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Identification of 4-4'-(1-methylethylidene)-bisphenol from an Endophytic Fungus *Cladosporium oxysporum* derived from *Aglaia odorata*

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ABSTRACT

Endophytic fungi has an economic potential as raw material for biologically active compounds. *Cladosporium oxysporum* is one of the endophytic fungi isolated from Indonesian medicinal plant *Aglaia odorata* Lour (Local name: Pacarcina). This planth has been used for fever, cough, diarrhea, inflammation and injury. In our previous study, the ethyl acetate extract and several fractions of the extract of *C. oxysporum* showed antimicrobial activity against *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*. The objective of the currents study is to investigate the chemical constituent of the active fraction. Purification of the metabolite was achieved by using column chromatography followed by preparative thin layer chromatography. Identification of the metabolite was conducted by using TLC densitometry, GC-FID and GC-MS. Compound 1 was isolated from fraction 12. The purity of this compound was determined by 2D-TLC and GC-FID. The UV-Vis profile of compound 1 indicates a phenolic compound. Further analysis by using GC-MS shows one peak at a retention time (Rt) of 23.80 minutes, predicted as 4-4'-(1-methylethylidene)-bisphenol. The chemical constituent of the sub fraction 12.2.7 (fraction **a**) is identified as 4-4'-(1-methylethylidene)-bisphenol.

Keywords: *aglaia odorata* lour, *cladosporium oxysporum*, endophytic fungi, 4-4'-(1-methylethylidene)-bisphenol.

INTRODUCTION

Fungi provide a large potential as a source for the discovery of new drugs such as various types of antibiotics (Schulz *et al*., 2002). Endophytic fungi are microbes that live in plant tissues without causing any symptoms in its host plant. Bills *et al*. (2002) reported that endophytic fungi from tropical region provide more active metabolites and vary significantly compared to those of subtropical region. Indonesian biodiversity, especially endophytic microbes have not been well studied, whereas its potency as rich sources of valuable active substances is very promising. Most of endophytic fungi are able to produce the bioactive metabolites. As continuation of our work on endophytic fungi, some endophytic fungi have

been isolated, such as *Lecythophora* sp., *Hypocrea cf.koningii*, *Kabatiella caulivora* strain A and B, *Cladosporium oxysporum*, *Aspergilus penicilloides* from *Alyxia reinwardtii*, whilst *Eurotium rubrum*, *Aspergilus penicilloides*, and *Cladosporium oxysporum* derived from *Aglaia odorata* Lour (Sugijanto, 2011). Several studies have reported that compounds produced by endophytic fungi have shown biological activities such as antimicrobial, antifungi (Sugijanto *et al*., 2011), antiviral, antiparasitic, and antitumor (Astuti *et al*., 2014; Santiago *et al*., 2014).

A. odorata Lour (*Camunium sinense* Rumph), belonging to the family of *Meliaceae*, one of the traditional herbs used as "*jamu"* has been trusted as fever therapy, cough, diarrhea, inflammation, and injury (Kinghorn *et al.*, 2011). *A. odorata* Lour has a vascular tissue, living in a tropical climate, allowing their endophytic microbes that grow and live in it.

Previous study had reported the isolation of an endophytic fungus *C. oxysporum* from *A. odorata* Lour derived from Purwodadi Botanical Garden, East Java, Indonesia. The fungus was cultivated in liquid malt extract medium. Cultures were incubated in static condition at room temperature for 28 days, followed by extraction with ethyl acetate. The initial testing of the ethyl acetate extract showed antimicrobial activity (Sugijanto *et al*., 2005). Bioassay guided fractionation was further conducted. The ethyl acetate extract was fractionated by column chromatography using silica gel 60 with gradient elution *n* hexane, ethyl acetate, and methanol. The resulted 13 fractions were subjected to antimicrobial assay. The results showed that several fractions *C. oxysporum* have antimicrobial activity, six of 13 fractions gave inhibition against *S. aureus* ATCC 6538, *E. coli* ATCC 8739, and *C. albicans* ATCC 10231 at 24 h incubation. The eight fractions of 13 fractions giving inhibition against *S. aureus* ATCC 6538; six fractions inhibit *E. coli* ATCC 8739 and nine fractions provide inhibition zones against *C. albicans* ATCC 10231. Among all of the fractions, the 7th fraction exhibit the highest inhibition zone against *S. aureus* ATCC 6538, and *C. albicans* ATCC 10231 while the 12th fraction exhibit the inhibition zone against *C. albicans* ATCC 10231 (Sugijanto and Dorra, 2016). Based on these data then the current study focus on the isolation of secondary metabolites from fraction 12.

Some species of *Cladosporium* that live as endophytes have been able to produce bioactive metabolites such as aconitine, bulgarein, cladosporol, huperzine A, paclitaxol, 3-phenyl-2-propenoic acid, bis (2-ethyl-hexyl) phthalate, Sumiki's Acid, acetyl Sumiki's acid, brefeldin A, pandangolide 1a, pandangolide 1, pandangolide 2, and isocladospolide B (Yang *et al.*, 2012; Hua Qi *et al*., 2008; Assante *et al.*, 2005; Jadulco *et al*., 2001; Raj *et al*., 2014; Gesner *et al.*, 2005).

To the best of our knowledge, there is no report of the identification of 4-4'-(1-methylethylidene)-bisphenol from an endophytic fungus *Cladosporium* sp.

METHOD

Plant material and isolation of the endophytic fungi

C. oxysporum was isolated from stem of the host plant *A. odorata* Lour collected from Purwodadi Botanical Garden, East Java, Indonesia as described by Sugijanto *et al*., 2009. Determination of the *C. oxysporum* was done by Dr. Arnulf Diesel, Institut für Pharmazeutische Biologie Universität Düsseldorf using biological molecular method (Sugijanto *et al*., 2008).

Cultivation and extraction of endophytic fungi

Cultivation of *C. oxysporum* was undertaken on 431 culture bottles each containing 40 mL malt extract liquid medium and was incubated for 28 days at room temperature (30±3 °C) with initial pH at 5.60 ± 0.05. The cultures broth and mycelia (13.2 L) were then extracted with ethyl acetate. The ethyl acetate layers were collected, combined, and concentrated in rotary evaporator at 35 °C to obtain a yellowish brown extract (5.52 g).

Fractionation and purification metabolites of endophytic fungi

Ethyl acetate extract of *C. oxysporum* (2.80 g) was separated by column chromatography over 200 g silica gel 60 (62.5 x 3.5 cm) as a stationary phase and eluted with mixture of *n*-hexane, ethyl acetate, and methanol in increasing polarity, which yielded 13 fractions. Fraction 12 (250 mg) showed antimicrobial activity against *C. albicans* using disk diffusion method (Sugijanto and Dorra, 2016). Fraction 12 was further purified using column chromatography on Sephadex LH 20 (25 g) with methanol as mobile phase, and obtained three sub-fractions, namely 12.1 – 12.3. Sub-fraction 12.1 and 12.3 were not analysed due to low amount of samples. Sub-fraction 12.2 (52.3 mg) was then purified using preparative chromatography on silica gel 60 with dichloromethane and ethyl acetate (9:1v/v) as mobile phase. Eight sub-fractions were obtained, namely 12.2.1 to 12.2.8. Sub-fraction 12.2.7 (13.7 mg) was analyzed by TLC and obtained one spot which showed a red-purple spot with anisaldehyde-H_2SO_4 conc. reagent (R_f 0.34), whilst the other sub fractions the amount were too low to be analyzed and the spots were very weak. Purity of the fraction was analyzed by 2D-TLC. Sub fraction 12.2.7 (fraction **a**) was further analyzed using TLC-densitometry, GC-FID, and GC-MS.

Analytical condition

TLC analysis were performed on silica gel TLC plates 60 F254 aluminum sheets 20 x 20 cm (Merck). Visualization of the TLC spot were done with UV at a wavelength of 254 and 366 nm and anisaldehyde - sulfuric acid. GC-MS analysis was undertaken using GC-FID with Agilent Technologies 6890N and GC-MSD with the Agilent 6973 series equipped by Willey 7n.1 database (2004) on HP-5 column (30 m x 0,250 mm x 0,25 µm). Inlet temperature was set at 250 °C, split ratio 50:1, flow rate 1 mL/min (He), oven temperature was programmed from 100 °C – 250 °C, 5 °C/min for 30 minutes analysis. Transfer line temperature from oven to detector was 280°C; energy of ionization was set at 70 eV.

Method of Identification

EI MS of peak 1 was compared to the Willey 7n.1, NIST and Mass Bank databases according to the method of Commission Decision 2002/657/EC, the identity of a compound can be verified if its MS spectrum showed at least 4 identical fragments compared to standard MS.

RESULT AND DISCUSSION

TLC of sub-fraction 12.2 was presented in Figure 1 showed 5 spots (UV 254) and 3 spots respectively: based on this separation on these spots, preparative TLC chromatography was performed. Sub-fraction 12.2 (52.3 mg) was selected to purified using preparative chromatography on silica gel 60 with dichloromethane and ethyl acetate (9:1 v/v) as mobile phase.

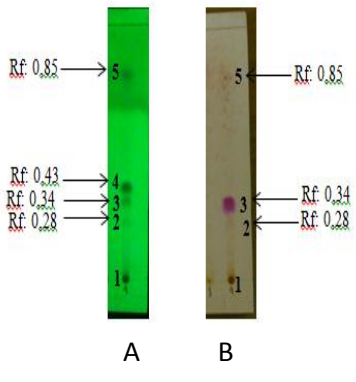

Figure 1. TLC fraction 12.2 *C. oxysporum* using silica gel 60 F254 as the stationary phase; the mobile phase is dichloromethane: ethyl acetate: 9:1. Visualized by UV254 (A) and anisaldehyde-sulfuric acid (B). Specification figures show the number of fractions.

Preparative thin layer chromatography for fractions 12.2

Fraction 12.2 was purified based on consideration of the TLC profiles using preparative thin layer chromatography (stationary phase: silica gel 60; mobile phase: dichloromethane : ethyl acetate = 9:1); fractions that have identical TLC chromatograms were collected and evaporated into dryness; eight sub fractions, namely 12.2.2 until 12.2.8 were recovered. Only sub fraction 12.2.7 (13.7 mg; R_f 0.34) showed one spot by TLC, therefore it was selected for purity test using 2D TLC and was further analysis.

Purity test

Based on the results of the 2D TLC sub fraction 12.2.7 (fraction **a**) already pure or just a single spot, so that further analysis can be done by TLC-densitometry and GC.

Characterization of isolates

TLC-densitometry chromatogram profile of fraction **a** indicated there was a dominant peak at R_f 0.34, which was observed in the UV spectrum at λ_{max} 273 nm. UV absorption at a wavelength of approximately 270 nm can be indicative of phenolic compound (Skoog *et al.*, 2007). The result from Figure 2 showed that UV-visible absorption spectra profile from scanning spectrum fraction **a** were similarly to the UV visible absorption spectra of bisphenol A in previous publications (Zhuang *et al*., 2014).

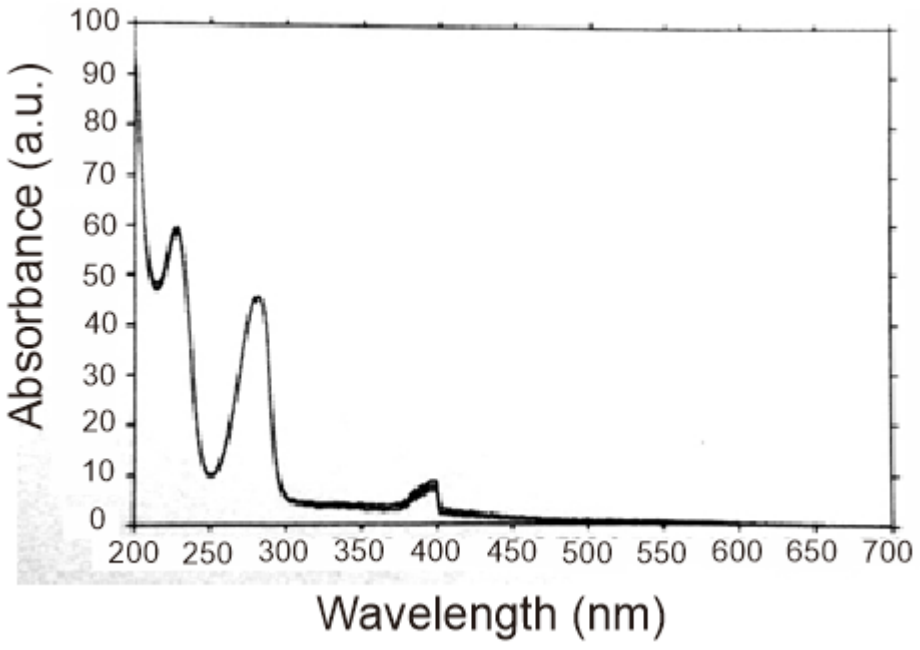

Figure 2. UV–visible absorption spectra of fraction **a** using TLC-densitometry.

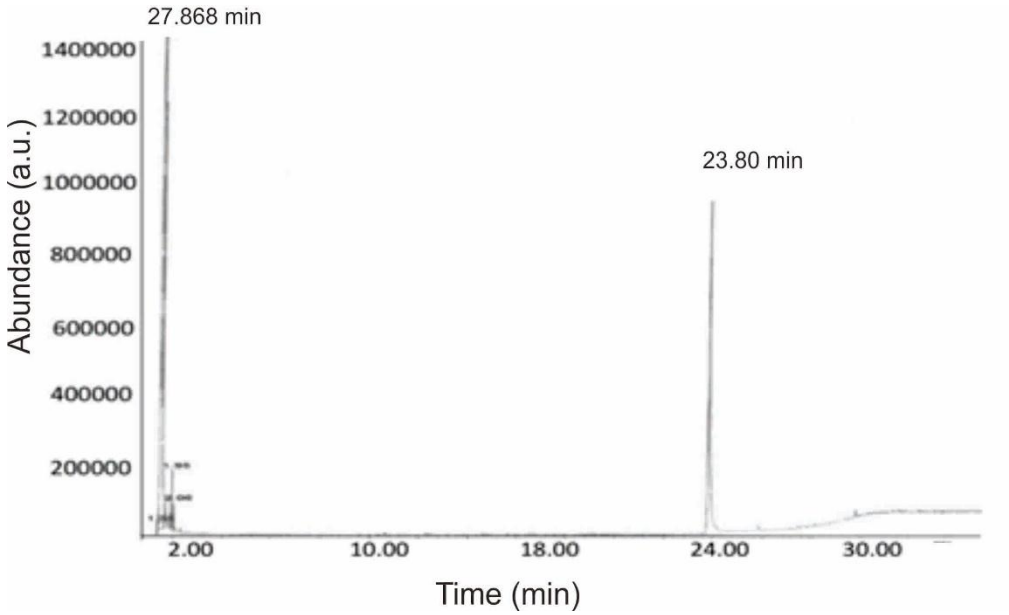

Figure 3. Total Ion Chromatogram of fraction **a**

The GC-FID chromatogram of fraction **a** exhibited peak (**1**) at a retention time (R_t) of 27.868 min was further analyzed using GC-MS, showed there is one peak (**1**) at a retention time (R_t) of 23.80 minutes **(**Figure 3**).**

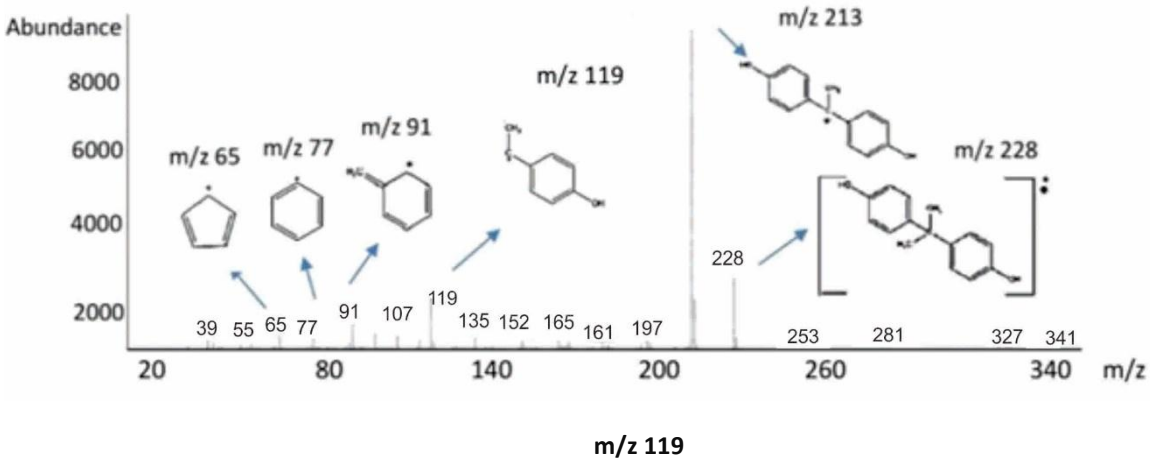

Figure 4. EI-MS of peak 1 of fraction a.

EI-MS of peak 1(see Figure 4) showed molecular ion $[M]^+$ at *m/z* 228, other peaks were observed at *m/z* 213 [M-15] (base peak), *m/z* 119 [M-109], 91 [M-137], 77 [M-151], and 65 [M-163]. Wiley data base predicted that peak 1 was 4-4'-(1-methylethylidene)-bisphenol, with the highest quality score (87%). The EI-MS of peak 1 was identical with the EI-MS spectra of 4-4'-(1-methylethylidene)-bisphenol mass bank (www.massbank.jp) with a similarity score of 88.31%. Criteria resemblance to the database can be considered equal if > 80% (Odchimar *et al*., 2016). According to Commission Decision 2002/657/EC, the

identity of a compound can be verified if its MS spectrum showed at least 4 identical fragments compared to standard MS. Identification was conduct by comparing mass spektrums with databases from Wiley 7n.1, National Institute of Standart and Technology (NIST), and mass bank spectra (www.massbank.jp) that peak 1 was 4-4'-(1-methylethylidene)-bisphenol also supported by data fraction **a** showed similarly to the UV visible absorption spectra of bisphenol A in previous publications (Zhuang *et al*., 2014).

Compound 4-4'-(1-methylethylidene)-bisphenol or bisphenol A is an essential chemical compound consisting two groups of phenol, and primarily used in industry for the manufacture of plastic polycarbonate and epoxy resins. Bisphenol-sesquiterpenes have been isolated from fungus *Kionochaeta ramifera* BCC 7585. Bisphenol-sesquiterpenes isolated is ramiferin which demonstrated antimalarial activity, antifungal against *C. albicans* and antituberculosis (Bunyapaiboonsri *et al*., 2008). Based on literature above, it is suggested that 4-4'-(1-methylethylidene)-bisphenol or bisphenol A may be responsible for antifungal activity given by fraction of 12 of the ethyl acetate extracts of *C. oxysporum* endophytic fungi from *A. odorata* Lour. The amount of the fraction **a** is too low after analysis thus the antifungal assay could not be done.

CONCLUSIONS

4,4'-(1-methylethylidene)-bisphenol or bisphenol A was identified for the first time in *C. oxysporum* endophytic fungus isolated from *A. odorata* Lour, by GC-MS and additionally compared with databases from Wiley 7n.1, National Institute of Standart and Technology (NIST), and mass bank spectra .

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