

Isolation of Antioxidant Compound from Endophytic Fungi *Acremonium sp.* from the Twigs of Kandis Gajah

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Abstract

The endophytic fungi *Chrisonilia sitophila*, *Acremonium sp.*, and *Penicillium sp.* have been isolated from the tissues of the twigs of kandis gajah. The fungal strains were grown in 3 l potato dextrose broth medium (PDB) at room temperature for 28 days. To extract the antioxidant compounds, the cultures broth were filtered for mycelia removal followed by extraction and evaporation. All of the extracts were evaluated for their antioxidant activities by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The extract of *Acremonium sp.* had strong activity with IC₅₀ value of 10.3 µg/ml, which is equivalent to ascorbic acid activity with IC₅₀ value of 9.8 µg/ml. The extract was subjected to column chromatography on Si gel twice to obtain a high purity antioxidant compound in the form of yellow oil. The molecular structure was determined based on spectroscopic data, including ¹H-NMR, ¹³C-NMR, HMQC, HMBC, and COSY. The compound was determined as sesquiterpene 3,5-dihydroxy-2,5-dimethyltrideca-2,9,11-triene-4,8-dione.

Abstrak

Isolasi Senyawa Antioksidan dari Jamur Endofitik *Acremonium sp.* dari Ranting Kandis Gajah (*Garcinia griffithii*). Jamur endofitik *Chrisonilia sitophila*, *Acremonium sp.*, dan *Penicillium sp.* telah diisolasi dari jaringan ranting tumbuhan kandis gajah. Ketiga strain kapang tersebut ditumbuhkan dalam 3 l medium *potato dextrose broth* (PDB) pada temperatur kamar selama 28 hari. Masing-masing biakan disaring untuk memisahkan miselium dan dilanjutkan dengan ekstraksi dan evaporasi. Semua ekstrak dilakukan uji aktivitas antioksidan berdasarkan aktivitas peredaman radikal bebas 1,1-diphenyl-2-picrylhydrazyl (DPPH). Ekstrak *Acremonium sp.* memiliki aktivitas yang kuat dengan nilai IC₅₀ 10,3 µg/ml yaitu setara dengan aktivitas asam askorbat dengan nilai IC₅₀ 9,8 µg/ml. Ekstrak aktif selanjutnya dikromatografi kolom dan diteruskan dengan rekromatografi hingga diperoleh senyawa antioksidan murni berupa minyak berwarna kuning. Struktur molekul ditentukan berdasarkan data spektroskopi yang meliputi ¹H-NMR, ¹³C-NMR, HMQC, HMBC, dan COSY. Senyawa hasil isolasi adalah golongan seskuiterpen yaitu 3,5-dihidroksi-2,5-dimetiltrideka-2,9,11-triena-4,8-dion.

Keywords: Acremonium sp., antioxidant compound, endophytic fungi, Garcinia griffithii, sesquiterpene

1. Introduction

Plant endophytes are a group of microorganisms living within plant internal tissues or organs without causing any apparent symptoms or diseases in the hosts. They can serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts [1]. Endophytic fungi are rich sources of novel organic compounds with interesting biological activities

and a high level of biodiversity. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance [2-3].

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. Added to this are enormous difficulties in raising enough food on certain areas of

the earth to support local human populations. Environmental degradation, loss of biodiversity and spoilage of land and water also add to problems facing mankind. Endophytes are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry [4-6].

In the previous studies, it was found that bioactive compounds are potentially more active compared to commercially available drugs. However, the problem is that the compounds were found with low rendement and their sources are mainly from endemic plants which are difficult to cultivate, harvested for such a long time, and believed to have a socio-religious function for people living in their habitat. One kg dry powder of stem barks of kandis gajah (*Garcinia griffithii*) can only produce 5 mg of antioxidant compounds of 1,6,7-trihydroxyxanthone, 5 mg guttiferone I, and 7 mg isoxanthochymol. Those compounds have antioxidant activities twice higher than a synthetic antioxidant butyl hydroxy anisole (BHA). Besides, it can also produce 14 mg of 1,5-dihydroxy-3,6-dimethoxy-2,7-diprenylxanthone (antimalaria); 110 mg of 1,7-dihydroxyxanthone (antibacterial); 34 mg β -sitosterol -3-O- β -D-glucoside, 21 mg of stigmaterol-3-O- β -D-glucoside. Furthermore, it can also produce 12 mg of the compound 1,6-dihydroxy-3-methoxy-4,7-diprenylxanthone which shows antioxidant activity lower than standard BHA [7-12].

In this paper, we report the isolation of antioxidant compound extracted from *Acremonium sp.*, an endophytic fungi from the twigs of kandis gajah. It is also reported the structure elucidation of the compound as well as its antioxidant activity.

2. Methods

Source of endophytic fungi. Plant materials of kandis gajah were collected in April 2010 from Sarasah Bonta, Lembah Arau, Kabupaten Lima Puluh Kota, West Sumatra. Voucher specimens have been deposited in the Laboratory of Herbarium Universitas Andalas, Padang.

Isolation of endophytic fungi. The twigs were cut into small rods (about 5 cm in length) and rinsed in running tap water followed by successive surface sterilization in 70% ethanol and 0.2% HgCl₂ for 2 min (twice). The twigs rods were rinsed three times in sterilized distilled H₂O and cleaved aseptically into small segments, which were deposited on a Petri dish containing potato dextrose agar medium (PDA) (200 g potato, 20 g dextrose, and 15 g agar in 1 l of H₂O, supplemented with 100 mg/l of chloramphenicol to suppress bacterial growth) and then incubated at room temperature (25 °C). The germinating *hypha* tips were observed and transferred to new PDA plates and then subcultured until pure cultures were obtained [13-14].

Identification of the fungi. The fungi strains were identified base on their colony and their cell morphology characteristic. Macroscopic characterization based on colony morphology was done by growing the isolates in three different media; *czapez dox agar* (CDA), *malt extract agar* (MEA), and *potato dekstrose agar* (PDA) and observing the size, the growth, the color, and color of the colony reverse. IN addition, microscopic characterization was also done by observing the non-reproductive structures, reproductive structures, conidia, and conidiophores by comparing its morphological characteristics [15].

Fermentation, extraction, and antioxidant activity test. The purified fungi (a small park) were transferred under a sterile condition to the PDB medium. For chemical investigations, the fungal strains were static cultivated in 3 l PDB medium for 28 days at room temperature [16]. The culture broth was filtered to remove mycelia. The culture was extracted with 3 l ethyl acetate (twice), after removal of the solvent in vacuum, the resulting residue was subjected to antioxidant activity test.

Isolation of antioxidant compound and structure elucidation. The extract which has antioxidant activity was subjected to column chromatography on Si gel eluted by a gradient of n-hexane : ethyl acetate from 1:0 to 0:1 (v/v), continued with recolumn to afford antioxidant compound. The molecular structure is identified by spectroscopic methods including ¹H-NMR, ¹³C-NMR, HMQC, HMBC, and COSY.

Antioxidant activity test. The free radical scavenging activity was tested as bleaching of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The reaction mixture (4 ml) contained 3.8 ml of daily prepared DPPH solution (0.05 mM) and 0.2 ml various concentrations of tested compound or of positive control (ascorbic acid) dissolved in dimethyl sulphoxide (DMSO). After 30 min in the dark at room temperature, the absorbance was recorded at 517 nm. Radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula [17].

$$\% \text{ inhibition} = \left\{ \frac{A_k - A_s}{A_k} \right\} \times 100 \quad (1)$$

A_k = control absorbance
 A_s = sample absorbance

3. Results and Discussion

Three fungi (R1-R3) were isolated from the twigs of kandis gajah (Figure 1). The strains were identified as R1= *Chrisonilia sitophila*, R2= *Acremonium sp.*, and R3= *Penicillium sp.* by the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya.

All of the extracts were evaluated for their radical scavenging activities by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. DPPH is a free radical molecule with a purple color that can be changed into a stable compound in yellow by the reaction with antioxidant. Antioxidant compound give a single electron in DPPH resulting in reduction in free radical DPPH [18]. Extract R2 showed strong activities with IC_{50} values of 10.3 $\mu\text{g/ml}$, which was equivalent of the well-known synthetic antioxidant ascorbic acid ($IC_{50} = 9.8 \mu\text{g/ml}$). However, extract R3 were not active ($IC_{50} = 272 \mu\text{g/ml}$) and extract R2 only showed weak or moderate activities ($IC_{50} = 23,8 \mu\text{g/ml}$).

Ethyl acetate extracts of *Acremonium sp.* (3.8 g) was subjected to a column chromatography (CC) over silica gel and eluted with n-hexane-ethyl acetate by increasing polarity, to yield four fractions (F1-F4). F4 was further fractionated by and eluted with n-hexane-ethyl acetate (2:8) to obtain four fractions (F4.1-F4.4). F4.4 was subjected to yield compound **1** (374 mg) in the form of a yellow oil. Compound **1** showed strong activity with IC_{50} value of 10.8 $\mu\text{g/ml}$. Minami *et al.* classify the power of antioxidant activity based on IC_{50} values. A sample is grouped to strong, moderate, and not active antioxidant if the IC_{50} value are less than 10, less than 100 and more than 100 $\mu\text{g/ml}$ respectively [19]. Deachathai *et al.* reported that the antioxidant compounds with IC_{50} values $\leq 11.4 \mu\text{g/ml}$ was grouped as strong [20]. The isolation procedure for compound **1** is showed in Figure 1.

The $^1\text{H-NMR}$ spectrum reveal the presence of signals due to four methyl at δ_H 1.25 (3H, s), 1.48 (3H, s), 1.69 (3H, s), and 1.89 ppm (3H; d; 6.2). Three methyl signal (s) indicated that the three methyl groups are bound to the quaternary carbon. Furthermore, one methyl signal (d) showed that the methyl group attached to carbon methyne. There are also two signals for methylene protons are not chemical shift, at $\{\delta_H$ 2.66 (1H, m) and 2.49 ppm (1H, m)} and $\{2.17$ (1H, m) and 2.13 ppm (1H, m)}. In addition, revealed the presence of signals to four vinylic protons at δ_H 7.18 (1H; dd: 10.4: 4.9), 6.27 (1H, m), 6.21 (1H, m), 6.21 (1H, d: 10.4), and 6.04 ppm (1H, d; 15.3).

The $^{13}\text{C-NMR}$ spectrum exhibited the presence of 15 carbon signals attributed to 4 methyls, 2 methylenes, 4 methines, and 5 quaternary carbon atoms according to the HMQC spectrum. The complete assignment of carbon and proton made by 2D NMR analysis is showed in Table 1.

The HMQC spectrum shows that the vinylic protons at δ_H 7.18 (1H; dd: 10.4: 4.9), 6.27 (1H, m), 6.21 (1H, d; 10.4), and 6.04 ppm (1H, d; 15.3), each is bound to carbon at δ_C 145.5; 142.8; 130.2, and 127.0 ppm. Furthermore, two signal for methylene protons at δ_H $\{2.66$ (1H, m) and 2.49 ppm (1H, m)} and $\{2.17$ (1H, m) and 2.13 ppm (1H, m)}, each bound to carbon at δ_C 34.1 and 30.9 ppm. Four signals for methyl protons at δ_H 1.25 (3H, s), 1.48 (3H, s), 1.69 (3H, s), and 1.89 ppm (3H, d; 6.2), each bound to carbon at δ_C 29.9; 23.5; 6.2; and 19.1 ppm.

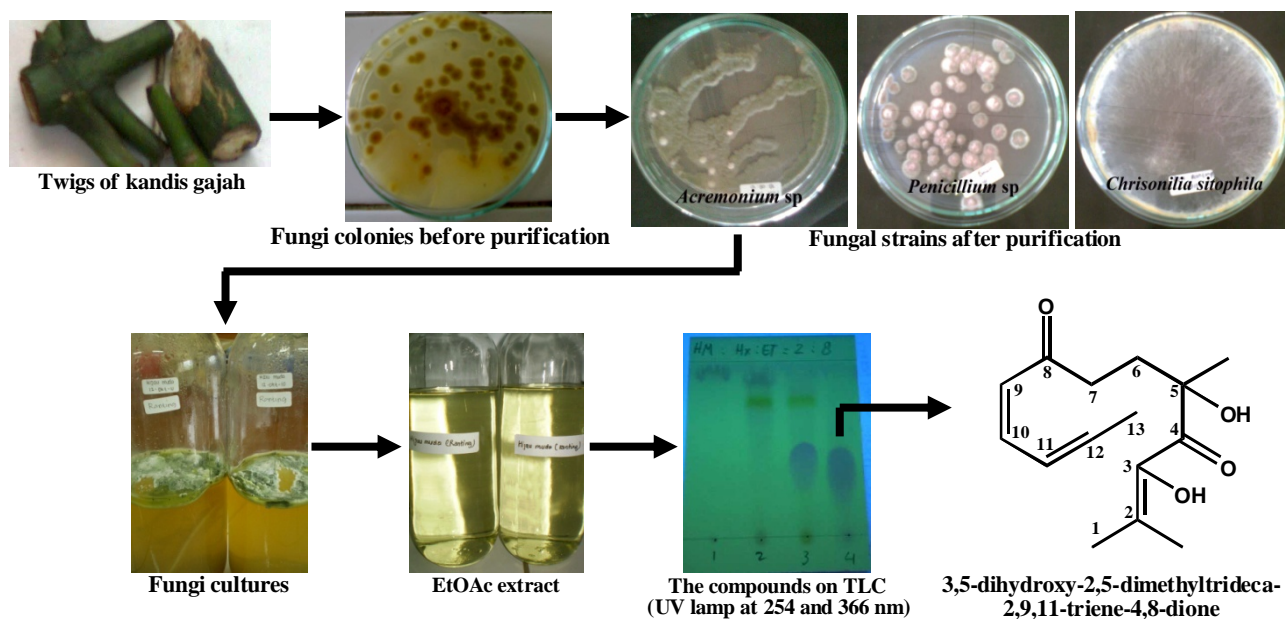


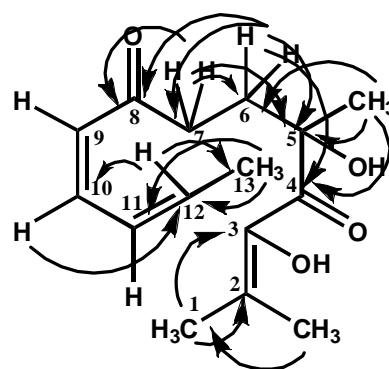
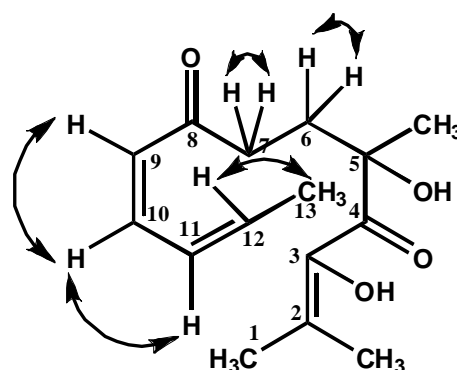
Figure 1. Brief Procedures to Isolation of Compound **1** from Endophytic Fungi *Acremonium sp.* from the Twigs of Kandis Gajah

Table 1. The NMR Data of Compound 1, Recorded at ^1H -500MHz; ^{13}C -125 MHz in CDCl_3

No. C	δ_C (ppm)	δ_H (ppm), ΣH , multiplicity, J (Hz)	HMBC	COSY
1	6.2	1.69 (3H;s)	C-3; C-2	
2	97.8			
3	174.7			
4	201.2			
5	82.8			
6	30.9	A=2.17 (1H;m) B=2.13 (1H;m)	C-4; C-8; C-5; C-7	H-6B H-6A
7	34.1	A=2.66 (1H;m) B=2.49 (1H;m)	C-8; C-5; C-6	H-7B H-7A
8	201.8			
9	127.0	6.04 (1H;d;15.3)		H-10
10	145.5	7.18 (1H;m)	C-12	H-11; H-9
11	130.2	6.21 (1H;m)		H-10
12	142.8	6.27 (1H;m)	C-10; C-13	H-13
13	19.1	1.89 (3H;d; 6.2)	C-11; C-12	
2-CH ₃	29.9	1.25 (3H;s)	C-1	
5-CH ₃	23.5	1.48 (3H;s)	C-4; C-5; C-6	

The HMBC spectrum of the compound **1**, vinylic protons at δ_H 7.18 ppm (H-10) correlated with the carbon at δ_C 142.8 ppm (C-12) and 201.8 (C-8), then protons at δ_H 6.27 ppm (H-12) correlated with the carbon at δ_C 145.5 ppm (C-10). Similarly to that seen with proton signal at δ_H 6.04 ppm (H-9) correlated with the carbon at δ_C 130.2 ppm (C-11) and 201.8 (C-8). Furthermore, methylene protons at δ_H 2.66 (H-7A) and 2.49 ppm (H-7B) correlated with carbon at δ_C 201.8 (C-8), 82.8 (C-8), 30.9 (C-6) ppm. The emergence of two methylene protons at two different chemical shifts indicates that both protons are rigid. Similarly, the methylene protons attached to C-6, each of which appears at 2.17 (H-6A) and 2.13 ppm (H-6B) correlated with the carbon at δ_C 201, 8 (C-5), 82.8 (C-5), 34.1 (C-7) ppm, while the H-6B continued its correlation with carbon C-4. Correlation of two pairs of methylene protons indicates that the proton is bound through two and three bonds to the carbonyl carbon. The methyl protons at 1.89 ppm (C-13) correlated with the two carbon atoms at 130.2 (C-11) and 142.8 ppm (C-12). Similarly, the methyl protons at 1.69 ppm (C-1) correlated with carbon at 174.7 (C-3) and 97.8 (C-2), and methyl protons at 1.25 ppm (2-CH₃) correlated with carbon at 6.2 (C-1). This indicates that both the methyl group is geminal. Furthermore, the methyl protons at 1.48 ppm (5-CH₃) correlated with the carbon at 201.2 ppm (C-4). The HMBC correlation of compound **1** is showed in Figure 2.

The ^1H - ^1H COSY spectrum revealed the presence of three vinylic protons. The proton at δ_H 7.18 ppm (H-10) showed correlation with vinylic proton at 6.21 ppm (H-11) and 6.04 ppm (H-9). Similarly, the vinylic protons

**Figure 2. The HMBC Correlation of Compound 1****Figure 3. The ^1H - ^1H COSY Correlation of Compound 1**

at δ_H 6.27 ppm (H-12) correlated with methyl proton at 1.89 ppm (H-13). Furthermore, two pairs of methylene protons attached to the same carbon atom, which is not

chemical shift (H-6A; H-6B and H-7A; H-7B) are correlated each other, where the correlation continued with beside methylene protons. This indicates that the two methylene protons located on beside to carbon atom. The COSY correlation of compound **1** is showed in Figure 3.

The antioxidant activity test of pure compounds showed strong activities with IC₅₀ values of 10.8 µg/ml, but slightly lower when compared to the antioxidant activity of extracts (IC₅₀ = 10.3 µg/ml). This might be due to the occurrence of synergistic compounds in the extracts. When compound **1** was reacted with DPPH, the radical (3-O.) may be stabilized as in the form of epoxide (C2-O-C3) by radical at C-3 and in the form diketone (on C3 and C4) by leaving the tertiary radical at C-2. Besides the radical (3-O.) may be stabilized through the binding hydrogen of 5-OH, so that the radical move to (5-O.). This can lead to radical stability due to the distribution of radical in the molecule.

4. Conclusions

The endophytic fungi *Acremonium sp.* from the twigs of kandis gajah (*G. griffithii* T. Anders) produces a pure compound 374 mg from 3 l of PDB medium at room temperature for 28 days. The compound had strong antioxidant activity equivalent to ascorbic acid. Based on the spectroscopic data (¹H-NMR, ¹³C-NMR, HMQC, HMBC, and COSY), the compound was identified as 3,5-dihydroxy-2,5-dimetiltrideka-2,9,11-triene-4,8-dione.

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