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Antioxidant compounds produced by *Pseudocercospora* sp. ESL 02, an endophytic fungus isolated from *Elaeocarpus sylvestris*

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ABSTRACT

Objective: To isolate endophytic fungi from *Elaeocarpus sylvestris* (*E. sylvestris*) and to isolate antioxidant compounds from a potential source fungus.

Methods: Endophytic fungi were isolated from fresh leaves and stems of *E. sylvestris* and identified based on DNA analysis. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was used to evaluate the antioxidant activity of the fungi. The potential antioxidant fungus was further studied to isolate antioxidant compounds. The isolated compounds were identified by melting point analysis, optical rotation, spectral analysis using a UV spectrophotometer, high resolution fast atom bombardment mass spectrometry, X-ray crystallography analysis, ^{1}H nuclear magnetic resonance analysis and ^{13}C nuclear magnetic resonance analysis. The isolated compounds were evaluated with DPPH radical scavenging, reducing power, and β-carotene bleaching assays.

Results: Seven endophytic fungi were successfully isolated from *E. sylvestris* and identified as *Pestalotiopsis* sp. EST 01, *Pestalotiopsis* sp. EST 02, *Diaporthales* sp. EST 03, *Meyerozyma* sp. EST 04, *Diaporthales* sp. EST 05, *Pestalotiopsis* sp. ESL 01, and *Pseudocercospora* sp. ESL 02. Of the seven fungi, *Pseudocercospora* sp. ESL 02 had the highest antioxidant activity [IC_{50} = (30.54 ± 0.88) μg/mL]. From that fungus, two compounds identified as terreic acid (1) and 6-methylsalicylic acid (2) were isolated with an IC_{50} of DPPH radical scavenging activity of (0.22 ± 0.02) mmol/L and (3.87 ± 0.27) mmol/L, respectively. The compounds also had good activities from the reducing power and β-carotene bleaching assays.

Conclusions: The *Pseudocercospora* sp. ESL 02 fungus isolated from *E. sylvestris* looks promising as a novel source of terreic acid.

1. Introduction

It is considered that treating thousands of human diseases may rely on products extracted from natural sources. Moreover, isolating bioactive compounds from plants has attracted much attention. *Elaeocarpus sylvestris* (*E. sylvestris*), a tropical and subtropical evergreen tree distributed in South Korea, Southern China, and Japan [1], has been used as a natural medicine and reported as a potential antioxidant plant [1,2]. In our previous study, we successfully isolated three antioxidant compounds (ellagic acid, gallic acid, and methyl gallate) from a methanolic extract of the leaves of *E. sylvestris* [3]. However, to the best of our knowledge, there have been no studies of endophytic fungi from *E. sylvestris*.

In addition to the extensive research on bioactive compounds from plants, research on the biodiversity of fungal endophytes has also received much attention. Endophytic fungi are microorganisms living inside plants and are considered a promising

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source of novel and natural biologically active compounds [4,5]. Some endophytes produce special secondary metabolites such as alternariol derivatives with strong antimicrobial and cytotoxic activities from the fungus *Alternaria* sp. [6,7], penialidins A–C with strong antibacterial activities from *Penicillium* sp. [8], and eupenicinicols A and B that contain decalin moiety isolated from *Eupenicillium* sp. LG41 [9]. Not only can endophytic fungi produce specific metabolites, they also promote identical or similar bioactive compounds from their host plants [10]. For instance, paeonol was produced by *Chaetomium* sp. isolated from *Paeonia suffruticosa* [11], piperine was produced from *Colletotrichum gloeosporioides* isolated from *Piper nigrum* [12], and huperzine A was produced from *Trichoderma* sp. isolated from *Huperzia serrata* [13]. In addition, endophytic fungi may also produce other general secondary metabolites, such as altersolanol derivative produced by *Nigrospora oryzae* isolated from the leaves of *Combretum dolichopetalum* [14], and adenosine, which exhibited potential 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, was isolated from *Penicillium* sp. YY-20, an endophytic fungus isolated from *Ginkgo biloba* [15].

In the present study, the endophytic fungi from *E. sylvestris* were isolated and evaluated for their antioxidant potency. Furthermore, secondary metabolites having antioxidant activity were isolated and identified from the fungus that exhibited the highest antioxidant potency. This study can complement the research on the antioxidant potency of *E. sylvestris* as the host plant of the endophytes.

2. Materials and methods

2.1. Materials and general instruments

Fresh leaves and stems of *E. sylvestris* were collected from the plant collection at Ehime University, Japan in March 2014. Quercetin, gallic acid, ascorbic acid, Tween 40, DPPH, β-carotene, potassium ferricyanide, trichloroacetic acid, and ferric chloride were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All solvents used in this study were purchased from Wako Pure Chemical Industries Ltd.

UV spectra were measured using a UV–vis 1200 spectrophotometer (Shimadzu Corp., Kyoto, Japan). Melting points were determined with a micro melting point apparatus MP-J3 (Yanaco Co., Ltd., Kyoto, Japan). Mass spectra were recorded using a GCMS-QP5050A (Shimadzu Corp., Kyoto, Japan) at an electron energy of 70 eV with a direct inlet and fast atomic bombardment mass spectrometer (FAB-MS; Shimadzu Corp., Kyoto, Japan). Optical rotation was determined using a P-2100 polarimeter (Jasco, Tokyo, Japan). Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a JEOL JNM-AL500 spectrometer.

2.2. Isolation of endophytic fungi

Endophytic fungi were isolated by washing the fresh leaves and stems with soap and tap water, followed by soaking the samples in 70% ethanol for 1 min and then 1% sodium hypochlorite for 20 min. After rinsing with distilled water three times, the leaves were cut and placed in a Petri dish containing potato dextrose agar (PDA) medium (20% potatoes, 2% glucose and 2% agar). After the fungi had grown, they were purified and placed in another Petri dish containing PDA medium. This process was repeated until the purified fungi were successfully isolated.

Molecular identification of the isolated endophytic fungi was conducted using a nucleotide sequencing of internal transcribed spacer (ITS) region of rRNA. The isolated fungi were cultivated in a Petri dish containing PDA medium and incubated for 7 days at room temperature. DNA was extracted from the fungi based on a previous method [16] with a slight modification. The extracted DNA was used as a template for PCR to amplify the ITS1-F and ITS4-B regions. The products were then sequenced using two PCR primers and an automated ABI Prism DNA sequence. The ITS sequences were compared with the available data in the NCBI GenBank database. A phylogenetic relationship between the isolated fungi was constructed using MEGA software (version 5.2.2).

2.3. Fermentation and extraction

Two mycelia plugs of each isolated fungus were inoculated into a 500 mL Erlenmeyer flask containing 200 mL potato dextrose broth medium and incubated for two weeks at room temperature, in a static condition and shaking condition (TAH-RS-12 shaker; Thomas Scientific Co. Ltd., Tokyo, Japan; 75 r/min). Afterwards, the fermented broth was filtered to separate the mycelia and the filtrate. The filtrate was extracted with ethyl acetate; the mycelia were homogenized prior to extraction with ethyl acetate. The ethyl acetate extracts were concentrated under a rotary evaporator to obtain a crude extract used for screening of the DPPH radical scavenging activity assay.

2.4. Large scale fermentation and isolation of antioxidant compounds from the potential fungus

The culture filtrates of the potential antioxidant fungus (total volume 20 L) were extracted with ethyl acetate and evaporated. The crude extract (2.5 g) was fractionated with *n*-hexane followed by chloroform, ethyl acetate, and methanol. Each fraction was evaluated for DPPH radical scavenging activity. The chloroform fraction, which had high activity and the highest yield, was further separated by silica gel column chromatography eluted with a combination of *n*-hexane and chloroform (100% *n*-hexane to 100% chloroform) followed by a combination of chloroform and methanol (100% chloroform to 100% methanol), resulting in eight fractions. From fraction 2, compound **1** (13 mg) was isolated as a pale yellow needle crystal. From fraction 1, a colorless needle crystal was isolated as compound **2** (5 mg). The isolated compounds were identified using thin layer chromatography, melting point, UV maximum absorption, high resolution fast atom bombardment mass spectrometry (HR-FABMS), X-ray crystallography, and ^{1}H NMR and ^{13}C NMR analysis.

2.5. DPPH radical scavenging activity assay

The DPPH radicals scavenging activity was conducted based on a previous method [17] with minor modifications. Various concentrations of samples were mixed with 0.5 mL of the 1 mmol/L DPPH radical solution in methanol. A similar solution without a sample was used as control. Absorbance (A) was measured using a UV–vis spectrophotometer at 517 nm after incubation at room temperature under dark condition for 30 min. Quercetin was used as a positive standard. The assay

was carried out in triplicate. The percentage of scavenging activity was determined using the following formula:

Scavenging activity (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$

2.6. Reducing power assay

The reducing power assay was performed according to a previous report [18] with slight modifications. Approximately 0.5 mL of samples in methanol (1 mg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). Trichloroacetic acid (2.5 mL, 10%) was added to the mixture after 20 min incubation at 50 °C. The mixture was then centrifuged at 3000 r/min for 10 min. The upper layer solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride solution (0.5 mL, 0.1%). Absorbance was then measured with a spectrophotometer at 700 nm. The reducing power assay was measured as gallic acid equivalent (GAE).

2.7. β-Carotene bleaching assay

The β-carotene bleaching assay was conducted based on a previous report [19] with slight modifications. Approximately 0.2 mg of β-carotene, 20 mg of linoleic acid, and 200 mg of Tween 40 were mixed in 10 mL of chloroform, which was then removed using a rotary evaporator at 40 °C. The resulting mixture was diluted with distilled water up to 50 mL and mixed vigorously. From the solution, aliquots (4.8 mL) were added to test tubes containing 0.2 mL of sample (1 mg/mL) and methanol as a blank. A similar solution without β-carotene was prepared as background. The tubes were incubated at 50 °C. The absorbance at 470 nm was measured periodically at 0, 30, 60, 90 and 120 min. Ascorbic acid was used as a standard.

2.8. Statistical analysis

All assays were performed in triplicate in three independent experiments. Data were expressed as the mean ± SD. Statistical analysis was carried out using SPSS version 16.0 for Windows followed by Duncan's *post hoc* test. Differences at $P < 0.05$ were considered to be significant.

3. Results

3.1. Isolation and evaluation of antioxidant activity of endophytic fungi

In this study, we successfully isolated seven endophytic fungi, of which, five isolates (EST 01–EST 05) were extracted from stems and two isolates (ESL 01 and ESL 02) were from the leaves of *E. sylvestris*. The molecular identification of seven fungi was done by amplifying ITS region using ITS1-F and ITS4-B, and the sequence was determined. Sequence similarity search was done with NCBI BLAST resulted in several closely related sequences, which identified EST 01–EST 05 as *Pestalotiopsis* sp. EST 01, *Pestalotiopsis* sp. EST 02, *Diaporthales* sp. EST 03, *Meyerozyma* sp. EST 04 and *Diaporthales* sp. EST 05, respectively. Meanwhile, ESL 01–ESL 02 were identified as *Pestalotiopsis* sp. ESL 01 and *Pseudocercospora* sp. ESL 02. Of the fungi, the filtrate of *Pseudocercospora* sp. ESL 02 from a shaking culture had the highest DPPH scavenging activity with an IC_{50} of (30.54 ± 0.88) μg/mL followed by the filtrate of *Pestalotiopsis* sp. EST 02 from a static culture and the mycelium of *Pseudocercospora* sp. ESL 02 from a shaking culture with an IC_{50} of (34.26 ± 3.90) and (58.80 ± 1.06) μg/mL, respectively.

3.2. Isolation of antioxidant compounds from the potential fungus

From the fungus showing the highest antioxidant potential, *Pseudocercospora* sp. ESL 02, the antioxidant activity-guided isolation resulted in two compounds from the chloroform fraction after conducting large scale fermentation on the shaking culture. The recrystallization of the compounds followed by analysis using ^{1}H NMR and ^{13}C NMR and other instrumental analysis resulted in identifying compounds **1** and **2** as terreic acid and 6-methylsalicylic acid, respectively (Figure 1).

Compound **1** (terreic acid): pale yellow needle crystal; melting point 120–121 °C; UV spectra (MeOH) λ max (log ε) 214 nm (4.03) and 316 nm (3.88); $[\alpha]^{15}D$ + 27.65° (c: 0.15, $CHCl_3$). ^{1}H NMR (500 MHz, CD_3OD): δ 1.94 (s, 3H); 3.88 (d, 1H, J = 3.6); 3.91 (d, 1H, J = 3.55); 6.81 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD): δ 8.76; 51.56; 53.79; 120.38; 151.83; 187.48; 190.66. HR-FABMS: *m/z* 154 for $C_7H_6O_4$. The structure was confirmed using X-ray crystallographic analysis (Figure 2).

Compound **2** (6-methylsalicylic acid): colorless needle crystal; melting point 167–169 °C; UV spectra (MeOH) λ max (log ε) 214 nm (4.15), 240.5 nm (3.77), and 306.5 nm (3.51). ^{1}H NMR (500 MHz, CD_3OD): δ 11.07 (1H, s, 1-COOH, 2-OH); 7.35 (1H, t, J = 7.561 Hz, 8.29 Hz, 4-H); 6.87 (1H, d, J = 8.54 Hz, 5-H); 6.77 (1H, d, J = 7.32 Hz, 6-H); 2.63 (3H, s, 3-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 175.866 (COOH); 163.764 (C2); 142.886 (C-6); 135.477 (C-4); 123.235 (C-5); 115.867 (C-3); 110.890 (C-1); 24.088 (CH_3). HR-FABMS: *m/z* 152 for $C_8H_8O_3$. The chemical structure was confirmed using X-ray crystallographic analysis (Figure 2).

3.3. Evaluation of antioxidant activity of the isolated compounds

The antioxidant activity of the isolated compounds was evaluated using three different assays: DPPH radical scavenging activity, reducing power assay and β-carotene bleaching assay. The results of the DPPH radical scavenging activity and reducing power assay of the isolated compounds are shown in Table 1.

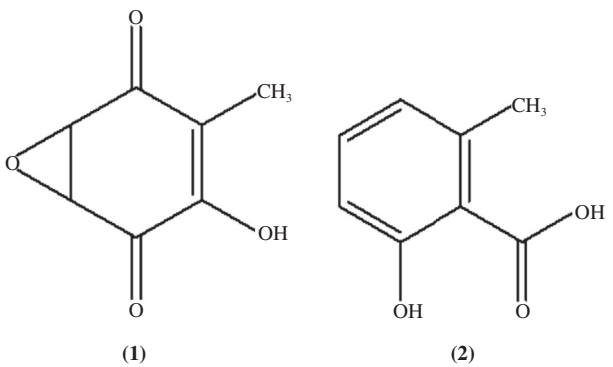

Figure 1. Chemical structure of the isolated compounds. **(1)**: Terreic acid; **(2)**: 6-Methylsalicylic acid.

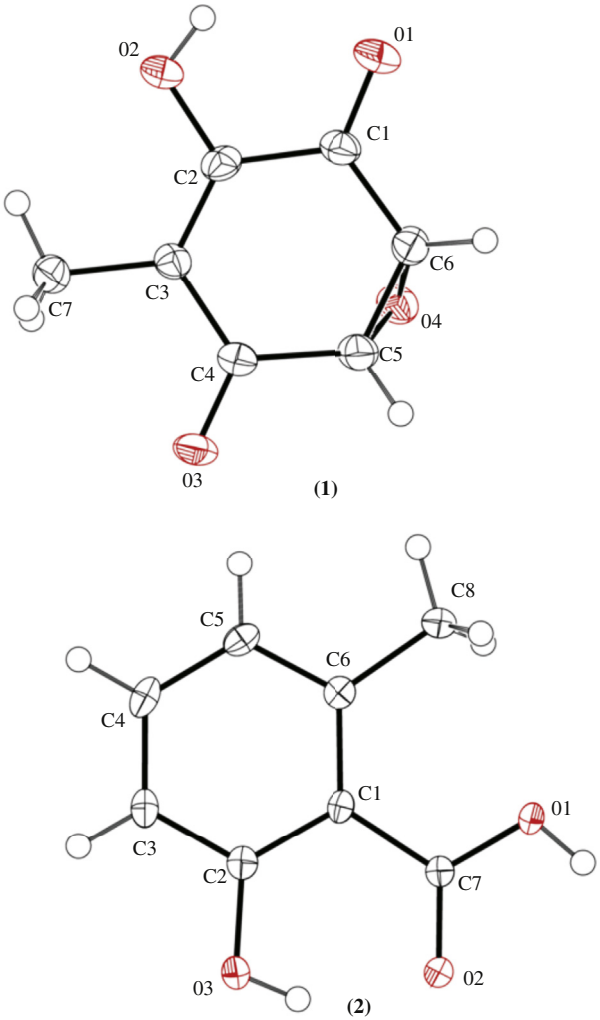

Figure 2. The structure of the isolated compounds as determined by X-ray crystallography.
(1): Terreic acid; (2): 6-Methylsalicylic acid.

The DPPH radical scavenging activity assay showed that compound **1** had higher activity compared with compound **2** with an IC_{50} of (0.22 ± 0.02) and (3.87 ± 0.27) mmol/L, respectively. Moreover, the activity of the compounds was significantly different at $P < 0.05$ using statistical analysis with ANOVA followed by Duncan's test. However, all the isolated compounds had lower activity than quercetin as a positive standard with an IC_{50} of (0.03 ± 0.02) mmol/L. The reducing power assay of the isolated compounds showed the activity of **1** and **2** as (17.43 ± 4.01) and (7.16 ± 2.48) mg GAE/g, respectively.

The result of β-carotene bleaching assay of the isolated compounds is presented in Figure 3, compared with ascorbic acid as standard. The result showed that compound **2** has higher activity than compound **1** in inhibiting the discoloration of β-carotene with increasing incubation time. However, both compounds were higher than ascorbic acid and blank.

Table 1
Evaluation of DPPH radical scavenging and reducing power activity of the isolated compounds.

Compounds	IC_{50} of DPPH radical scavenging activity (mmol/L)	Reducing power activity (mg GAE/g)
Quercetin	0.03 ± 0.02[a]	–
1	0.22 ± 0.02[b]	17.43 ± 4.01
2	3.87 ± 0.27[c]	7.16 ± 2.48

Data are expressed as mean ± SD. Different letters in the same column indicate significant differences ($P < 0.05$). –: Not tested.

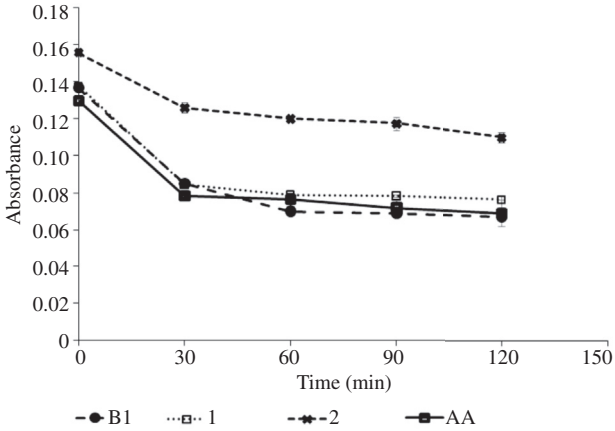

Figure 3. β-Carotene bleaching activity of the two isolated compounds compared with ascorbic acid (AA) and a blank without sample (Bl).

4. Discussion

Seven fungal strains were isolated from the leaves and stems of *E. sylvestris*. Strains of the genus *Pestalotiopsis* were found in both the stems and leaves. To our knowledge, this report is the first on endophytic fungi isolated from an *E. sylvestris* plant. Other endophytic fungi from genus *Elaeocarpus* were found in *Elaeocarpus sphaericus*, such as *Acremonium*, *Chaetomium*, *Cladosporium*, *Oidendron*, *Mortierella*, *Humicola*, *Cochliobolus*, *Nigrospora*, and *Trichothecium* [20]. From *Elaeocarpus serratus*, the endophytic fungi *Arthrinium* sp. and *Cladosporium* sp. were isolated, while *Botryosphaeria australis* was isolated from *Elaeocarpus holopetalus* [21,22].

Among the isolated fungi, *Pestalotiopsis* sp. EST 02 and *Pseudocercospora* sp. ESL 02 showed good antioxidant activity with the filtrate of *Pseudocercospora* sp. in the shaking culture having the highest DPPH radical scavenging activity. An ethyl acetate extract of *Pseudocercospora kaki* isolated from *Camellia sinensis* has been reported to have antimicrobial activity [23]. Furthermore, glycerol, 4-hydroxyphenyl ethanol, 4-chlorobenzyl alcohol, 1,2,3,4-tetrahydroxy valeraldehyde, β-D-galactopyranose and 1,2-benzenedicarboxylic acid were detected from the extract using gas chromatography-mass spectrometer. In addition, the study reported that the dual culture of *Pseudocercospora kaki* and *Penicillium sclerotiorum* showed significant antagonistic activity against the rice blast pathogen *Magnaporthe grisea*. However, few studies have reported on secondary metabolites from the genus *Pseudocercospora*.

Pseudocercospora belonging to the Mycosphaerellaceae family, is distributed widely in subtropical and tropical countries [24], and is a genus of phytopathogenic fungi associated with a wide range of plants [24–26]. Although *Pseudocercospora* are phytopathogenic fungi, some species in Brazil have been recognized as having the potential for to be used as biological control agents against invasive weeds [24]. In addition, it has been reported that *Pseudocercospora elaeocarpi* has been isolated from *Elaeocarpus* sp. in Japan [25].

As well as the seven isolated fungi, more endophytes could live inside *E. sylvestris*. The choice of medium and sterilizing

agent may affect the growth of fungal endophytes. Moreover, endophytic fungi are diverse. Endophytes that grow faster could quickly cover a plate resulting in slower-growing endophytes not being isolated [11].

Isolating the antioxidant compounds from the potential fungus *Pseudocercospora* sp. ESL 02 resulted in terreic acid and 6-methylsalicylic acid as compared with reported data [27–29]. Recently, terreic acid has been commonly isolated from *Aspergillus terreus* [27,30,31]. However, in this study we report on terreic acid isolated from the endophytic fungus *Pseudocercospora* sp. ESL 02. Hence, our study supports the diversity of secondary metabolites that can be produced by endophytic fungi. On the other hand, 6-methylsalicylic acid bears a structural resemblance to salicylic acid, and it has been reported that it mimics salicylic acid by enhancing the accumulation of pathogenesis-related proteins and resistance to tobacco mosaic virus [29]. This is the first study to report the isolation of terreic acid and 6-methylsalicylic acid from *Pseudocercospora* sp.

Terreic acid is commonly derived from 6-methylsalicylic acid. Isolating those compounds from *Pseudocercospora* sp. gave an indication that the biosynthesis pathway of terreic acid in the study could involve 6-methylsalicylic acid, which is a pathway that has already been studied [30,32]. Other than terreic acid, some compounds are commonly derived from 6-methylsalicylic acid such as patulin from *Penicillium expansum* and yanuthone D from *Aspergillus niger* [30].

Secondary metabolites from endophytic fungi can play a role in plant defense, and some to have pharmaceutical potential. The biological activities of terreic acid have been extensively studied, with one study reporting on its antibiotic action [29]. Terreic acid also has been reported to selectively inhibit the catalytic activity of Bruton's tyrosine kinase and has been used as a chemical probe to examine the function of Bruton's tyrosine kinase [33].

Antioxidant efficacy cannot be comprehensively predicted with only a single antioxidant assay [34]. Therefore, several methods are necessary to evaluate antioxidant activities. In this study, we evaluated antioxidant activity using several assays: DPPH radicals scavenging activity, reducing power, and β-carotene bleaching assays.

The DPPH assay is one of the most commonly used methods to evaluate free radical scavenging activity. DPPH is a stable radical that produces a purple solution in methanol. The antioxidant activity in this study was measured by the discoloration to yellow as the stable molecule 2,2-diphenyl-1-hydrazine formed. Antioxidant compounds can scavenge the radicals by donating their hydrogen [35], and the hydroxyl group of the isolated compounds may play a role in the activity. Moreover, particularly for compound **1**, the presence of the C=C double bond configured with the keto arrangement of terreic acid is considered responsible for electron delocalization, which increases the scavenging activity against radicals, as has been studied in luteolin [34]. Terreic acid is an epoxy quinone, and it is known that quinones can inhibit free radical chain reactions [36].

The reducing power assay may show the antioxidant capacity of the compounds to have an enhanced ability to donate electrons, resulting in the reduction of Fe^{3+} to Fe^{2+} [34]. The higher gallic acid equivalent value indicates the higher reducing power of the samples. Compound **1** had higher activity than **2**.

The β-carotene bleaching assay is typically used to determine the ability of antioxidants to reduce discoloration of β-carotene, which could be caused by the attack of free radicals against the double bonds of β-carotene. Antioxidants reduce the discoloration by stabilizing the linoleate-free radical and other radicals formed in the system. Compound **2** showed higher activity in inhibiting β-carotene bleaching than **1**, in which after 120 min, the antioxidant activity was still retained at (64.18 ± 4.22)% and (14.18 ± 3.17)%, respectively.

The different results might be due to the different mechanisms involved in the DPPH radical scavenging assay and β-carotene assay. In the DPPH radical scavenging assay, antioxidants donate an electron or hydrogen radical to the DPPH radical to become a stable molecule causing discoloration of the DPPH radical [37]. In the β-carotene bleaching assay, radicals generated from the autoxidation of linoleic acid by heating under air atmosphere attack the C=C double bond of β-carotene causing discoloration of the yellowish β-carotene solution. When the antioxidant is added to the solution, the discoloration can be retarded by a competing reaction between β-carotene and antioxidant with the subjected radicals [38]. In addition, the DPPH assay is used in organic solvent systems such as methanol or ethanol, whereas the β-carotene bleaching assay is used in an aqueous emulsion system. Therefore, a complex interfacial phenomenon that influenced the antioxidant behavior resulted in a phenomenon formulated as “polar paradox”. Nonpolar antioxidants are thought to exhibit stronger antioxidant properties in emulsion because they concentrate at the lipid:air surface ensuring high protection of the emulsion itself. Meanwhile, polar antioxidants remaining in the aqueous phase are more diluted and less effective in protecting the lipid [39] with compound **1** being more polar than **2**. Moreover, the DPPH radical scavenging assay shows chemical antioxidant activity, whereas the β-carotene assay shows an antioxidant activity more likely to be of practical use. However, despite the different activities, both compounds had higher activity than ascorbic acid, which retained antioxidant at (2.98 ± 1.23)% after 120 min.

In general, compound **1** showed stronger antioxidant activities than **2**, and compound **1** is considered derived to be derived from **2**, a bio-metabolism process from a weaker antioxidant active compound to a stronger antioxidant active compound. The results suggested that compound **1** contributes to the antioxidant activity of *Pseudocercospora* sp., although other compounds could play a role in the antioxidant activity. As previously reported [27], terreic acid showed promising antioxidant activity.

This study successfully isolated seven endophytic fungi from *E. sylvestris*, with the fungus *Pseudocercospora* sp. ESL 02 showing the highest antioxidant activity. Isolating the antioxidant compound from *Pseudocercospora* sp. ESL 02 resulted terreic acid **(1)** and 6-methylsalicylic acid **(2)**, with terreic acid having strong antioxidant activity. The results indicate the potential of *Pseudocercospora* sp. as a novel source of terreic acid. The study also complements the study of the antioxidant potency of *E. sylvestris* as the host plant of the fungus.

Conflict of interest statement

We declare that we have no conflict of interest.

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