Antioxidant activity of endophytic fungi isolated from the stem bark of *Swietenia mahagoni* (L.) Jacq

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**Abstract.** Endophytic fungi are microbes that live to form colonies in plant tissue without harming their host plants. Each higher plant has the potential to contain several endophytic fungi that produce secondary metabolites as a result of coevolution or genetic recombination of the host plant. Endophytic fungi derived from medicinal plants are candidates for producing bioactive compounds. *Swietenia mahagoni* known as mahoni, is a medicinal plant that has been used to treat various diseases such as to prevent degenerative diseases. Almost all degenerative diseases are caused by free radicals. The endophytic fungus of *S. mahagoni* is a potential source for the discovery of antioxidant compounds. In the present study, we report an antioxidant compound from endophytic fungal isolated from the stem bark of *S. mahagoni*. Antioxidant activity was tested by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method. The compound was identified by the spectroscopic method as phenolic compound. The endophytic fungi were identified by molecularly based on genetic analysis as *Cladosporium tenuissimum*

1. Introduction
Endophytic fungi are microscopic organisms that live in plant tissues such as leaves, roots, fruit, and stems. The fungus lives in mutual symbiosis with its host [1]. Some endophytic fungi produce the same phytochemical compounds as their host plants. Taxol is a well-known anticancer agent found in every species of the yew tree (*Taxus spp.*). Stierle et al. (1993) found the endophytic fungus *Taxomyces andreanae* from the *Taxus brevifolia*, which can produce taxol compounds [2]. Currently, several taxol-producing endophytic fungi have been identified. Podophyllumotoxin compound is an anticancer, antiviral, antioxidant, antibacterial, immunostimulant, and antirheumatic. The main supply of podophyllotoxin is from the *Sinopodophyllum*. Yang et al. (2003) reported six endophytic fungi obtained from *Sinopodophyllum hexandrum*, *Diphyleia sinensis*, and *Dysosma veitchii*, which can produce podophyllotoxin [2-5].

Sources of natural antioxidants, such as vegetables, fruits, spices, and medicinal plants, are widely known because the presence of phytochemicals, flavonoid and phenolic compounds. Recently, many studies have revealed that endophytic fungi also produced of phenolic compounds which have antioxidant activity. Recently, endophytes have gained as novel sources of medicinally important...
metabolites including antioxidant, antimalarial, anticancer, antibacterial, and other activities that have attracted much attention from researchers worldwide [6-8].

*Swietenia mahagoni* has been widely used in various countries including in Asia as a cure for various diseases such as hypertension, diabetes, malaria, HIV, bleeding, nerve disorders, anti-inflammatory, skincare, treating fever, as a tonic, astringent, etc. This plant possesses numerous pharmacological effects including anti-inflammatory, neuropharmacological, anti-HIV, hepatoprotective, antioxidant, immunomodulatory and antimicrobial [9-12]. The ability of endophytic fungi to produce secondary metabolites depends on the host plant and the environment of the host plant to grow. The host plants that are used as drugs with antioxidant effects are candidates for getting endophytic fungi that produce antioxidant compounds [13,15]. In the previous studies, we have reported the isolation of endophytic fungi from the stalks of *S. mahagoni* and their antioxidant activity. The extract of endophytic fungus *Cladosporium tenuissimum* has a good antioxidant activity, but the compound (5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate which have isolated from that extract not shows antioxidants activity [12]. In this paper, we reported that *C. tenuissimum* has isolated from *S. mahagoni* stem bark. Based on this, this study aimed to isolate antioxidant compounds from *C. tenuissimum*.

2. Materials and methods

2.1. Sampling and identification

The stem bark of *S. mahagoni* (L.) Jacq were taken from around the Sriwijaya University campus, Indralaya, Ogan Ilir district, South Sumatra. Plant identification was carried out at the Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University.

2.2. Chemicals

The Chemicals used in this study include Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), distilled water, alcohol 70%, KLT kiessel gel 60 F254 20 x 20 cm, silica gel G 60 70-230 mesh, sodium hypochlorite (NaOCl) 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), methanol, ethyl acetate, chloroform, n-hexane (Merck).

2.3. Isolation of endophytic fungal strains

Fresh sample (2 x 4 cm) was rinsed with tap water and sterilized by immersion in alcohol 70% for 3 minutes, followed by immersion in NaOCl 2% and then rinsed three times with sterile water. The sample (0.5 x 1 cm) was placed on three PDA plates containing 150 mg/L of chloramphenicol. The plates were incubated at 27 ± 2 °C for 5 to 14 days. The hyphal tip from the segments were transferred into new PDA plates containing 150 mg/L of chloramphenicol and further incubated for 5 to 14 days to obtain pure culture [12,16]. The culture of endophytic fungal was deposited for culture collection of the Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University.

2.4. Identification of endophytic fungal *C. tenuissimum*

Endophytic fungal was identified based on molecular analysis at the Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Indonesia [12].

2.5. Cultivation and isolation of pure compound

Six plugs (5 x 5 mm2) of endophytic fungus culture on PDA were transferred onto culture flasks containing 200 mL PDB and incubated at room temperature under static conditions. After four weeks of incubation, mycelium was separated from the broth culture using a Whatman filter paper. The broth culture was partitioned using ethyl acetate (1:1, triplicate) to obtain ethyl acetate extracts and then evaporated using a rotary evaporator to obtain concentrated ethyl acetate extract [12,16]. The extract (1.47 g) was column chromatographed on a silica gel column (70-230 mesh) as a stationary phase and eluted with a gradient solvent system. Eluates were placed in vials (10 mL) and analyzed by thin layer chromatographed (TLC). The potential stain was continued to separation and purification by column
chromatographed to obtain pure compound. The identification of the compound was determined by spectroscopic analysis $^1$H-NMR and $^{13}$C-NMR. The stages of isolation of pure compound from endophytic fungal *C. tenuissimum* from the stem bark of the *S. mahagoni* were compared with the stages of previous studies of the same fungus from the *S. mahagoni* leaf stalks [12] shown in Figure 1.

2.6. **Antioxidant activity test by DPPH method**

Concentrations of DPPH solution 0.05 mM was prepared by dissolving 1.98 mg DPPH in 100 mL methanol. The samples were dissolved to make series concentrations of 1000; 500; 250; 125; 62.5; 31.25; and 15.625 μg/mL. The samples (200 μL) in various concentrations were added with 3.8 mL DPPH 0.5 mM and incubated at room temperature in the dark. After 30 minutes, the absorbance was measured using a UV-Vis spectrophotometer at λ max 517 nm. The antioxidant activity was expressed in IC$_{50}$ values by plotting % inhibition vs concentration series [17,18]. The percentage of the free radical scavenging activity was calculated as:

\[
\text{% inhibition} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100
\]

(1)

![Figure 1](image-url)

**Figure 1.** The stages of isolation of pure compound from endophytic fungal *C. tenuissimum* from the stem bark of the *S. mahagoni* (→) were compared with the stages of previous studies of the same fungus from the *S. mahagoni* leaf stalks (→).

3. **Results and discussion**

Isolation and purification of endophytic fungal from the stem bark of *S. mahagoni* produce endophytic fungi similar to those found in the leaf stalks of *S. mahagoni* [12]. Based on the phylogeny tree's analysis, the endophytic fungus has the highest homology with *C. tenuimum* strain APBSDSF6. The sequence was submitted to GenBank and deposited with the accession number MN379836. Ethyl acetate extract of the fungal *C. tenuissimum* isolated from the stem bark and the leaf stalk of *S. mahagoni* showed the same stain pattern on TLC. Fadhillah *et al.* (2019) have succeeded in isolating one compound, namely (5-hydroxy-2-oxo-2H-Piran-4-y) methyl acetate. The compound is not an active antioxidant [12]. However, the TLC results showed another purple stain which was possibly demonstrated antioxidant activity.

3.1. **Isolation and identification of pure compound**

The concentrated of ethyl acetate extract (1.47 g) was separated by column chromatography on silica gel (70-230 mesh, 30 g) and eluted with n-Hexane-ethyl acetate (10:0→0:10) and ethyl acetate-methanol (10:0→0:10), yielded four subfractions (A-D). Fraction C (0.36 g) was re-chromatographed with a
solvent system of n-hexane-ethyl acetate= 7:3→0:10 to obtain three fractions (C1–C3). Fraction C3 (71 mg) was further purified as same as previous with n-hexane-ethyl acetate = 2: 8 as mobile phase, to obtain pure compound 1 (38 mg). To determine the structure of compound 1, a 1D-NMR spectrometric analysis was performed.

![Figure 2](image.png)

**Figure 2.** The $^1$H-NMR spectrum of pure compound from *C. tenuimum* ($^1$H-500 MHz, in CD$_3$OD).

The spectrum of $^1$H-NMR (figure 2) showed the presence of five proton signals. The signal indicated two aromatic protons at 6.36 ppm $\delta^H$ (1H, d, $J = 2.5$ Hz) and 6.34 ppm (1H, d, $J = 2.5$ Hz). The protons by the doublet multiplicity and the coupling constant $J = 2.5$ Hz indicated the meta position. In the spectrum appeared one signal of methoxy protons at $\delta^H$ 3.78 ppm (3H, s), one signal of methylene proton at 3.69 ppm (2H, s), and one signal of methyl proton at $\delta^H$ 2.53 ppm (3H, s).

The spectrum of $^{13}$C-NMR (Figure 3) showed 11 carbon. The aromatic groups indicated six signals at $\delta^C$ 101.17, 110.74, 121.92, 137.85, 160.54, and 163.72 ppm. The Signals at 160.54 and 163.72 ppm indicated the presence of two oxyaryl carbon atoms (= C-O). Based on the $^1$H-NMR spectrum, there are two protons in the aromatic ring, so the methoxyl and hydroxyl groups were attached to the aromatic ring. The methoxyl carbon atom appears at $\delta^C$ 55.85 ppm. The signal in downfield carbons at chemical
shift $\delta_C$ 206.38 ppm indicated the presence of ketone carbonyl carbon. Thus, the methyl ketone group is attached to the aromatic ring. The presence of this methyl ketone group was supported by the methyl signal's appearance at the spectrum of $^1$H-NMR $\delta_H$ 2.53 ppm with singlet multiplicity and the spectrum of $^{13}$C-NMR at $\delta_C$ 32.44 ppm. The other signal in downfield carbons at $\delta_C$ 175.41 ppm is the area for the carbonyl ester or carbonyl carboxylate groups. The signal is thought to be a carbonyl carboxylate and not an ester, because one methoxyl group was attached to the aromatic ring.

Figure 3. The $^{13}$C-NMR spectrum of pure compound from C. tenuimum ($^{13}$C-125 MHz, in CD$_3$OD).

Based on the analysis of H-NMR and C-NMR spectra, the compound 1 was identified as phenolic derivatives with four substitutes consisting of hydroxyl, methoxyl, methyl ketone, and acetic acid groups. The structure of compound 1 was shown in Figure 4.

Figure 4. The structure of phenolic derivatives from C. tenuimum.

3.2. Antioxidant activity of phenolic compound from C. tenuimum

The antioxidant activity test of ethyl acetate extract and compound 1 were compared with the previous extract and compound (5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate) of endophytic fungal C. tenuissimum from the S. mahagoni leaf stalks [12] as shown in Table 1.

Both of the ethyl acetate extract has good antioxidant activity with the DPPH test method. On the other hand, compound 1 and 5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate is not active showed different activity. Based on the structure of the two pure compounds, the abstraction of the proton phenolic compound by DPPH free radicals is much more comfortable and can form new free radicals that can be stabilized by distribution and delocalization electron in the molecule [19, 20]. Khiralla et al.
(2015) reported that the phenolic compounds as potential sources of natural antioxidants [21]. In contrast, the (5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate compound is inactivated antioxidant because the abstraction of the proton by DPPH free radicals produces new free radicals that are less stabilized. The presence of heteroatoms in the ring seems to inhibit free radical resonance in molecules.

Table 1. The IC₅₀ value of ethyl acetate extract and pure compound of endophytic fungal *C. tenuissimum* from the stem bark of *S. mahagoni* and from the leaf stalks of *S. mahagoni* with ascorbic acid as a positive control using DPPH method.

| Sample tested                       | Stem bark (µg/mL) | Leaf stalk (*) |
|-------------------------------------|-------------------|----------------|
| Ethyl acetate extract               | 86.2              | 85.4           |
| Compound 1                          | 37.8              | -              |
| 5-hydroxy-2-oxo-2H-Piran-4-yl methyl acetate | -              | > 100          |
| Ascorbic acid                       | 22.2              | 22.2           |

(*)[12]

4. Conclusion

The endophytic fungal *C. tenuissimum* has been found in stem bark and the leaf stalk of *S. mahagoni*. However, the chemical contents are the same. This study showed that *C. tenuissimum* could be used as a new source of antioxidants. Further research is needed to isolate other active compounds in *C. tenuissimum*.

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