ANTIMICROBIAL METABOLITE FROM THE CULTURE OF ENDOPHYTIC FUNGUS AFK-8 ISOLATED FROM KAYU KUNING [*Archangelisia flava* (L.) Merr.]

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ABSTRACT

The fungus AFK-8 is one of endophytic filamentous fungus isolated from young stems of kayu kuning [*Archangelesia flava* (L.) Merr.]. The ethyl acetate extract derived from fungus culture shows their antibiotic activity tested against bacteria and fungi on a disc diffusion method. Scaling up cultivation of endophytic fungus AFK-8 on 1L PDA for 3 weeks yielded 143.3 mg of chloroform extract. Fractionation guided assay of these extract showed that the fraction 6 is the most active fraction against tested microbes with MIC values 8, 32, 4 and 64 □g/ml against *B. subtilis, M. luteus, S. aureus* dan *E. coli* respectively. This fraction is also active against *C. albicans, R. minuta and A. niger* with MIC value 32 *m*g/ml. The proposed chemical structure of active metabolit in fraction 6 is 1,2-diamino-9,10-anthracenedione which was determined through a GC-MS analysis.

Key words : *Arcangelisia flava, endophytic fungi, metabolites, antimicrobial activity.*

INTRODUCTION

Microbial endophyte is a microbial colony which resident inside of healthy plant tissues. Endophytic microbes have been recognize as a chemical producer with a broad range biological activity (Tan and Zou, 2001). Some of endophytes could have ability to mimicking their plant resindent chemical constituents such as *Taxomyces andreanae* from *Taxus brevifolia* that also produce taxol (Stierle *et al*., 1993), an endophyte fungus from the plant *Notapodytes foetida* that produce camptotecin (Puri *et al.*, 2005) in laboratory. Some of *Phomopsis* fungi species associate with the plant of *Salix gracilostyla* var. *melanostachys* (Horn *et al.*, 1995), the fungus *Cryptosporiopsis* cf. *quercina* associate with *Tripterigium wilfordii* (Strobel *et al*., 1999; Li *et al*., 2000), the fungus *Cytospora* sp. CR 200 and *Diaporthe* sp. CR 146 (Brady *et al*., 2000) have been reported produce various secondary metabolites that shows an antibacterial activity.

Kayu kuning [*Arcangelisia* flava (L.) Merr.] is a climbing plant belongs to Menispermaceae plant family used extensively as a medicinal plant. The biologically active chemical compounds of kayu kuning are protoberberin alkaloids i.e. berberine, palmatine and jarorrhizine. According to Arrogo and Sibel (2009) berberine can be used to prevent metabolic illness, related to cardiac disorder, also has antiinflammatory and antiproliferation properties. Based on their traditional used as a medicinal plant, we could interest to explore the bioprospecting of the endophytic fungi associated with kayu kuning.

MATERIAL AND METHOD

Plant Materials

The youg stems of kayu kuning (*Archangelisia flava*) were collected from Sambas, West Kalimantan in 2007 and identified by DR. Sudarmono, Bogor Botanical Garden.

Isolation of Endophytic Fungi

Young stems of kayu kuning were washed by tap water and then cut with the length of 1-5 cm, followed by sterilization by soaking the stems in the 70% alcohol for 2 minutes, then soaked in the NaOCl for 5 minutes and soaked again in 70% alcohol for 30 second. The sterilized stems were sliced with sterile knife and then put on Corn Meal Malt Agar (CMMC) media that had been added with 0.05 mg/ml chloramphenicol followed by incubation in the room temperature for 1 week. Every colony of endophitic fungi were serially transfered onto Potato Dextrose Agar (PDA) media untill pure colony were obtained. The obtained endophytic fungi were preserve in -80 ^{o}C at LIPI-MC.

Screening of Secondary Metabolites Production and Antibacterial Assay

All of isolated endophytic fungi were cultivated in 20 mL PDA, Potato Dextrose Broth (PDB) media and Glucose-Yeast extract-Pepton (GYP) in 100 mL test tube, then incubated at room temperature for 3 weeks. After 3 weeks of incubation, fungi cultures and its biomass were extracted twice with ethyl acetate : methanol (10 : 1), shaken

with vortex and allowed to form two layer. The upper layer was taken and concentrated with rotary evaporator. The extracts were then analysed by Thin Layer Chromatography (TLC) and eluted dichloromethane : methanol : acetic acid (6 : 1 : 1 drop). Then, the TLC chromatograms were monitored by UV light at the wavelength of 254 nm dan 365 nm, then sprayed with $Ce(SO_4)_2$.

Extract from each endophytic fungi were diluted in acetone and tested for its antibacterial activity by diffusion method at the concentration of 10 mg/mL. Bacteria isolates used for antibacterial activity were *Bacillus subtilis, Micrococcus luteus, Staphilococcus aureus* and *Eschericia coli,* inoculated in Nutrient Broth media and incubated for 18 hours at room temperature. After that the tested bacteria was taken with sterile micropippette (100 µL) and cultured on Mueller Hinton Agar (MHA) media. 10 µL of extract was dropped on sterile paperdisc then put on MHA media that had been inoculated with bacteria isolate followed by incubation at 37^{o}C for 24 hours. The growth inhibition zone around paperdisc was then measured.

Scaling up Cultivation of Fungus AFK-8

The endophytic fungus AFK-8 was cultivated onto 2 x 500 ml of PDA (in Erlenmeyer 5 L) and incubated at room temperature. After 3 weeks, all ofmedium and biomass were homogenazied and extracted with ethyl acetate-methanol (10:1, 3 times in equal volumes) and concentrated under reduce pressure at 30 ^{o}C to achieved 143.3 mg of yellowish extract.

Isolation of Active Metabolites

The whole fungus extract (143,3 mg) was subjected into a Sephadex LH-20 column chromatography and eluted with methanol to achieved 6 fractions. All of fractions were then subjected into TLC analysis and a disc diffusion antibacterial assay.

The fraction was disolved in aceton and tested for its antibacterial activity by diffusion method at the concentration of 10 mg/mL, while bacteria isolates used were *B. subtilis, M. luteus, S. aureus* and *E. coli*. Bacteria isolate was cultured on MHA media. The sterile paper disc that had been added with the 10 µL extract was then put on MHA media that had been inoculated with bacteria isolate, followed by incubation for 24

hours at 37 ^{o}C. The growth inhibitoin zone was the clear zone around the paper disc and the diameter of clear zone was measured.

Determination of MIC Value of Fraction 6

The MIC values were determined through a microdillution assay (Rodriguez-Tudela , 2002). Determination of MIC value was done by preparing of fraction 6 stock solution in DMSO at the concentration of 512 ug/ml. The medium for antifungi was Saburaud Broth (SB), while for antibacterial test was Mueller Hinton Broth (MHB). Fungi isolates used for the test were *C. albicans, R. minuta, A. niger, A. flavus* and *S. cerevisiae*, while bacteria isolates used were *M. luteus, E.coli, S. aureus, B. subtilis.* In each 96 *microwell titer* was added as : well 1 added with growth media 2x concentration (100 ul) and stock solution (100 ul). Well 2 – 14 added with growth media 1x concentration (100 ul). Well 1 homogenized with micropippette, then pippette 100 ul and put on well 2. The same method was done untill well 14. After that in each well was added with bacteria inoculum (100 ul). Well 15 was positive control (100 uL growth media and 100 uL inoculum), while well 16 was negative control (200 uL growth media). The 96 microwell titer was then incubated in the shaker incubator for 24 hours at 35-37^{o}C. The MIC was observed visually.

GC-MS Analysis of Fraction 6

The chemical constituents of fraction 6 from the fungus AFK 8 extract cultivated on PDA was analysed by an ion trap GC-MS (Varian, Saturn 2000) with the capillary column VM 17 (0.25 mm x 30 m, varian) Conditions of analysis were : injector temperature = 230 ^{o}C, column temperature was programmed from 80 ^{o}C (isothermal for 3 minutes) to 250 ^{o}C with temperature rate 5 ^{o}C/minute. Column pressure was 10,7 psi, carrier gas flow was 1,3 Ml/minute (Helium). Interfase temperature = 250 ^{o}C, trap temperature = 150 ^{o}C. The sample (in aceton solution) was injected 5 µL.

RESULT AND DISCUSSION

The isolation of endophytic fungi from kayu kuning (*Arcangelisia flava* (L.) Merr. stem resulted in 9 endophytic fungi named AFK -1 ~ AFK-9. The TLC analysis results showed that the TLC chromatograms oatterns of ethyl acetate-methanol extracts of fungi AFK-1, AFK-2, AFK-3, AFK-5, AFK-6 and AFK-7 are different each other. On the other hand, the TLC chromatogram pattern of AFK-4 is identical to AFK-2, while AFK-8 and AFK-9 are identical to those of AFK-7. The above result revealed that the fungus AFK-2 is chemotaxonomically identical species with fungus AFK-4, and fungi AFK-7, AFK-8 and AFK-9 are also chemotaxonomically identical species.

From the antibacterial screening tests of ethyl acetate-methanol extract of all the above endophytic fungi, it was found fungus AFKR-8 possess an antibacterial activity againts all tested bacteria *B. Subtilis, E. coli, M. luteus* and *S.aureus.* In order to isolate and characterize principle antibacterial metabolite(s) produce by the endophytic fungus AFK-8, the fungus was then cultivated on 2 x 500 ml of PDA (in 5 L size Erlenmeyer), and then extracted with ethyl acetate-methanol to achieve a yellowish extract.

Fractioation of AFK 8 extract with Sephadex LH-20 column chromatography yielded 6 fractions, i.e. F1 (55.1 mg), F2 (2.7 mg), F3 (25.5 mg), F4 (7.6 mg), F5 (34.8 mg) and F6 (8.1 mg) respectively.

The antibacteria activity assay on a disc diffusion method showed that the F6 (fraction 6) had hihger antibacterial activity compare to other fractions. The diameter of growth inhibition for fraction 6 was : *M. luteus* (17 mm), *B. subtilis* (17 mm), *S. aureus* (14 mm) at the concentration of 100 □g/10 □l, but failed to show their activity against E. coli at tested concentration (100 □g/10 □l).

M. luteus *B. subtilis* *S. aureus*

Figure 1. Growth inhibition of fraction 6 of AFK 8 extract to several bacteria isolates

Furthermore, the F6 was subjected into a subceptible antibacterial assay against 4 tested bacteria 5 tested fungi (Tabel 1) in a microdillution method. The results showed that fraction 6 (MIC= 4 mg/L) had higher activity compared to chloramphenicol (MIC= 16) against *S. aureus*, and its activity was similar to chloramphenicol against *B. subtilis* (MIC = 8 mg/L). Fraction 6 also had higher activity against *R. minuta* (MIC = 32 mg/L) dan *S. cerevisiae* (MIC = 32 mg/L) and similar to *C. albicans* (MIC = 32 mg/L) compared to nystatin and cabisidin. Based on the result, it was concluded that fraction 6 of AFK-8 extract capable of inhibiting the growth of tested bacteria and fungi, moreover its activity was better than comersial antibiotic against several bacteria and fungi.

GC-MS analysis result of F6 (Fig. 2) showed a single main peak on its chromatogram, indicate that F6 was almost pure. The MS spectrum of F6 shows base peak and ion molecule at m/z 238.0. Comparison of mass spectrum (Fig. 3) of F6 with known chemical mass spectra in *NIST Library* and *Wiley*, showed that the mass spectra of F6 is identical with 1,2-diamino-9,10-antrasenediona. This compound was classified to anthraquinone. According to Anonim (2010) antrasenediona has the ability as anticancer and known as anticancer antibiotic. Based on the GC-MS analysis , the possibility of molecule formula of fraction 6 AFK 8 PDA was $C_{14}H_{10}N_2O_2$.

Table 1**.** MIC value of F6 (fraction 6) against tested bacteria and fungi.

No	Microbe isolate	MIC (mg/L)				
		F6	chloramphenicol	erythromycin	nystatin	Cabisidin
1	*B. subtilis*	8	8	0,03	nt	nt
2	*M. luteus*	32	16	0,06	nt	nt
3	***S. aureus***	**4**	**16**	**32**	nt	nt
4	*E. coli*	64	16	16	nt	nt
5	*C. albicans*	32	nt	nt	32	32
6	***R. minuta***	**32**	nt	nt	**64**	**64**
7	*A. niger*	32	nt	nt	16	64
8	*A. flavus*	32	nt	nt	16	32
9	***S. cerevisiae***	**32**	nt	nt	**64**	**64**

nt : not tested

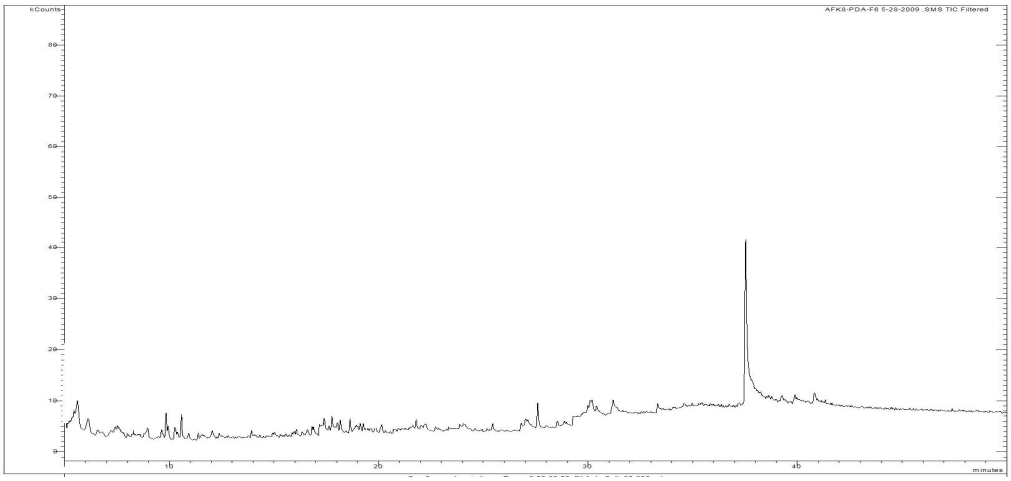
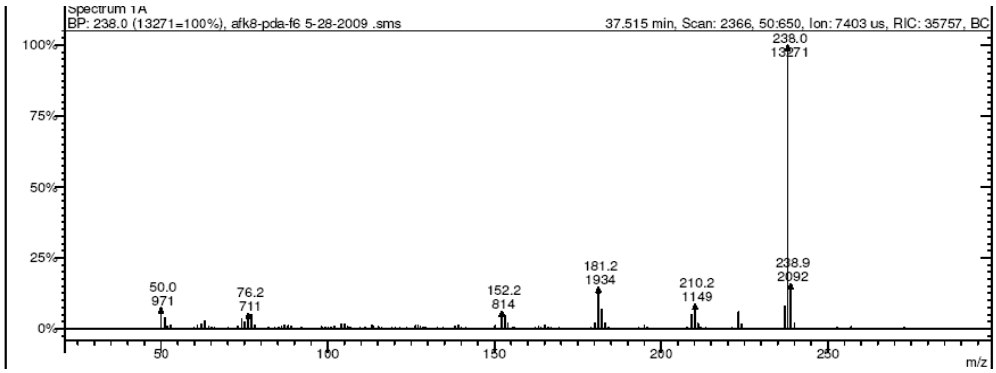
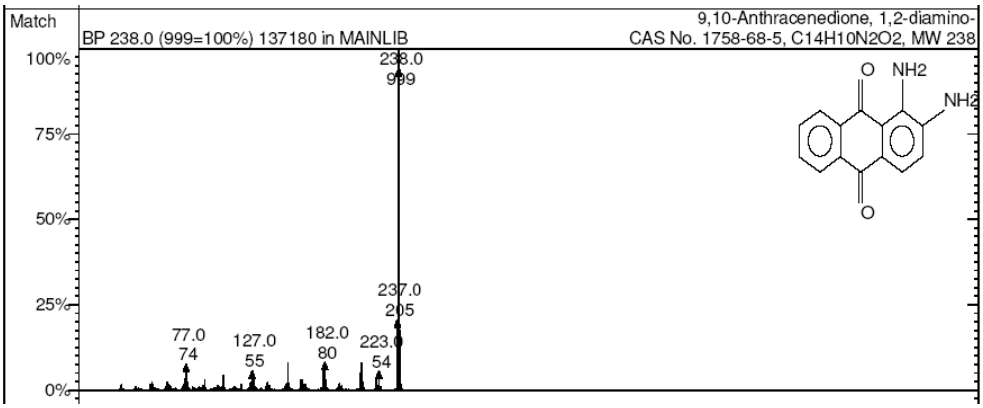


Figure 2. GC chromarogram of fraction 6



(a)



(b)

Figure 3. (a) MS spectrum of F6, (b) MS spectrum of 1,2-diamino-9,10-Antrasenediona derived from NIST Library.

CONCLUSION

Isolation of endophytic fungi from kayu kuning stem [*A. flava* (L) Merr.] from Sambas (West Kalimantan) yielded 9 endophytic fungi isolates which were chemotaxonomically classified into into six group. Fraction 6 (F6) of fungus AFK-8 extract had the ability to inhibit the growth of several bacteria and fungi isolates better than comersial antibiotic. The molecular structure of main constituent of fraction 6 was proposed as 1,2-diamino-9,10-antrasenediona with a molecular formula of $C_{14}H_{10}N_2O_2$. Further study to determine its absolut chemical structure need to be done by other spectroscopic analysis.

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