Chemical compound isolated from antioxidant active extract of endophytic fungus Cladosporium tenuissimum in Swietenia mahagoni leaf stalks

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Chemical compound isolated from antioxidant active extract of endophytic fungus Cladosporium tenuissimum in Swietenia mahagoni leaf stalks

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Abstract. Fadhillah, Elfita, Muharni, Yohandini H, Widjajanti. 2019. Chemical compound isolated from antioxidant active extract of endophytic fungus Cladosporium tenuissimum (59 wietenia mahagoni leaf stalks. Biodiversitas 20: 2645-2650. Swietenia mahagoni L. Jacq is a medicinal plant that widely used as an antimicrobial, anti-inflammatory, antimutagenic, anticancer, antitumor, and antidiabetic. Several bioactive compounds have been reported to be associated with this plant that supports its treatment activities. Flavonoid content of the S. mahagoni has antioxidant activity. Therefore the plant is widely used to treat degenerative diseases caused by free radical activity in the body. Scientists have proven that the medicinal plants have a symbiotic relationship with the number of microorganisms that can produce bioactive compounds. One of the symbiotic microorganisms is endophytic fungi believed to be associated with every plant on earth. In this paper, we report a secondary metabolite compound from 36 dophytic fungi isolated from S. mahagoni. The endophytic fungi (DMG1-DMG4) were isolated fro 27 leaf stalks of S. mahagoni. Antioxidant activity of endophytic fungi extracts was tested by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. 42 chemical compound from the active extract was isolated by the chromatographic method, and the structure was elucidated by spectroscopic methods including UV, IR, NMR 1D (H-NMR and 13C-NMR), NMR 2D (HMQC and HMBC). Antioxic 44 activity test showed the endophytic fungus (DMG3) has the highest activity. The chemical compound from DMG3 was identified as 5-hydroxy-2-oxo-2H-piran-4-yl) methyl acetate). The phylogenetic method used to molecular analysis of DMG3 confirmed the fungus as Cladosporium tenuissimum.

Keywords: Antioxidant, Cladosporium tenuissimum, endophytic fungi, Swietenia mahagoni

INTRODUCTION

Free radical can cause cell damages in the human body. The use of the antioxidant compound can handle it as these can stop or retard free radical activity (Yadav et al. 2014). Free radicals have unpair electrons as an agent to disrupt healthy cells to an abnormal cell. Antioxidants donate their electron to free rad 49 to become neutral molecules (Aguilar et al. 2016). There are two types of antioxidants, namely endogenous and exogenous. The antioxidant is a product of the body and obtained from outside the body through food intake (Ramayulis 2015). The body needs an exogenous antioxidant to increase its defenses from oxidative stress (Kasote et al. 2013). One type of plant that has been used by humans to treat diseases associated with free radical activity in the body is mahagoni (Swietenia mahagoni L. Jacq). It has been widely used in many countries, including Asia, as a cure for various diseases. S. mahagoni has many beneficial effects on body health because it contains antimicrobial, anti-inflammatory, hepatoprotective, antioxidant, neuropharmacological, antiimmunomodulatory agents pharmacological effects (Naveen et al. 2014). Biochemical compounds like flavonoids, quercetin, and isoflavones have

been reported in several studies from extract biomass of S. mahagoni seed as a source of antioxidant (Bera et al. 2015; Syahwiranto and Theresi 2018). Biomass of S. mahagoni is easy to obtain as it was available throughout on the season, but excessive use of biomass may disrupt its the natural balance and can cause damage to its natural ecosystem. Besides this, S. mahagoni has a long growth period, and it can be harvested up to 10 to 15 years or more. Thus we need an alternative source to produce antioxidant compounds apart from biomass. In this study, we used extracts of liquid media of endophytic fungi from S. mahagoni.

Endophytic fungi are mutualistic symbiotic microganisms living asymptomatically in the host plant tissue. These fungi have high ability to produce a variety of secondary metabolites depending upon their host plants and the environment around their host plants (Jalgaonwala et al. 2011; Ludwig-Muller 2015). The endophytic fungi live in the cell layer of the host p 631 without harming the host and have an essential ro55 as a new source of bioactive compounds (Muharni et al. 2014; Deepthi et al. 2018). The literature of endophy 60 fungi from S. mahagoni is still minimal. Dompeipen et al. (2011) reported that the biomass of endophytic fungus from S. mahagoni rods has higher antioxidant activity when compared to biomass extract.

Mutmainnah (2016) reported that endophytic fungi of *S. mahagoni* could be used as raw material to the medicine of diabetes mellitus, but the active compound of the product produced by endophytic fungi *S. mahagoni* has not been found.

MATERIALS AND METHODS

Materials

Leaf stalks of mahogany (*Swietenia mahagoni*) were obtained from Ogan Ilir, South Sumatra, Indonesia. The process of 52 ntifying plants was done in the Department of Biology, Sriwijaya University, Ogan Ilir District, South Sumatra Province, Indonesia.

Sample sterilization and isolation of endophytic fungi

Samples from leaf stalks of *S. mahagoni* was cleaned under running water. Alcohol 70% and HgCl₂ 5% (w / v) were used to sterilize surface of sample for 1 minute. The samples were then rinsed with sterile distilled water. Furthermore, sterilized samples were inoculated in a petri dish containing PDA medium and incubate for 3-7 days at 37°C (Budiono et al. 2019). Fungi with different morphological features were separated and purified. Each colony was then transferred to a new PDA media with a streak plate method (Elfita et al. 2015).

Cultivation and extraction of secondary metabolites of endophytic fungi

Actively growing hyphae of endophytic fungi were inoculated in 300 ml of media PDB (Potato Dextrose Broth) in the bottle and incubated at room temperature in static for six weeks. Endophytic fungi were inoculated into 30 bottles. The inoculation process was carried out in a sterile state with a PDB ratio of 1:10. After six weeks, the mycelium of Endophytic fungi and its culture media were separated and continued with partition by ethyl acetate 351). The extract was separated from medium and evaporated using a rotary evaporator at temperature 65°C to obtain concentrated ethyl acetate extract (Elfita et al. 2014).

Antioxidant activity test with DPPH method

The crude extracts of the endophytic fungi were diluted to 58 0, 500, 250, 125 and 62.5 ppm. To each contration, 0.2 mL was added to 3.8 mL DPPH 0.5 mM. The solution mixture was homogenized and left for 30 minutes in a dark place and absorption measured by UV-Vis spectrophotometer (λ = 517 nm). Antioxidant activity was determined based on absorption inhibiting percentage of DPPH and IC₅₀ value (Budiono et al. 2019).

% Inhibition =
$$\frac{Abs(Blank) - Abs(Sample)}{Abs(Blank)} \times 100\%$$

Identification of endophytic fungi

Isolates of endophytic fungi DMG3 was identified at the LIPI Cibinong biological research center. Internal transcribed spacer (ITS) of the ribosome DNA locus was used for

identification. PHYTO nucleons pure reagent was used to extract DNA of isolated fungi DMG3. Primary ITS-4 and ITS-5 were used in PCR amplification (Liang et al. 2012; Pansanit and Pripdeevech 2018). The 51 CR amplicon was sequenced and blasted by using National Center for Biotechnology Information (NCBI) (Mishra et al. 2017). Fungal DNA with the highest similarity selected and grouped using ClustalW program (Hannula and Veen 2016) in txt, MSF, and PHYLIP formats (Felsenstein 1989). File PHYLIP opened with SEQBOOT program by entering 1000 times replication. DNAdist and Neighbor program run by entering the numbers 1000 as the amount of data processed. The last processed, the file was entered to CONSENSE program. Files names altered and added extensions, phb or tre. Figure phylogenetic tree created by TreeView (Puillandre et al. 2017) and edited by Mega4 program (Clifton et al. 2018).

Isolation and identification of pure compounds from the active ethyl acetate fraction of endophytic fungi extract

The concentrated extr 40 was prepared and put into the chromatography column, then eluted with gradient solvent using n-hexane-ethyl acetate (10:0→0:10). Eluates were collected in vials (± 10 mL). The vials were detected by TLC then vials with the same stain pattern on the TLC were combined and concentrated with a rotary evaporator. The fraction that has a potential stain pattern was purified by chromatographic tects ques and recrystallized to obtain pure compounds. The compound was determined by UV, IR, ¹H-NMR, ¹³C-NMR, HSCQ, and HMBC spectroscopy.

RESULTS AND DISCUSSION

Sample sterilization and isolation of endophytic fungi

Leaf stalk of S. mahagoni was sterilized by surface sterilization method. In this case, alcohol and HgCl₂ were used as chemical sterilization agents. After the sample was incubated for 3-7 days, the growth of endophytic fungi began to appear. Four fungi had different physical characteristics labeled as DMG1-DMG4. Each fungus separated on different PDA medium.

Cultivation and extraction of secondary metabolites of endophytic fungi

The fungus growing on liquid PDB was harvested after an incubation period of 6 weeks by separating biomass from liquid media by filtration. The liquid media from the DMG1-DMG4 fungus were extracted by partition method in ethyl acetate and the extracts were evaporated to obtain crude extracts DMG1= 4.37; DMG2 = 1.74; DMG3 = 5.98; and DMG4 = 2.21 g, respectively.

Antioxidant activity test with DPPH method

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The endophytic fungi extracts from leaf stalk of S. mahagoni have 56 n evaluated of antioxidant activity by DPPH methods. The IC_{50} value of the extracts and ascorbic acid as standard antioxidant showed in Table 1. The results of antioxidant activity test showed that DMG3 had the highest IC_{50} value compared to the others. Furthermore, the pure compound was isolated from fungus DMG3.

Table 1. The IC₅₀ value of endophytic fungi from ethyl acetate extract of *S. mahagoni* leaf stalks and ascorbic acid using DPPH method

Test samples	Extract	IC ₅₀ (μg/mL)
	DMG1	2394.26
Endophytic fungi	DMG2	1037.45
	DMG3	85.35
	DMG4	575.10
Ascorbic acid	Ascorbic acid	22.23

Identification of endophytic fungi

Identification of DMG3 was carried out based on partial genetic analysis. DMG3 identified using the phylogenetic tree (Figure 2) by matching 1000 bootstrap. DMG3 fungi there was on the same clade as *Cladosporium tenuissimum* strain *APBSDSF6* with high bootstrap support (99%). It can be stated that DMG3 fungi is *Cladosporium tenuissimum*. The Counting of nucleotide bases from DMG3 fungi bellow:

TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAC
CTTAGAAATGGGTTTGTTTTACGGCGTAGCCTCCCGAGC
ACCCTTTAGCGAATAGTTTCCACAACGCTTAGGGGACAG
AAGACCCAGCCGGTCGATTTGAGGCACGCGGGGACCGC
GTTGCCCAATACCAAGCGAGGCTTGAGTGGAATAGAC
GCTCGAACAGGCATGCCCCCCGGAATACCAGGGGGCGCA
ATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCA
ATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCG

ATGCCAGAACCAAGAGATCCGTTGTTAAAAGTTTTAATT
TATTAATTAAGTTTACTCAGACTGCAAAGTTACGCAAGA
GTTTGAAGTGTCCACCCGGAGCCCCCGCCCGAAGGCAGG
GTCGCCCCGGAGGCAACAGAGTCGGACAACAAAGGGTTA
TGAACATCCCGGTGGTTAGACCGGGGTCACTTGTAATGA
TCCCTCCGCAGGTTCACCTACGGAGACCTTGTT.

Isolation and identification of pure compounds from the active ethyl acetate fraction of endophytic fungi extract

The concentrated extract of DMG3 was tested for stain pattern with TLC. Ratio solvent used on TLC is n-hexane: ethyl acetate (10: 0 ~ 0: 10). The extract was separated over 27 a gel (230-400 mesh) by vacuum liquid chromatography method using gradient solvent n-hexane: ethyl acetate (10: 0 →0: 10). Eluates were monitored using TLC to obtain four fractions (F1-F4). The potential fraction F3 was separated using a gravity column chromatography to obtain 57 vials. Each vial was analyzed for the stain patterns with TLC and obtained five frac 53s (F_{3.1}-F_{3.5}). Furthermore, the fraction F_{3.5} separated by column chromatography using eluent nhexane: ethyl acetate (2: 8). The vial 7 as white crystal was examined using TLC and showed a single stain which fluorescent at the UV lamp. The pure compound was identified by spectroscopy method. The 1H-NMR spectra showed the presence of four signal proton. The 13C-NMR spectra appeared the signal at eight chemical shift for eight carbon (Fig 1). The correlation between protons and carbon showed in HMQC and HSQC spectra (Figure 1 and Table 2).

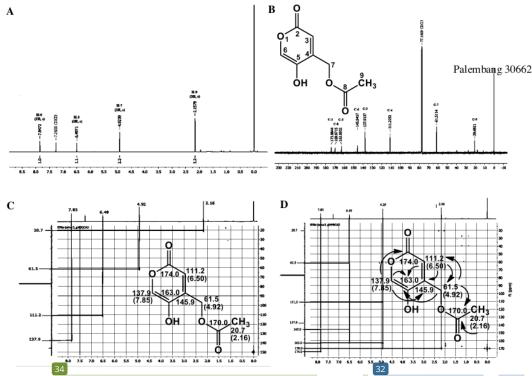


Figure 1. The H-NMR (A), 13C-NMR (B), HSQC (C), and HMBC (D) spectra of compound 1 (H-500 MHz, 13C-125 MHz, in CDCl₃)

[61] e 2. ¹³C and ¹H NMR of compound 1 (¹H-500 MHz, ¹³C-125 MHz, in CDCl₃) and 5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate (¹H-500 MHz, ¹³C-125 MHz, in CDCl₃) (Elfita et al. 2014)

	δ _C (ppm)		$\delta_H(ppm)$, ΣH , multiplicity		2 HMBC	
No. C	5-hydroxy-2-oxo-2H- Piran-4-yl) methyl acetate (*)	Compound 1	5-hydroxy-2-oxo-2H- Piran-4-yl) methyl acetate (*)	Compound 1	5-hydroxy-2-oxo-2H- Piran-4-yl) methyl acetate (+)	Compound 1
2	173.9	174.0				
3	111.2	111.2	6.50 (H; s)	6.50 (H; s)	145.8; 162.9	61.5; 145.9; 163.0
4	145.8	145.9	, , , ,	, , ,		, ,
5	162.9	163.0				
6	137.9	137.9	7.85 (H;s)	7.85 (H; s)	145.8; 162.9; 173.9	145.9; 163.0; 174.0
7	61.4	61.5	4.90 (2H; s)	4.92 (2H; s)	111.2; 162.9; 169.8	111.2; 163.0; 170.0
8	169.8	170.0				
9	20.6	20.7	2.15 (3H; s)	2.16(3H; s)	169.8	170.0

Note: *) Elfita et al. (2014)

Discussion

Sample sterilization and isolation of endophytic fungi

There are many microorganisms in one type of plant. Therefore, it needs methods to isolate endophytic fungi associated with plants. Several techniques were needed to get pure endophytic fungi. The first thing to do to isolating endophyt 25 fungi was to wash fresh S. mahagoni leaf. Samples washed with running water to remove impurities on the leaf surface and sterilized by surface sterilization method. It aims to remove epiphytic fungi and other microorganisms from the surface of plant tissues. Chemical sterilization in this was carried out by the use of alcohol 70% and HgCl2. The utilization of alcohol intends to remove fungi and bacteria found on the surface of the leaf stalk of S. Mahagoni. Whereas HgCl2 was used to maximize the sterilization process. HgCl₂ can remove microorganisms by inhibiting enzyme activity can cause protein precipitation. Its impact on damage cell triggers the death of microorganisms (Hyde and Soytong 2008; Fouda et al. 2015). In this isolation process, the PDA medium was used to grow the fungus because it has simple formulation, and that can support the growth of various fungi (Akharaiyi and Abiola 2016).

Cultivation and extraction of secondary metabolites of endophytic fungi

DMG1-DMG4 fractions were cultivated in slanted medium to multiply the fungi that have isolated. A homogenous suspension of the fungi was grown in distilled water. The fungus suspension inoculated into liquid media PDB made from potato powder and dextrose, which is a food source for fungi and yeast and used to grow and identify fungi (Pradeep et al. 2013). Furthermore, fungus culture incubated for six weeks. The fungi were harvested with filtering the medium to separating from its biomass. Liquid medium extracted by ethyl acetate. The extraction was aimed to separate a substance that was contained in the sample. That is based on differences in solubility and level of the polarity of the solvent to get bioactive compounds in the sample (Alternimi et al. 2017). The extraction was also carried out to attract antioxidant compounds. It was contained in extracts of endophytic fungal liquid medium

DMG1-DMG4. The ethyl acetate is a semi-polar solvent. The generally semi-polar solvent like phenol and flavonoid derivatives has antioxidant activity. The results of extraction concentrated into crude extracts containing a mixture of chemical compounds that were produced by endophytic fungi and had different activities. The purification needed to get the desired active compound.

Antioxidant activity test with DPPH method

Endophytic fungi extract DMG1-DMG4 made in concentration series by diluting it in methanol. Each concentration series is then added with a DPPH solution. The antioxidant activity of a compound is determined by interpreting experimental data from the antioxidant test method. DPPH is a free radical that can react with compounds that can donate hydrogen atoms. The nature of DPPH is useful for testing 46 antioxidant activity of specific components in an extract. Because of the impaired electrons, the DPPH gives a strong absorption at $\overline{517}$ nm. When the electrons become paired up by the presence of free radical scavengers, the absorbance decreases stoichiometrically according to the number of electrons taken. The presence of antioxidant compounds can change the color of the DPPH solution from purple to yellow (Molyneux 2004; Bougatef et al. 2009). The DPPH react with UV light. Therefore, the test of antioxidant activity samples mixed with DPPH stored in a dark room.

T23 DPPH test was showed that DMG3 samples have high antioxidant activity with an IC₅₀ value of 85.35 µg/mL and believed to be a strong antioxidant compared to other fungal extracts. The antioxidant activity of an extract can be categorized as strong (IC₅₀ < 200 µg/mL), moderate (IC₅₀ 200-1000 µg/mL), and weak (IC₅₀ > 1000 µg/mL) (Molyneux 2004). The pure compound from endophytic fungi DMG3 is a inactivated antioxidants (IC₅₀ > 100 µg/mL).

It is often found that the antioxidant activity of an extract is more active compared to its pure compound which may be caused by a synergistic effect. If the antioxidant activity of an extract is stronger than the pure compound, in further research to find the source of antioxidant raw materials used in the form of extracts.

Identification of endophytic fungi

The fungi of DMG3 which have antioxidant activity were identified. It was carried out using a phylogenetic analysis with 1000 times matching. Some DNA data on fungal species that sourced from NCBI compered with DNA data from DMG3 fungi. The phylogenetic method used to analyze the similarity of DNA. That is analysis represented as a branching system such as a tree diagram. That is known as a phylogenetic tree (Podani 2017). Phylogenetic trees are a diagram that shows kinship relations between organisms. Furthermore, determining the closeness of fungal DNA carried out by phylogenetic software. The phylogenetic tree of DMG3 is shown in Figure 2.

Phylogenetic tree of DMG3 was made by Mega software with the Neighbor-joining method. The phylogram topologies can indicate that the DMG3 is Cladosporium tenuissimum. It was located in the same clade location with C. tenuissimum strain APBSDSF6. That supported with high bootstrap (99%). C. tenuissimum is mycoparasites which infects plants by forming rust on plant organs. Sharma and Heather (1978) reported that C. tenuissimum Cooke was a hyperparasite of rust in Melampsora larici-populin. Nasini et al. (2004) reported that C. tenuissimum was produced 5 Cladosporlos compounds. That is Urediniospores agents from U. appendiculatus as peanut rust agents. that's fungi produced compound had the potential to inhibit rust in germination. Fujii et al. (1995) succeeded in isolating the caladospolide compound from C. tenuissimum. Compounds isolated from C. tenuissimum are generally derivatives of lactone. This study was showed that C. tenuissimum produce secondary

metabolites. Its biological activity is higher than the host. On this study revealed that *C. tenuissimum* associated in *S. mahagoni* were able to produce compounds ((5-hydroxy-2H-piran 20x0-4-yl) methyl acetate) (Compound 1). These compounds have not found in other *Cladosporium* sp.

Isolation and identification of pure compounds from the active ethyl acetate fraction of endophytic fungi extract

Compound 1 as a white crystal which has Rf value 0.46 with eluent n-hexane: ethyl acetate (2:8). It was analyzed by the spectroscopic method to determine the structure of the resulting compound. The UV spectrum of compound 1 has λ max 273 nm. After the addition of NaOH a bathochromic shift occurs which produces λ max 313 nm. The results of the analysis with UV-Vis spectroscopy showed that compound 1 had free OH group. It showed the wavelength had a greater shift when it added with NaOH shear reagent. The IR spectrum (KBr) of compound 1 indicates the presence of OH group at 3367.7 cm⁻¹. CHaromatic at 3111.2 cm⁻¹. CH-aliphatic at 2924.1 cm⁻¹, COester stretches at 1730.2 cm⁻¹ and 1662.6 cm⁻¹ with a sharp pick. C = C aromatic at 16269.9 cm⁻¹ and CO-ester bending at 1255.7 cm⁻¹. The presence of CO-ester-stretching and warping indicates that compound 1 has a lactone ring which has a side chain CO-ester. The 1H-NMR spectrum showed (Fig 1) that compound 1 has four protons. Then have a chemical environment with a methyl group signals at δ_H 2.16 (3H; s). The methylene signals at δ_H 4.92 (2H; s), and two aromatic protons at δ_H 6,50 (H; s) and δ_H 7.85 (H;

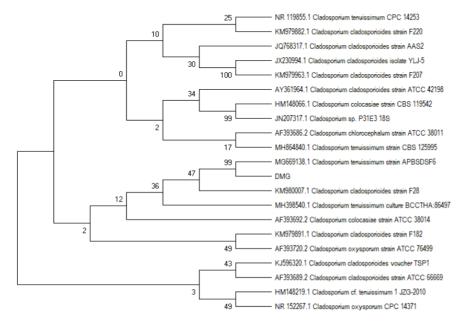


Figure 2. Phylogenetic trees of DMG3

The $^{13}\text{C-NMR}$ spectrum shows that compound 1 has 8 carbon chemical environments (Fig 1). The quaternary ester carbon on the lactone ring is at δ_{C} 174.0 (C-1). The quaternary carbon which binds the ester outside the lactone ring is at δ_{C} 170.0 (C-7). The quaternary carbon bound to the OH δ_{C} 163.0 (C-4), carbon quaternary bound to the ester outside the lactone ring in δ_{C} 145.9 (C-5). Methine carbon attached to the ring ester lactone at δ_{C} 137.9 (C-5), carbon methine that in the lactone ring at δ_{C} 111.2 (C-2). Carbon methylene outside the lactone ring at δ_{C} 61.5 (C-6) and the methyl carbon at δ_{C} 20.7 (C-8). The HSQC and HMBC correlations shown in table 2, as compared with table data in the literature (Elfita et al. 2014). compound 1 is a 5-hydroxy-2-oxo-2H-Piran-4-yl) methyl acetate.

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