4 were determined by five different methods namely $ABTS^+$, DPPH, hydroxyl radical scavenging ability, reducing power assay and ferrous ion chelating ability. The results of the assays indicated that the antioxidant activity of the extracts vary with the solvents used for extraction. The methanol extract had the highest antioxidant effect which was higher than the positive control BHT in the reducing power, DPPH and hydroxyl radical scavenging assays (Table 1). Therefore, the antioxidant ability of methanol extract was further confirmed via different

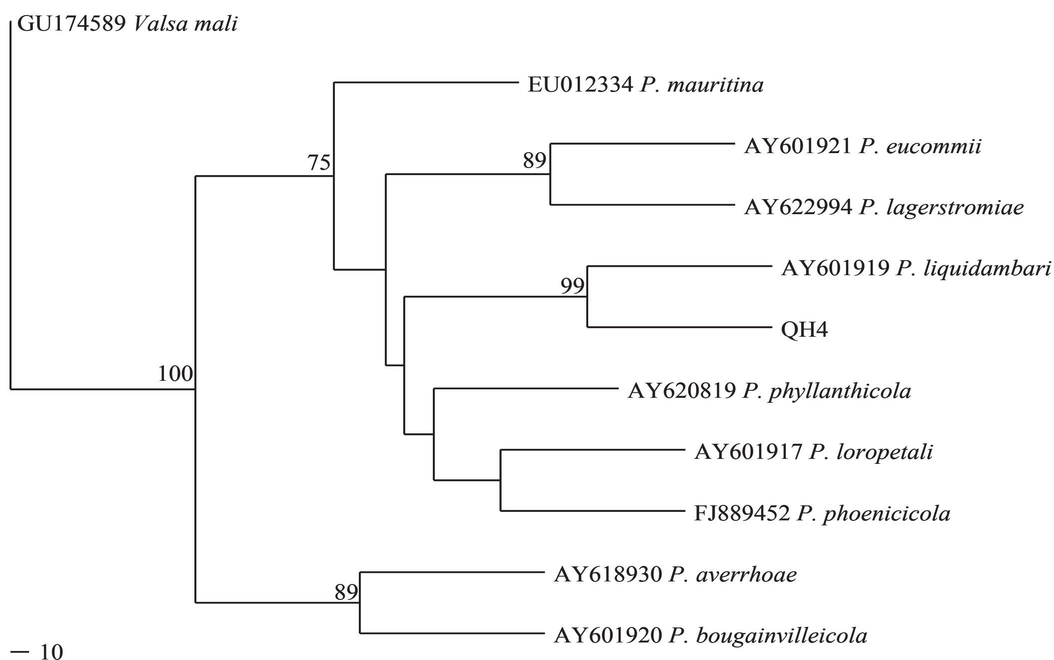

Figure 1. Phylogenetic tree based on maximum parsimony analysis of ITS rDNA data. Bootstrap support values less than 50% are not shown. The tree was rooted with *Valsa mali*.

assays. It was found that the reducing power, DPPH and hydroxyl radical scavenging activities of the methanol extract were dose-dependent and positively correlated (Table 2). When the concentration of the methanol extract was lower than 4.5 mg/ml, the ferrous ion chelating activity showed no significant change with increasing the methanol extract concentration. When the concentration of the methanol extract was between 4.5 mg/ml and 5.0 mg/ml, the ferrous ion chelating activity increased slightly (Table 2). According to the present results, antioxidant activity of extracts in various model systems was dependent on the polarity of extracts. The most polar extract (methanol), had the highest antioxidant activity.

3.3 Relationship Between Total Phenolic Contents and Antioxidant Activity

Endophytic fungi, a potential source of novel natural antioxidants [18, 24], have attracted more and more attention in recent years. Therefore, in this work, we determined the total phenolic contents (TPC) of the mycelia of *Phomopsis liquidambari* QH4 in different solvents. It was found that the amount of TPC in the mycelia of QH4 varied among different solvent extracts. The methanol extract contained the highest TPC, while TPC of hexane extracts were the lowest (Table 1). The results revealed that the methanol extract of QH4 contained significantly higher TPC than those of some plants which were commonly used as antioxidants. As shown in Figure 2, some correlation relationships were present between the TPC of the different extracts and each antioxidant model. With further correlation analysis, the relationship between the TPC and chelating ability had a coefficient of R^2 = 0.4836 (Figure 2A). It was found that there were two positive relationships between the TPC of the different extracts and DPPH

Table 1. Total phenolic contents and antioxidant activities of different extracts of *Phomopsis liquidambari* QH4 with different solvents.

Samples	TPC (mg TE/g DW of the mycelia)	TEAC (mg trolox/g DW of the mycelia)	DPPH scavenging activity (%)	Reducing power (A)	Hydroxyl radical scavenging (%)	Ferrous Ion Chelating (%)
Methanol extract	60.07 ± 0.68a	109.98 ± 0.56a	61.77 ±0.03a	0.313 ± 0.00a	63.07 ± 0.42a	76.84 ± 1.07b
Acetone extract	33.96 ± 0.90b	82.02 ± 1.49b	22.92 ± 1.25d	0.114 ± 0.01c	30.43 ± 0.06c	40.00 ± 1.11d
Ethyl acetate extract	18.44 ± 0.21c	47.22 ± 1.95c	27.89 ± 0.08c	0.121 ± 0.01c	29.53 ± 0.29c	70.23 ± 0.72c
Chloroform extract	10.38 ± 0.95d	26.60 ± 1.92d	19.71 ± 0.26e	0.106 ± 0.00d	14.91 ± 0.17d	36.50 ± 0.81e
Hexane extract	6.54 ± 0.75e	18.60 ± 1.13e	12.93 ± 1.16f	0.090 ± 0.01d	10.33 ± 0.05e	28.02 ± 1.02f
BHT/EDTA-2Na	-	-	54.66 ± 0.09b*	0.204 ± 0.00b*	46.92 ± 0.08b*	100.00 ± 0.42a[#]

Data expressed as means ± standard deviation (SD). Means within each column with different letters (a-f) differ significantly ($P<0.05$).*Comparison with BHT. [#]Comparison with EDTA-2Na.

radical scavenging (R^2 = 0.8490) and reducing power capacity (R^2 = 0.8266) (Figure 2B and 2C), whereas two higher correlations between the TPC and $ABTS^+$ and hydroxyl radical scavenging capacity was determined to be R^2 = 0.9651 and R^2 = 0.9438 (Figure 2D and 2E).

3.4 Antitumor Activity

In vitro screening results of the different solvent extracts of *P. liquidambari* QH4 showed various antitumor activities against the different tumor cell lines (Table 3). The hexane extract was inactive (less than 50 % growth inhibition) against all tested cell lines. The chloroform extract (20 μg/ml) showed growth inhibition of 54.89 % and 83.09 % on MCF-7 breast and COLO205 colon cell lines respectively. The methanol and acetone

Table 2. Antioxidant activities of methanol extract of *Phomopsis liquidambari* QH4 with different concentrations in hydroxyl radical scavenging, DPPH, reducing power assay and chelating assay.

Antioxidant activity models	Antioxidant activity ± SD (%)				
	50 (μg/ml)	100 (μg/ml)	200 (μg/ml)	300 (μg/ml)	400 (μg/ml)
DPPH scavenging activity (%)	29.14 ± 0.08	44.95 ± 0.24	61.77 ± 0.03	70.36 ± 0.11	80.58 ± 0.06
Reducing power (A)	0.107 ± 0.06	0.168 ± 0.00	0.313 ± 0.00	0.340 ± 0.00	0.387 ± 0.00
	3.0 (mg/ml)	3.5 (mg/ml)	4.0 (mg/ml)	4.5 (mg/ml)	5.0 (mg/ml)
Hydroxyl radical scavenging (%)	24.62 ± 0.08	39.29 ± 0.05	63.07 ± 0.42	73.95 ± 0.03	78.34 ±0.04
Ferrous Ion Chelating (%)	72.70 ± 0.31	75.18 ± 0.06	76.84 ± 1.07	72.34 ± 0.08	80.42 ± 0.09

Data expressed as means ± standard deviation (SD).

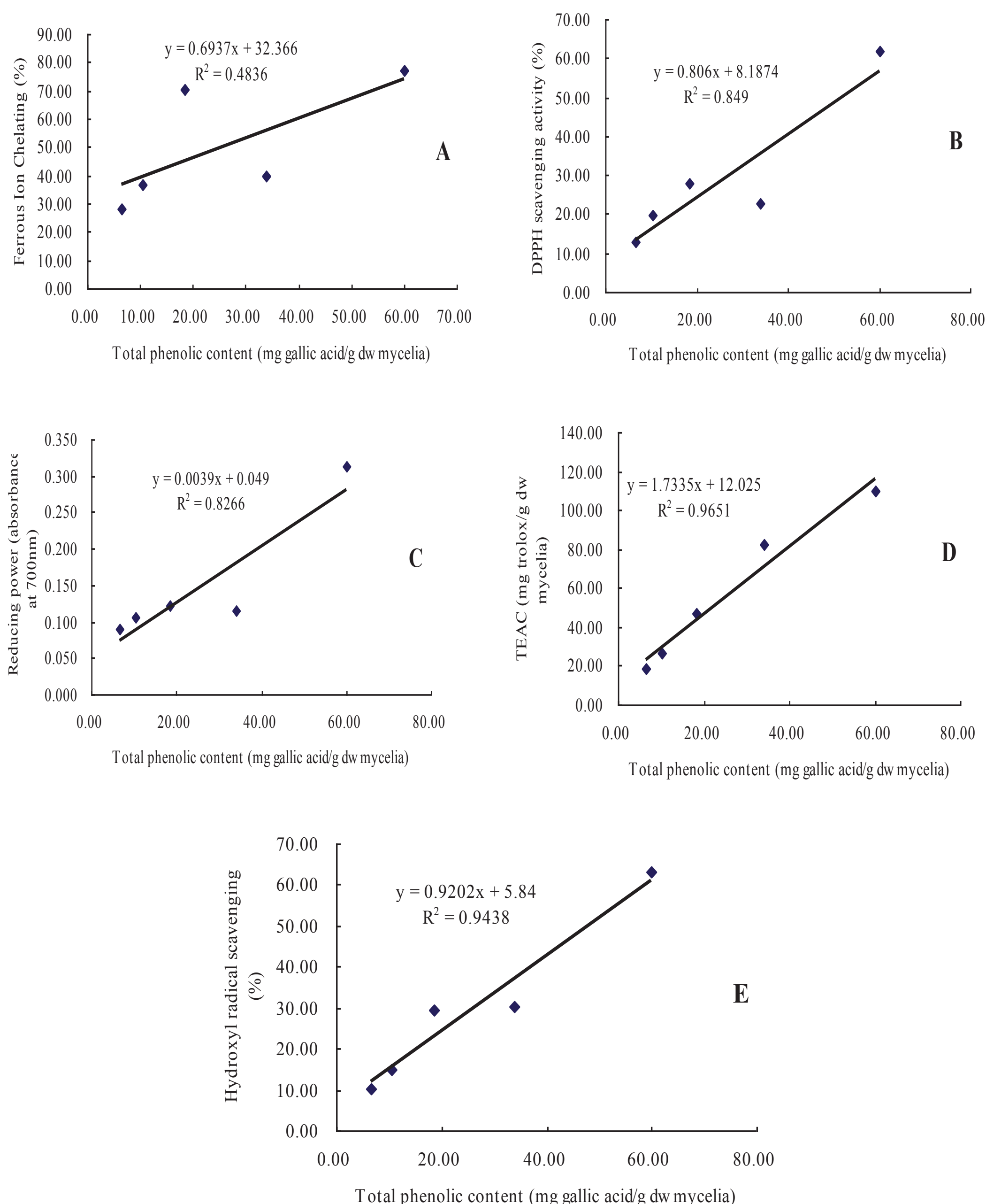

Figure 2. Relation between total phenolic contents of different extracts of *Phomopsis liquidambari* QH4 obtained by different solvents and each antioxidant activity models of (A) ferrous ion chelating assay, (B) DPPH radical scavenging activity, (C) reducing power assay, (D) $ABTS^+$ radical scavenging activity and (E) hydroxyl radical scavenging activity.

Table 3. *In vitro* cytotoxicities of different extracts from *Phomopsis liquidambari* QH4 obtained in different solvents.

Samples	Concentration (μg/ml)	Growth inhibition ± SD (%)		
		Leukemia (HL-60)	Breast (MCF-7)	Colon (COLO205)
Methanol extract	20	54.15 ± 0.13	16.65 ± 2.08	49.32 ± 1.05
	200	83.76 ± 2.56	95.98 ± 0.81	50.23 ± 3.22
Acetone extract	20	71.50 ± 0.49	28.11 ± 2.10	13.76 ± 1.70
	200	76.66 ± 2.83	49.90 ± 1.15	36.57 ± 3.22
Ethyl acetate extract	20	71.99 ± 3.21	53.66 ± 0.65	92.72 ± 1.34
	200	93.07 ± 3.00	95.56 ± 0.75	96.73 ± 1.20
Chloroform extract	20	12.81 ± 2.79	54.89 ± 0.09	84.02 ± 1.23
	200	27.89 ± 1.66	55.63 ± 0.17	82.13 ± 1.02
Hexane extract	20	12.70 ± 1.29	13.08 ± 0.01	25.64 ± 0.05
	200	21.69 ± 1.06	13.82 ± 0.26	33.83 ± 2.18
Adriamycin	1.00×10^{-6} (mol/l)	78.75 ± 1.12	85.80 ± 0.32	72.61 ± 0.69

Data expressed as means ± standard deviation (SD)

extracts (20 μg/ml) was active against HL-60 leukemia cell lines with growth inhibition of 54.16 % and 71.50 % respectively. In particular, the ethyl acetate extract (20 μg/ml) from QH4 was active against all tested cell lines, with 71.99 %, 53.66 % and 92.70 % growth inhibition of HL-60 leukemia, MCF-7 breast and COLO205 colon cell lines respectively. The ethyl acetate and chloroform extracts exhibited potential antitumor activity against COLO205 colon cell lines in a concentration-dependent manner, with the lower IC_{50} value (3.84 μg/ml and 7.33 μg/ml) (Table 4).

4. Discussion

In this study, endophytic fungus QH4 isolated from *Artemisia annua* was identified as *Phomopsis liquidambari* via phylogenetic analysis of ITS rDNA sequences, and our research finding demonstrated that the mycelia extracts of *Phomopsis liquidambari* QH4 had antioxidant and antitumor activities. Species of *Phomopsis* are prevalent as endophytes of many hosts in both temperate and tropical regions and are especially common in the sapwood of angiosperms [25]. Endophytic species of *Phomopsis* were present in the sapwood of

Table 4. In *vitro* cytotoxicities of ethyl acetate and chloroform extracts from *Phomopsis liquidambari* QH4 with a series of low concentrations against COLO205 colon tumor cell line and their IC_{50} value.

Samples	Growth inhibition ± SD (%)					IC_{50} (μg/ml)
	1.25 (μg/ml)	2.5 (μg/ml)	5.0 (μg/ml)	10.0 (μg/ml)	20.0 (μg/ml)	
Ethyl acetate extract	24.55 ± 0.86	31.40 ± 0.85	53.09 ± 1.60	72.67 ± 2.45	92.72 ± 1.34	3.84
Chloroform extract	0.00 ± 0.00	0.00 ± 0.00	38.66 ± 2.01	60.59 ± 1.57	84.02 ± 1.23	7.33

Data expressed as means ± standard deviation (SD)

almost all angiosperm endophytes examined by Boddy and Griffith [26]. The discoveries of biologically active fungal metabolites including new antibiotics, chemothereputic agents, and agrochemicals have been the focus of the scientific community worldwide. These fungal metabolites are generally recognized as highly effective, possess low toxicity, and have a minor environmental impact [27]. Like *Pestalotiopsis* which has been shown to be highly creative with more than 130 novel potentially medicinal metabolites discovered [28], *Phomopsis* is a similarly creative genus with several important discoveries including exclusive and structurally significant, physiologically active fungal metabolites. Kumaran *et al.* [29] screened the endophytic fungus *Phomopsis* for production of the anticancer drug taxol, and suggested that *Phomopsis* could be an excellent alternative source for taxol and may serve as a potential genetic-engineered species for the enhanced production of taxol. Hemtasin *et al*. [30] reported that three new sesquiterpenes secondary metabolites with cytotoxic activity were isolated from ethyl acetate extract of the fungus *Phomopsis archeri* collected from cortex stem of *Vanilla albidia*. Bunyapaiboonsri *et al.* [31] isolated six new oblongolides secondary metabolites with cytotoxic activity from ethyl acetate extract of liquid culture of *Phomopsis* sp. BCC 9789 from a wild banana (*Musa acuminate*) leaf. To our knowledge, this is the first report on the antioxidant and antitumor activities of endophytic fungus *Phomopsis liquidambari*.

So far, a number of fungi have been reported as endophytes in different *Artemisia* species. Huang *et al.* [32] have investigated the taxonomic identities and molecular phylogenetic relationships of fungal endophytes isolated from three plant species, namely *Artemisia capillaris*, *A. indica*, and *A. lactiflora*. Their results suggested that *Alternaria*, *Colletotrichum*, *Phomopsis*, and *Xylaria* species are the dominant fungal endophytes in the *Artemisia* hosts, and some of these endophytes exhibited host and tissue specificity. Meanwhile their study also showed that some antioxidant phenolic metabolites were produced by the fungal endophytes from these three *Artemisia* species [3], and AcapF1 (*Alternaria* sp.), AcapF3-4 (*Phomopsis* sp.), AilL3 (*Xylaria* sp.), AilL1 (*Colletotrichum* sp.) and AilL4 (*Diaporthe* sp.) were found to have antioxidant activity. It was reported that the chloroform extracts of *Rhodotorula* sp. and *Fusarium* sp. from *Artemisia argyi* had significant cytotoxic effects (<20 μg/ml) with IC_{50} values of 0.1-19.8 μg/ml [33]. In the present work, the ethyl acetate and chloroform extracts exhibited potential antitumor activity against COLO205 colon cell lines in a concentration-dependent manner, with the lower IC_{50} value (3.84 μg/ml and 7.33 μg/ml) (Table 4).. Recently, research group of Tan [13, 34-37] reported some novel and bioactive metabolites produced by endophytic fungi including *Colletotrichum* sp., *Hypoxylon* sp. and *Myrothecium* sp. from the *A. annua*, namely 3â,5á-dihydroxy-6â-phenylcetyloxy-ergosta-7,22-dieneÿ7,8-dimethylalloxazine, 3â,5á-dihydroxy-6â-acetoxyergosta-7,22-diene, paranolin, leptosphaeric acid, isoprenylindole-3-arboxylic acid and new cytotoxic 10,13-cyclotrichothecane-derived macrolides. Zhang *et al.* [11] found that three *Aspergillus* strains SPS-01, SPS-02 and SPS-04 from *A. annua* showed strong antimicrobial activities against *Escherichia coli*, *Staphylococcus aureus* and *Trichophyton rubrum* respectively. And an endophytic *Mucor* sp. SPS-11 had antimicrobial effect on *Rhizoctonia cerealis*. Two strains namely *Aspergillus* sp. SPS-02 and *Cephalosporium* sp. SPS-08 exhibited strong antimicrobial activities against *Magnaporthe grisea*. Masroor Qadri *et al.* [38] identified and screened 36 endophytic fungi in six different genera including *Fusarium*, *Gibberella*, *Sordaria*, *Chaetomium*, *Alternaria* and *Paraphoma* from *Artemisia annua*. And the extracts from three

strains *Fusarium tricinctum* Art, *Gibberella avenacea* Art2 and *Alternaria* sp.Art9, showed significant activity against the fungal pathogen *Candida albicans* with an IC_{50} of 50, 15 and 50 μg/ml, respectively. The extracts from two strains *Sordaria superba* Art3 and *Fusarium redolens* Art4 were found to have immunosuppressive properties. The findings of our research are in agreement with the above reports. Endophytic fungi from *Artemisia* plants are therefore believed to be potential sources of bioactive components.

There were previous studies on the antioxidant activity of endophytic fungi from other medicinal plants. For example, Strobel *et al.* [39] and Harper *et al.* [40] obtained two antioxidants, pestacin and isopestacin, from the endophytic fungi *Pestalotiopsis microspora*. Phongpaichit *et al.* [41] reported that 22.5% of the extracts of endophytic fungi from garcinia plants exhibited remarkable antioxidant activities. Phenolic compounds that seem to have important role in stabilizing lipid oxidation and to be associated with antioxidant activity were emphasized in several reports [42-43]. In this study, the methanol extract of QH4 contained significantly more TPC than those of some plants which were commonly used as antioxidants. Proestos *et al.* [44] have reported a total phenolic content in the range of 2.9-28.2 mg/g in Greek aromatic plants. Chuanphongpanich *et al.* [45] determined total phenolic content to be 32.92-48.71 mg/g in Thailand broccoli seeds.The correlation relationships between the TPCs of the different extracts of QH4 and each antioxidant model were analyzed. Theantana *et al.* [46] reported that the TPC of endophytic fungi from Thai medicinal plants showed a positive correlation with $ABTS^{+}$ radical scavenging activity (R^2 = 0.4009) and reducing power (R^2 = 0.7247) but was not correlated with DPPH radical scavenging activity. They suggested that phenolic compounds are the main antioxidant components of reducing power and other secondary metabolites may play a role as the main free radical scavengers. Huang *et al.* [3] demonstrated a strong positive correlation (R^2 = 0.9041) between TPC and total antioxidant capacity ($ABTS^{+}$) in 292 endophytic fungi from 29 Chinese medicinal plants. At the same time, they reported that phenolic compounds were the main free radical scavengers. In an endophytic *Xylaria* sp. from *Ginkgo biloba,* a higher correlation between DPPH radical scavenging activity and flavonoids contents (R^2 = 0.9392) than that with phenolic contents (R^2 = 0.7336) was showed [47]. There was a wide range of variation in the antioxidant effects of different phenolic compounds [48]. The unclear relationship between the antioxidant activity and total phenolics may be explained in various ways, for example, the antioxidant activity of the extract depends not only on the concentration, the structure and the interaction of the phenolics, but also on other secondary metabolites. The high free radical scavenging activity and total antioxidant activity of *Phomopsis liquidambari* could be resulted from the coexistence of phenolic compounds and other secondary metabolites. The results of our study were similar to those of previous reports and indicated that *Phomopsis liquidambari* QH4 from *Artemisia annua* may serve as a potential source of antioxidants.

Since the discovery that an endophytic fungus (*Taxomyces andreanae*) produced the anti-cancer drug paclitaxel (Taxol®) derived from Pacific yew (*Taxus brevifolia*) [6], much attention has been paid to finding novel, safe and efficient antitumor compounds from endophytic fungi. It was reported that plant-derived extracts containing antioxidant principle showed cytotoxicity toward tumor cells [49]. Gu *et al.* [50] reported that two new benzo[j]fluoranthene-based secondary metabolites with cytotoxic activity against

SW1116 cells were isolated from the $CHCl_3$/MeOH (1:1) extract of a solid culture of the endophytic *Hypoxylon truncatum* IFB-18 associated with *Artemisia annua*. Shen *et al.* [37] isolated a cytotoxic compound (lumichrome) from ethyl acetate extract of liquid culture of endophytic *Myrothecium roridum* IFB-E012 from *Artemisia annua*. The findings of our research were in agreement with the above reports. It is therefore suggested that ethyl acetate extract is likely to be a valuable source of natural substances with potential antitumor activity, whereas methanol extract possess moderate antioxidant activity.

In summary, the results presented in this study demonstrated that different extracts possessed different antioxidant and/or antitumor activities. There was a significant and linear relationship between the total phenolic contents and the antioxidant activities ($ABTS^+$ and hydroxyl radical scavenging capacity). The methanol extract with the highest phenolic contents showed excellent antioxidant activity via five different assays. The activity of methanol extract was significantly higher than that of the positive control BHT at the same concentration in three antioxidant activity models (reducing power, DPPH and hydroxyl radical scavenging assays), respectively. To our knowledge, this is the first report on the antioxidant activity of endophytic fungus from *Artemisia annua*. On the other hand, the ethyl acetate extract was active against all tested cell lines used here, especially COLO205 colon cell line. Meanwhile the ethyl acetate and the chloroform extracts were active against COLO205 colon cell line with the lower IC_{50} value of 3.84 μg/ml and 7.33 μg/ml respectively, which indicated that the antitumor compounds in mycelia of endophytic fungus *Phomopsis liquidambari* QH4 may be extracted by ethyl acetate or chloroform. The above results suggest that endophytic fungus *Phomopsis liquidambari* QH4 is a potential source of natural antioxidant and antitumor agents. However, the isolation, purification and characterization of the antioxidant and antitumor constituents in methanol, ethyl acetate and chloroform extracts of QH4 are awaiting further study to facilitate their development into novel antioxidant and/or anticancer agents for pharmaceutical industry in the future.

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