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Pachybasin, a Major Metabolite from Culture Broth of Endophytic Coelomyceteous AFKR-18 Fungus isolated from a Yellow Moonsheed Plant, *Arcangelisia flava* (L.) Merr.

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Endophytic fungi have been known to produce a broad range of biologically active secondary metabolites. One endophytic filamentous fungus, Coelomycetes AFKR-18, isolated from the young stems of a yellow moonsheed plant, *Arcangelisia flava*, has been found to produce pachybasin when placed in a liquid medium. The chemical structure of pachybasin was deduced from MS, 1D-, 2D-NMR spectrum analysis, and from reference data. On a micro-dilution test, pachybasin showed antimicrobial activities against *E. coli*, *B. subtilis*, *M. luteus*, *S. cerevisiae*, *C. albicans*, *A. niger*, and *A. flavus*, with MIC values of 64.0 µg/mL, and against *S. aureus* and *F. oxysporum* with MIC values of 32.0 and 16.0 µg/mL respectively.

Keywords: *Arcangelesia flava*, endophytic fungi, Coelomycetes AFKR-18, pachybasin, antimicrobial activity

INTRODUCTION

Endophytes are microorganisms that colonize healthy plant tissues without causing any direct obvious negative effects, and they are notable sources of bioactive natural products (Strobel & Daisy 2003). Various kinds of secondary metabolites, including alkaloids, indole derivatives, steroids, terpenoids, quinones, flavonoids, and many more, have been successfully isolated from endophytes (Tan & Zou 2001). Those metabolites exhibit different functions and benefit human in many areas including agronomy and medicine.

Many endophytes have been reported to produce substances with medicinal potential, due to their biological activity. One example is colletric acid, an active antimicrobial agent, which was isolated from a liquid culture of endophytic fungus *Colletotrichum gloeosporioides* (Zou *et al*. 2000). Some endophytes, isolated from *Calluna vulgaris*, *Emperitum nigrum*, *Vaccinium vitis-idaea*, and *Vaccinium myrtillus*, produced substances which were actively anti-mycobacterial (Gordien *et al*. 2010). Several anti-cancer agents such as taxol, camptotthecin, vinca alkaloids, and phodophyllotoxin can also be produced by endophytes. A comprehensive review of these anti-cancer agent-producing endophytic fungi was conducted by (Chandra 2012).

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In the field of agronomy, endophytes are important as potential biocontrol agents against plant diseases. *Trichoderma* endophytic isolates were reported to delay development of disease caused by *Phytophtora capsici* in hot pepper (*Capsicum annuum*) (Bae *et al*. 2010). In a field trial in Panama, application of suspension of *Colletotrichum gloeosporioides* spore on flowers and pods of cacao crops significantly reduced pod loss caused by *Moniliophthora roreri* and *Phytophthora* spp. (Mejía *et al*. 2008). Several endophytes isolated from live oaks (*Quercus fusiformis*) also showed potential as disease control agents against "oak wilt", as they inhibit the pathogen *Ceratocystis fagacearum* (Brooks *et al*. 1994).

Several approaches for host plant selection can be used to increase the likelihood of success in isolating bioprospective endophytes to discover new bioactive compounds. Selection decisions can be based on the particular environment where plants grow, the ethnobotanical history of plant uses or applications, the biodiversity of a plant's habitat, or its endemicity (Strobel & Daisy 2003). High endophyte diversity is most likely to be found in areas of great biodiversity overall, such as tropical rain forest.

The yellow moonsheed plant (*Arcangelisia flava* (L.) Merr.), a liana in the Menispermaceae family, grows throughout South East Asia. It has been used traditionally to treat malaria, dysentery, and fever (Subeki *et al*. 2005; Larisu 2011). The plant has

also been reported to be used as a tonic, abortive, expectorant, and emmenagogue (Verpoorte *et al*. 1982). Major secondary metabolites isolated from *A. flava* include quaternary protoberberine alkaloids berberine and jatorrhizine, and isoquinoline alkaloid pycnarrhine (Verpoorte *et al*. 1982). Alkaloid dehydrocorydalamine, thalifendine, and palmatine have been isolated as minor compounds (Verpoorte *et al*. 1982). Some of the alkaloids, including palmatine, berberine, jatorrhizine, dihydroberberine, and 20-hydroxyecdysone, proved to be active agents against *Babesia gibsoni*, a parasite which causes disease in domestic animals (Subeki *et al*. 2005).

In our laboratory, we conduct the isolation of the endophytic fungi from yellow moonsheed plant samples collected from different location in Indonesia. Isolation of endophytes of yellow moonsheed plants from Kalimantan has already led to the discovery of one potential antimicrobial compound (Praptiwi *et al*. 2010). In addition, the endophytes of a yellow moonsheed plant from Sukabumi produced phloroglucinol, a substance with many potential applications including use as an antispasmodic, antioxidant, dye, and as a precursor for many synthetic substances (Jamal *et al*. 2011). The endophytes were able to produce phloroglucinol 14.9 mg/L in a potato dextrose broth medium. In this study, we report on a major compound produced by one endophytic fungi, Coelomycetes AFKR-18, isolated from the young stem of *A. flava* collected from Bogor Botanical Garden.

MATERIALS AND METHODS

Source and Slection of Coelomycetes. Fresh young stems of *A. flava* were collected from Bogor Botanical Garden in 2008 and morphologically identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences. The young stems were rinsed in running water, cut into rods (1-5 cm), and sterilized externally by successive soaking in 70% alcohol for 2 min, NaOCl for 5 min, and 70% alcohol for 30 sec. The sterilized stems were sliced aseptically into small pieces. The inner parts of the slices were placed on Corn Meal Malt Agar (CMMA) medium, supplemented by 0.05 mg/mL chloramphenicol, and were incubated at room temperature for 1 week. Each colony of endophytic fungi was then serially transferred onto Potato Dextrose Agar (PDA) medium to obtain a pure colony. A resulting 18 total fungal colonies were obtained. The glycerol stocks of the colonies were stored at -80 ^{o}C in the Indonesian Culture Collection (INACC), Research Center for Biology, Indonesian Institute of Sciences.

Screening of Secondary Metabolites Production and Antibacterial Assay. All of the endophytic fungi obtained were screened for secondary metabolites and antibacterial activity according to the methods in Praptiwi *et al*. (2010). In brief, the fungal colonies were cultivated in Potato Dextrose Broth (PDB) and Glucose-Yeast extract-Pepton (GYP). After 3 weeks of incubation at room temperature, the fungal cultures and their biomass were extracted twice with ethyl acetate-methanol (10:1). The ethyl acetate extracts were concentrated under reduce pressure using a rotary evaporator, and analyzed by Thin Layer Chromatography (TLC) using eluent of dichloromethane : methanol : acetic acid (6:1:1 drop). Antibacterial activity tests were performed against *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Escherichia coli*, by the disc diffusion method (Schwalbe *et al*. 2007) at extract concentration of 10 mg/mL. The fungal isolate that showed the strongest antibacterial activity was selected for scaled-up cultivation.

Scaled-up Cultivation of AFKR-18 Fungus. Fresh AFKR-18 fungus grown on PDA medium was inoculated into 500 mL Erlenmeyer flasks containing 200 mL of PDB medium. After still incubation at room temperature for 3 weeks, the medium and biomass were homogenized and extracted three times with ethyl acetate-methanol (4:1) in equal volume. The ethyl acetate extract was concentrated under vacuum using a rotary evaporator at 30 ^{o}C to obtain yellowish extract.

Isolation and Spectroscopy Characterization of Active Metabolites. The AFKR-18 fungal extract was subjected into Sephadex LH-20 column chromatography and eluted with methanol. Based on the TLC monitoring outcomes, the collected fractions were grouped into sixteen fraction samples (F.1–F.16). Preparative TLC of F.10 and F.11 yielded 20.1 mg substance 1. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a JEOL JNM-ECA-500 spectrometer at 500 and 125 MHz, respectively. Chemical shifts were expressed in δ values relative to an internal standard of TMS. Mass spectra were recorded in an ion trap GC-MS (Varian, Saturn 2000) with the capillary column VM-17 MS (0.25 x 30 mm, varian). Injector temperature was 250 ^{o}C, column temperature was programmed from 100 ^{o}C (isothermal for 3 min) to 270 ^{o}C with temperature change rate of 5 ^{o}C/min.

Column pressure was 10.7 psi, carrier gas flow was 2 mL/min (Helium). Interphase temperature was 270 °C, and trap temperature was 150 °C. Solvent cut time was 3 min and scan MS was 50-600 (M/Z).

Antimicrobial Activity. MICs of 1 were determined by microdilution method, performed in 96 well plates. Each well contained 100 µL of Saburaud Broth (SB) media for antifungal assay or Mueller Hinton Broth (MHB) for antibacterial assay, 100 µL of microorganism suspension, and 100 µL of tested substance. The test microorganisms were from the Indonesian Culture Colection (InaCC) and consisted of *E. coli* InaCC B5, *S. aureus* InaCC B4, *B. subtilis* InaCC B1, *M. Luteus* LIPIMC 0076, *Candida albicans* LIPIMC y0382, *Saccharomyces cerevisiae* LIPIMC y0070, *Aspergillus niger* InaCC F234, *Aspergillus flavus* InaCC F44, and *Fusarium oxysporum* InaCC F78. Chloramphenicol, nystatin, and cabicidin were used as positive controls.

RESULTS

Eighteen endophytic fungi were isolated from young stems of *A. flava* (AFKR-1 to AFKR-18 isolates). Based on screening for antimicrobial activity by disc diffusion method, the fungus AFKR-18 isolate, later identified as belonging to the fungal class Coelomycetes, was selected for bioactive metabolites isolation.

Scaled-up cultivation of AFKR-18 in 5 L of PDB media for 3 weeks yielded 191 mg of ethyl acetate extract which was then fractionated on Sephadex-LH20 column to obtain 16 fractions (F.1-F.16). On the basis of TLC monitoring, one major compound (1, Figure 1) of AFKR-18 extract was observed in F.10 and F.11. It was characterized as a thick yellow spot on TLC silica plate. Further purification on Silica Gel 60 preparative TLC resulted in yellow powder of 1 (11.6 mg).

Identification of Isolated Compound. Compound 1 was subjected to MS, 1D, and 2D-NMR studies; the molecular mass was found to be 238 amu. The ^{1}H-NMR spectrum of 1 (Table 1) showed the presence of aromatic protons at δH 8.23 (1H, m), two aromatic ortho-coupled protons at δH 7.93 (2H, m), a singlet aromatic proton at δH 7.16 (1H, s) and δH 7.59 (1H, s), one hydroxyl group at δH 12.52 (1H, s), and a methyl group at δH 2.48 (3H, s). The 13C-NMR spectra of 1 showed 15 carbon signals including a methyl carbon at δC 22.2 and two ketone carbonyls at δC 182.9 and 189.2. Using the DEPT experiment protocol, it can be inferred that signals at δC 114.9, 134.2, 134.5, 149.9, 163.6, 182.9, and 189.2 are quarternary carbon atoms. Based on those

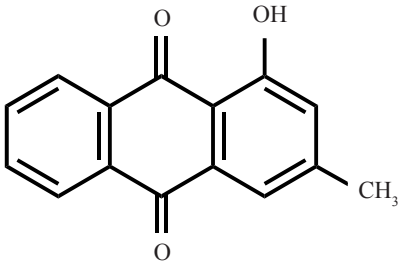

Figure 1. Chemical structure of compound1 (pachybasin).

Table 1. ^{1}H- and ^{13}C-NMR data of compound1 and Pachybasin in CDCL3, 500 MHz for ^{1}H and 125 MHz for ^{13}C

Position	1		Pachybasin*	
	δH	δC	δH	δC
1	-	163.6	-	162.7 (s)
2	7.16 (1H, s)	124.6	7.04 (1H, s)	124.1 (d)
3	-	114.9	-	114.0 (s)
4	7.59 (1H, s)	121.2	7.57 (1H, s)	120.7 (d)
4a	-	149.9	-	148.6 (s)
5	8.28 (1H, m)	127.5	8.23 (1H, t J=6.0Hz)	126.1(d)
6	7.93 (2H, m)	135.3	7.75 (2H, d J=5.0 Hz)	134.0 (d)
7		135.7		134.4 (d)
8	8.23 (1H, m)	127.9	8.23 (1H, t J=6.0Hz)	127.3 (d)
8a	-	134.5	-	133.5 (s)
9	-	189.2	-	187.9 (s)
9a	-	134.2	-	133.1 (s)
10	-	182.9	-	182.6 (s)
10a	-	134.2	-	133.2 (s)
Me-	2.48 (3H, s)	22.2	2.41 (3H, s)	22.2 (q)
OH-	12.52 (1H, s)	-	12.50 (1H, s)	

* Liu *et al*. 2007.

Table 2. HMBC of compound 1

C	H-2	H-4	H-5	H-8	Me-
1					
2		124.6 (7.59)			124.6 (2.48)
3	114.9 (7.16)	114.9 (7.59)			
4	121.2 (7.16)				121.2 (2.48)
4a					149.9 (2.48)
5					
6				135.3 (8.23)	
7			135.7(8.28)		
8					
9					
9a					
10		182.9 (7.59)			
10a					
Me-	22.2 (7.16)	22.2 (7.59)			

Table 3. Minimum inhibitory concentrations of 1 isolated from Coelomycetes AFKR-18 against some fungi and bacteria

Microorganism	MICs (µg/mL)			
	1 (Pachybasin)	Chloramphenicol	Nystatin	Cabicidin
Escherichia coli InaCC B5	64.0	8.0	not tested	not tested
Staphylococcus aureus InaCC B4	32.0	16.0	not tested	not tested
Bacillus subtilis InaCC B1	64.0	8.0	not tested	not tested
Micrococcus luteus LIPIMC 0076	64.0	8.0	not tested	not tested
Candida albicans LIPIMC y0382	64.0	not tested	32.0	32.0
Saccharomyces cerevisiae LIPIMC y0070	64.0	not tested	64.0	64.0
Aspergillus niger InaCC F234	64.0	not tested	16.0	64.0
Aspergillus flavus InaCC F44	64.0	not tested	16.0	32.0
Fusarium oxysporum InaCC F78	16.0	not tested	32.0	16.0

data, we deduced the presence of an anthraquinone with one hydroxyl and one methyl substitutes. The precise positions of the substitutes were confirmed by HMBC experiment (Table 2). In an HMBC experiment, there is a correlation between the methyl proton signals at δH 2.48 (3H, s) and three aromatic carbons at δC 124.6 (C-2), δC 121.2 (C-4), and δC 149.9 (C-4a). Other correlations were seen between the single aromatic proton signal at δH 7.16 (1H, s) and carbon signals at δC 114.9 (C-3), 121.2 (C-4), and methyl carbon signal at δC 22.2. In addition, there were also correlations between the single aromatic proton signal at δH 7.59 (1H, s) and carbon signals at δC 114.9 (C-3), 124.6 (C-2), 182.9 (C-1), and the methyl carbon signal at δC 22.2. These correlations indicate hydroxyl substitution at C-1 and methyl substitution at C-3. The structure of compound 1 was identified as pachybasin.

Antimicrobial Activity. MICs of compound 1 were determined by dilution method in 96 well plates against various bacteria and fungi. The MICs were listed in Table 3. Compound 1 was active against all microorganisms tested, although the MICs were still higher than positive controls (except for *Fusarium oxysporum*). The MIC of 1 against *F. oxysporum* is similar to cabicidin (16.0 µg/ml) and lower than nystatin (32.0 µg/mL).

DISCUSSION

In this study, pachybasin was isolated from a liquid culture of endophytic fungus of *A. flava* AFKR-18. The fungus species has not been fully identified, but morphologically, it belongs to the class of Coelomycetes (unpublished data). Pachybasin is known as fungal anthraquinone derivative. Its structure, with only one α-hydroxy substitution, makes pachybasin the simplest fungal anthraquinone (Shibata *et al*. 1955). Pachybasin was isolated for the first time from a living organism, *Pachybasium candidum*, by Shibata *et al*. (1955). Other fungi *T. harzianum*, *Phoma foveata*, and a marine-derived fungus of *Monodictys* were also reported to produce pachybasin (Shibata & Takido 1955; El-Beih *et al*. 2007; Liu *et al*. 2009).

Pachybasin was tested for its activity against various pathogenic bacteria and fungi. The results revealed that its activity was not as high as positive controls in inhibiting some pathogenic bacteria and fungi growth. Nevertheless, a notable result was obtained for its activity against *F. oxysporum* with an MIC value of 16.0 µg/mL, lower than the MIC of nystatin (32.0 µg/mL) and equal to that of cabicidin. *Fusarium oxysporum* is an *Ascomycete* fungus and a plant pathogen. It causes wilt disease in plants including cotton, banana, and date palm (Michielse & Rep 2009).

This result accords with the work of Liu *et al*. (2007) who first isolated pachybasin from *T. harzianum*, a biocontrol agent of plant pathogenic fungi. *Trichoderma* proved to control *Rhizoctonia* diseases in some crops as well as to induce plant growth (Lo & Lin 2002). The presence of pachybasin and chrysophanol as secondary metabolites in *T. harzianum* suggested their possible contribution to the agent's biocontrol function (Liu *et al*. 2007). The antifungal mechanism of these substances was speculated to derive from the formation of radicals cleaving proteins. Further investigation of the role of pachybasin revealed that this compound, along with emodin, enlarges the number of *Trichoderma* mycoparasitic coils via cAMP signaling (Lin *et al*. 2012). Therefore, both compounds were directly involved in the biocontrol mechanism of *Trichoderma*.

Similar to *T. harzianum*, the endophytic fungus Coelomycetes AFKR-18 may play an important role in the defence system of its host plant, *A. flava*, against pathogenic fungi, via production of pachybasin. Coelomycetes AFKR-18 may also be a potential biocontrol agent for other crops that are vulnerable to fungal disease, especially *F. oxysporum*, which causes disease in banana plants. However, further lab experiments are needed to evaluate the ability of the endophyte to colonize other plant tissue, as well as a field trial to assess the effectiveness of the endophyte treatment in reducing the incidence of this plant disease.

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