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ANTIBACTERIAL ACTIVITY OF RED PIGMENT ISOLATED FROM COASTAL ENDOPHYTIC FUNGI AGAINST MULTI-DRUG RESISTANT BACTERIA

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ABSTRACT

Multidrug-resistant (MDR) bacteria infections become a serious problem for these several decades. To solve this issue, finding of new antibiotics candidate in an urgency. Natural pigment is known to has biological activity against pathogenic bacteria. Coastal fungi are unexplored source of natural pigment to fight MDR bacteria. This research was aimed to isolate coastal endophytic fungi from smooth ant plant (*Hydophytum formicarum*), to screen endophytic fungi which produce red pigment, to extract the red pigment, to determine antibacterial activity of the red pigment and to identify the coastal endophytic fungi producing the red pigment. In this study, 7 fungi were isolated as endophytic fungi from *H. formicarum*. There were 3 isolates which produced extracellular pigment i.e. RS 1A which produced red pigment, RS 3 produced black pigment and RS 6A produced yellow pigment. Our study focused on red pigment which is produced by endophytic fungus strain RS 1A. The yield of red pigment was 8.8657% (w/w). This study showed that red pigment had antibacterial activity against *Escherichia coli*, *Acinetobacter baumannii* and *Proteus mirabilis* strain MDR. Judging from molecular and morphological identification, the endophytic fungus strain RS 1A was identified as *Aspergillus versicolor*.

Keywords: Antibacterial, endophytic fungi, MDR, pigment

INTRODUCTION

Bacteria having resistance to several antibiotics used to treat the infections are referred to as Multidrug-Resistant (MDR) bacteria (Cornaglia 2009; Magiorakos *et al.* 2012). MDR is a serious problem for medical world and public health. Several bacteria already reported as MDR are *Escherichia coli*, *Staphylococcus* spp., *Acinetobacter baumanni* and *Proteus mirabilis* (Ahmed *et al.* 2015; Korytny *et al.* 2016; Panda *et al.* 2016). Therefore, it is urgent to find new antibiotics. As a megabiodiversity and maritime country, Indonesia has many coastal resources which are potential to be sources of new antibiotics against MDR bacteria. Among coastal resources, smooth ant plant (*Hydnophytum formicarum*) is an neglected source for new antibiotic candidate. *H. formicarum* is a member of myrmecophytes plant which provides nesting cavities for ants (Lok & Tan 2009; Defossez *et al.* 2009). This plant was found as epiphytic plant in *Avicennia* sp. (mangrove plant) in Sorong, Papua. The utilization of epiphytic plant as source of

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bioactive compounds is hindered by environmental issues. Exploration of endophytic fungi is expected to overcome these issues. Information related to the biodiversity of endophytic fungi from *Hydnophytum formicarum* is rarely found. The first report about associated fungi from genus *Hyndophytum* was reported in 1911 (Defossez *et al.* 2009). Therefore, our study will add more information about endophytic fungi from *Hydnophytum formicarum*.

Coastal endophytic fungi are commonly reported as potential source of bioactive compounds. However, there is rare report regarding its anti-multidrug resistant bacteria activity. Endophytic fungi refer to microfungi which live inside plant tissues without causing any symptoms to the host (Thirunavukkarasu *et al.* 2015; Sibero *et al.* 2016a). In addition, Kusari *et al.* (2013) stated that endophytic fungi could produce the same or similar bioactive compounds like its hosts. Endophytic fungi are known to produce metabolites with various biological activities (Meng *et al.* 2015; Wong *et al.* 2015; Cao *et al.* 2016; Rahaweman *et al.* 2016; Sibero *et al.* 2016a). In contrary, fungal pigment is a neglected metabolite even though several researchers reported its bioactivity as antibacterial agent (Geweely 2011; Mani *et al.* 2015; Patil *et al.* 2015). Our study was aimed to isolate coastal endophytic fungi from smooth ant plant (*H. formicarum*), to screen endophytic fungi which produce red pigment, to extract the red pigment, to determine antibacterial activity of the red pigment and to identify the coastal endophytic fungi producing the red pigment.

MATERIALS AND METHODS

Sampling Preparation

Smooth ant plant (*H. formicarum*) was collected by Dr Kustiariyah Tarman, as epiphytic plant on *Avicennia* sp. in mangrove forest located in Sorong, West Papua Province (Fig. 1). The domatia part (hollow structure part) of smooth ant plant was collected by cutting the plant from the host plant using metal cutter. The plant was then put inside zipped plastic bag. The sample was taken to the Laboratory of Aquatic Microorganisms, Department of Aquatic Products Technology, Faculty of Fisheries and Marine Science, Institut Pertanian Bogor for isolating the endophytic fungi.

Fungal Isolation, Cultivation and Pigment Screening

Endophytic fungal isolation was carried out using surface sterilization method (Kjer *et al.* 2010). *H. formicarum* was cut approximately 1 cm^2

Figure 1 *Hydnophytum formicarum* as epiphytic plant on *Avicennia* sp. (mangrove tree) found in mangrove forest in Sorong, West Papua Province

and washed with distilled water followed by 70% ethanol (EtOH) for 1 minute and then re-washed with distilled water. After that, the pieces of samples were placed on Potato Dextrose Agar (PDA) without the addition of antibiotics and were incubated at 28 °C until growth was initiated. During isolation, a petri dish with PDA was left open as environmental control. After 7 days, there were several fungi growth on the PDA media. The fungus which grown nearby the sample but not found in environmental control petri dish was separated and placed into new PDA media as single colony. Each single colony was cultivated on PDA for 7 days at room temperature (27 – 28 °C). Every day the color change of media was observed. The fungus producing red pigment in PDA media was used for this research.

Pigment Extraction

Pigment was extracted using solid liquid extraction (Manikkam *et al.* 2015; Sibero *et al.* 2016b). PDA media were separated from the mycelia using sterilized metal cutter. The PDA media were then weighed and chopped until the segments became smaller and placed into Erlenmeyer flasks. A quantity of 100 mL acetone was poured into the flasks and shaken using shaker for 24 hours at 27 °C. The contents of the flasks were filtered through filter paper (Macherey-Nagel 640d·Ø 1125 mm). The filtrates were concentrated using rotary evaporator (30 – 33 °C, 30 minutes). The yield was obtained according to the following formula:

$$\%\ Yield = \frac{Yield}{PDA\ weight} \text{ x } 100\%$$

Antibacterial Activity

Antibacterial assay was conducted based on Sibero *et al.* (2016b) with several modifications. The pigment extracts were used to test against clinical MDR gram-negative bacteria, including *Acinetobacter baumannii*, *Escherichia coli* and *Proteus mirabilis* with two replications. These bacteria were clinical isolates and considered as MDR strains from RSUP Dr Kariadi (Dr Kariadi General Hospital Medical Center) and Rumah Sakit Nasional Diponegoro (Diponegoro National Hospital), both located in Semarang, Central Java Province, Indonesia. Gram negative bacteria were refreshed on MacConkey for 24 hours at 37 °C. Antibacterial assay was carried out using paper disk. The extracts concentrations were 50 µg/mL, 100 µg/mL, 250 µg/mL, 350 µg/mL and 500 µg/mL. Antimicrobial susceptibility disk for *Amoxicillin* (AML) 10 µg (Oxoid™) was used as positive control, while acetone was used as negative control. The bacteria turbidity standard was equivalent to a 0.5 McFarland. The bacteria were inoculated on Muller Hinton Agar (MHA) using cotton swab with rotation inoculation. After that, the positive control was placed in the middle, while the negative control and paper disks with extracts were placed around the positive control by forming a circular pattern and incubated at 36 – 37 °C for 24 h. The results of antibacterial assay were analyzed using factorial Analysis of Variance (ANOVA) with SPSS software. The confidence interval was 95%, while the significant difference was analyzed using Duncan test.

Morphology Observation

Slide culture method was performed for fungus cultivation with modifications (Qiu *et al.* 2005; Sibero *et al.* 2016b; Sibero *et al.* 2017). PDA media was prepared and cut approximately $2 \times 2\ cm^2$ and placed on sterilized object glass. The mycelia were inoculated on each side of the PDA and a cover glass was placed on the PDA, then put into sterilized petri dish for incubation. After 3 days, each side of PDA had been overgrown by the fungus and the mycelia already covered the inner side of the cover and object glass. The PDA was then removed, while the object and cover glasses were observed under a compound microscope. Morphological characteristics of the fungus were compared to Huh *et al.* (2013), Visagie *et al.* (2014) and Ama (2016).

Molecular Identification

Chelex method with several modifications was used for DNA extraction (Sibero *et al.* 2017). The red pigmented fungus was cultured on PDA for 7 days. Mycelium of the red pigmented fungus was taken and put into Eppendorf microtube, added with 100 µL ddH_2O and 1, 000µL of 0.5% saponin, then kept for overnight. After that, the mixture was centrifuged (12,000 rpm, 10 minutes at 5 °C). Supernatant were discarded, then 100 µL ddH_2O and 50 µL of 20% chelax 100 were added. The mixture was kept on water bath (80 °C, 10 minutes). In the first 5 minutes, the mixture was

mixed using vortex. The final mixture was centrifuged (12,000 rpm, 10 minutes at 5 °C). The supernatant was taken and stored at -20 °C.

The Internal Transcribed Spacer (ITS) region of fungus were amplified by PCR using universal primers internal transcribed spacer (ITS) 1 (5'-TCC GTA GGT GAA CCT GCG G-3') as forward and ITS 4 (5'-TCC TCC GCT TAT TGA TAT GC-3') as reverse (Sibero *et al.* 2017). PCR mixture consisted of GoTaq Green Master Mix Promega (12.5 µL), primer ITS 1 (1 µL), primer ITS 4 (1 µL), DNA extract (0.5 µL) and ddH_2O (10 µL). The PCR reaction condition consisted of preheat at 95 °C for 3 minutes, denaturation at 95 °C for 1 minute, annealing (gradient from 51.4 °C to 67.4 °C) for 1 minute, extension at 72 °C for 1 minute, final extention at 72 °C for 7 minutes and holding temperature at 16 °C. Denaturation, annealing and extension stages were performed for 30 cycles. The PCR product was loaded in agarose gel (1%) electrophoresis. The product of electrophoresis was visualized by UVI Doc HD5 (UVITEC Cambridge).

PCR product which performed clear DNA band was used for DNA sequencing. This stage was conducted at 1st Base Laboratories Sdn Bhd, Malaysia. The sequence was compared and aligned by Basic Local Alignment Search Tool (BLAST), then analyzed to its homology to other fungi obtained from Gene Bank. This research used neighbor-joining for statistical method, bootstrap method for test of phylogeny with number of bootstrap replications was 1,000. The phylogenetic analysis was constructed using MEGA 7 software package (Tamura *et al.* 2011).

RESULTS AND DISCUSSION

Endophytic Fungi from *H. formicarum*

There were seven fungi isolates which were successfully isolated from *H. formicarum* (Fig. 2). Each fungus had different macroscopic characteristics based on colony forms, mycelia colors and reverse media colors. Biodiversity of culturable fungi is influenced by nutrient content in media and isolation method (Kjer *et al.* 2010; Toma & Abdulla 2013). Surface sterilization is the most important procedure in endophytic fungal isolation. The aim of surface sterilization using ethanol 70% is to eliminate spores and other microbial contaminants attached to the sample surface (Kjer *et al.* 2010). Environmental control petri dish is also important to minimize the possibility of improper isolation. Fungi grown in environmental control petri dish and sample petri dishes were suspected as contaminant.

From endophytic fungal isolation, there were seven fungal isolates obtained (Fig. 2). Each fungus has different morphological characteristics. Among the seven endophytic fungi isolated, there were three fungal isolates produced extracellular pigment. They were RS 1A which produced red pigment, RS 3 produced black pigment and RS 6A produced yellow pigment. Pigment production was shown by the color change of the medium. Sibero *et al.* (2016[a]) successfully characterized black pigment from RS 3 as melanin.

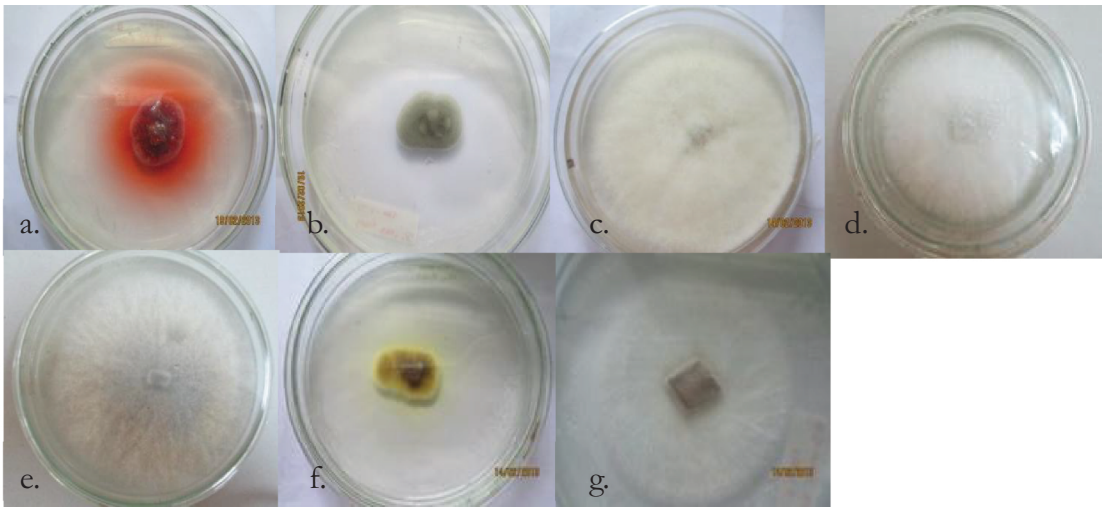

Figure 2 Endophytic fungal isolates obtained from *Hydnophytum formicarum* grown on PDA a) RS 1A; b) RS 1B; c) RS 2A; d) RS 2B; e) RS 3; f) RS 6A; g) RS 6B

Nutrient content in media has important role in fungal pigments. Mugesh *et al.* (2014) stimulated biopigment production of several endophytic fungi by diversifying growth media. As a result, the MECV01 fungus isolate produced red biopigment on Czapek-Dox Agar (CDA) and Czapek-Dox Yeast Autolysate Agar (CYA). On Malt Glucose Yeast Peptone Agar (MGYP) and Yeast Glucose Trace (YGT) media, the MECV01 fungus isolate produced yellowish red biopigment. Mugesh *et al.* (2014) stated that carbon source (sucrose) and trace elements gave impact to growth and pigment production.

Our study focused on red pigment needed in many industries such as food, textile and cosmeceutical industries. Based on macroscopic observation, fungus RS 1A had green colony and grew well at room temperature (28 °C). Green color was produced by the colony, while the mycelia had white color. Fungus RS 1A produced extracellular red pigment since the fourth day of being on PDA media at room temperature (28 °C). Production of red pigment increased and the pigment was released not only to the PDA media, but also to the mycelia (Fig. 3). Red pigment from this fungus was judged as extracellular pigment, because it was released outside the cells. Several fungi produced extracellular pigments and known to have biological activity (Dong & Yao 2012; Mani *et al.* 2015; a; Sibero *et al.* 2016b).

Antibacterial Activity of Red Pigment Produced from Fungus RS 1A

Extracellular pigment is extracted from media because it is spread outside the cells (Xiong *et al.* 2015; Akilandeswari & Pradeep 2016). Pigment production depends on several conditions, such as nutrient in media, light intensity, pH, trace elements, temperature and agitation (Mugesh *et al.* 2014; Bühler *et al.* 2015; Patil *et al.* 2015; Shi *et al.* 2015). This pigment was extracted directly from solid media and performed by maceration method with acetone. The yield of red pigment from fungus RS 1A was 8.8657 % (% w/w). Organic solvent such as methanol, acetone, chloroform and ethyl acetate are commonly used to extract natural pigment (Robinson *et al.* 2014; Vora *et al.* 2015). The pigment was tested against several MDR bacteria. Results of antibacterial assay are presented in Table 1 and Figure 4.

Enterobacteriaceae members are known as gram negative pathogenic bacteria such as *E. coli*, *Proteus*, *Salmonella* and *Shigella* (Shaikh *et al.* 2015; Dutta *et al.* 2016; CLSI 2016). These pathogenic bacteria cause urinary tract infection, nosocomial infection, blood stream infection and meningitis, causing death to human (Harrish *et al.* 2015; Shaikh *et al.* 2015; Iqbal *et al.* 2016). Based on the result of antibacterial assay, red pigment had activity to combat clinical MDR gram-negative bacteria. The best antibacterial activity was performed at concentration 500 μg/mL against MDR *E. coli* with inhibition zone of 19.8±1.13 mm. We highlighted the diameter of inhibition zone of Amoxicillin against *E. coli* and *P. mirabilis*. Amoxicillin had inhibition zone ≤ 13 mm. Therefore, according to CLSI (2016) these bacteria were resistant to Amoxicillin. Wong *et al.* (2013) and Dutta *et al.* (2016) successfully isolated and characterized *E. coli* from hospital and *P. mirabilis* from chicken carcasses as MDR which

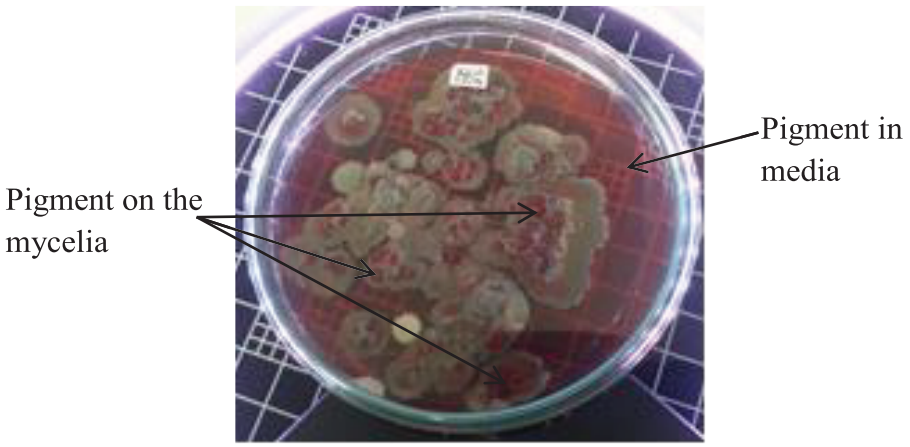

Figure 3 Fungus RS 1A after ten days of cultivation on PDA

Table 1 Antibacterial activity from fungal red pigment RS 1A against MDR bacteria

MDR bacteria	Concentration of red pigment extract (μg/mL)	Inhibition zone (mm)
Acinetobacter baumannii	50	4.0±0.42[a]
	100	4.25±0.35[a]
	250	4.6±0.00[a]
	350	5.25±0.63[ab]
	500	6.75±0.91[ab]
	Amoxicillin 10 μg	12.00±0.00[c*]
Escherichia coli	50	5.80±0.28[a]
	100	6.35±0.49[a]
	250	7.05±0.49[a]
	350	7.45±0.07[a]
	500	19.8±1.13[c*]
	Amoxicillin 10 μg	10.60±0.00[b]
Proteus mirabilis	50	5.15±2.1[a]
	100	6.15±0.63[ab]
	250	6.55±0.35[ab]
	350	6.85±0.21[ab]
	500	9.15±0.21[b]
	Amoxicillin 10 μg	12.00±0.00[c*]

Note: Data presented are mean±SD Values at the same column followed by the same letters are not significantly different at $p < 0.05$
Values with * shared letters denote significant difference at $p < 0.05$

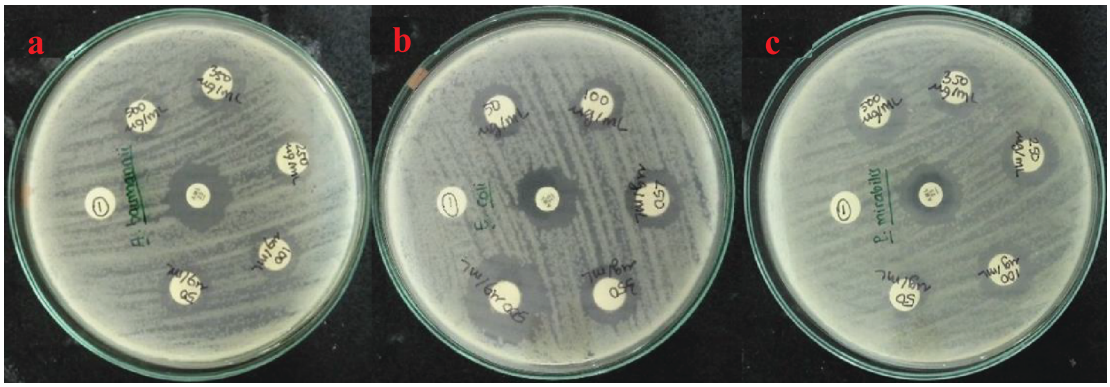

Figure 4 Inhibition zones of red pigment produced from fungus RS 1A against MDR bacteria: (a) *Acinetobacter baumannii*; (b) *Escherichia coli*; (c) *Proteus mirabilis*

resistant to Amoxicillin using CLSI standard. Somwanshi and Bodhankar (2015) reported that the endophytic fungi had antibacterial activity against MDR human pathogens such as *Escherichia coli, Acinetobacter baumannii*, *Salmonella typhi* and *Klebsiella pneumoniae*. On the other hand, Sibero *et al.* (2017) used marine fungi against *E. coli* strain MDR. In addition, Zhao et al. (2016) reported that *Monascus* pigment had activity against *E. coli* with MIC 2.5 mg/mL.

Fungus Identification

Fungus identification was carried out through molecular and microscopic observation approaches. For molecular identification, determination of optimum annealing temperature was conducted at temperature range from 51.4 °C to 67.4 °C in PCR.

Annealing temperature has important role on the success of PCR products. If the annealing temperature is too low, it will cause the amplification of non-specific DNA fragments. If the annealing temperature is too high, it will reduce the purity of PCR product (Rychlik *et al.* 1990). Figure 5 shows the result of PCR products visualization in agarose gel.

DNA of fungus RS 1A was well amplified from 51.40 to 60.2 °C. Temperature of 51.40 °C showed the brightest band in visualization. The

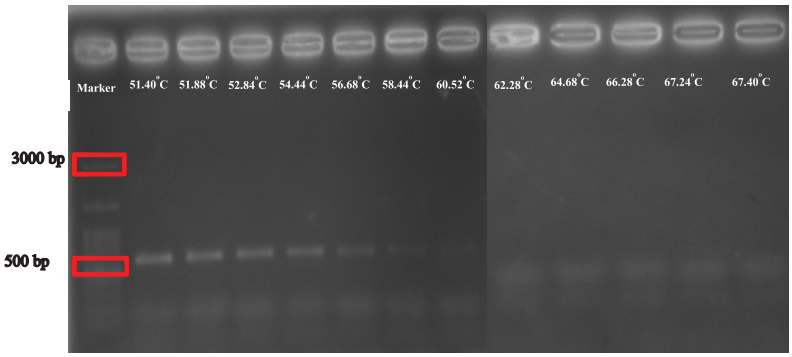

Figure 5 PCR products visualization from determination of annealing temperature

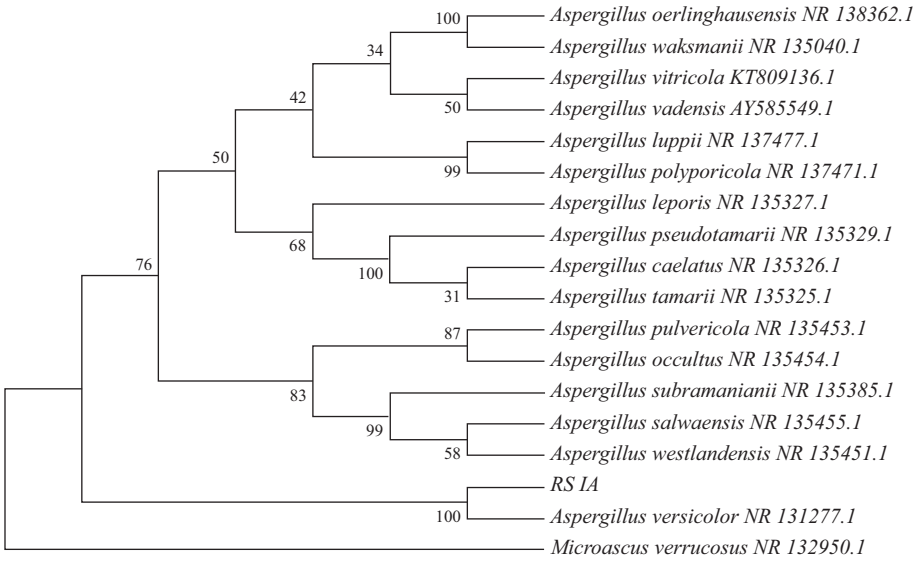

Figure 6 A cladiogram resulted from neighbor-joining tree of fungus RS 1A obtained from the ITS rDNA sequences analysis

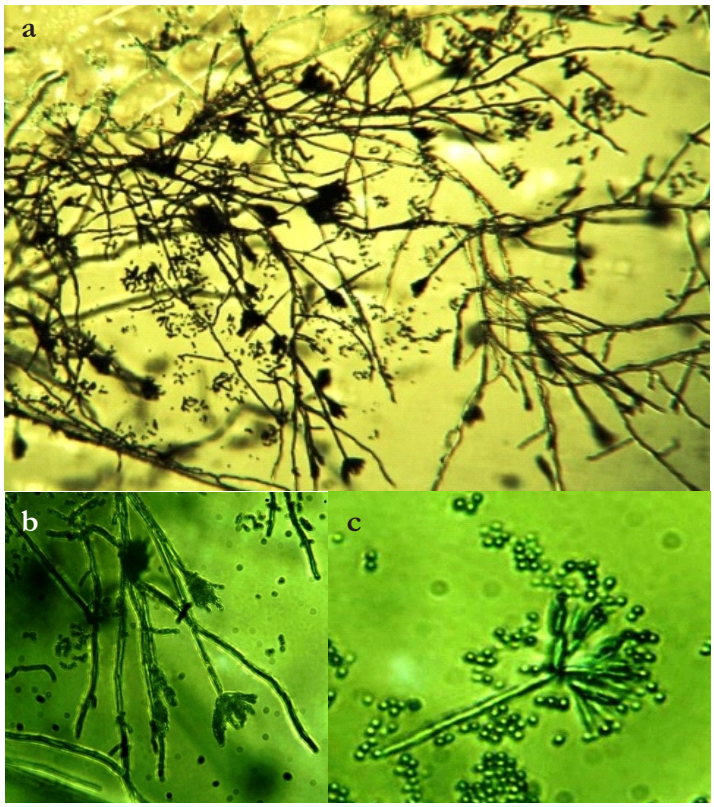

Figure 7 Microscopic morphology of fungus RS 1A identified as *Aspergillus versicolor*
(Note: a. Mycelia; b. Conidiophore; c. *Penicillium*-like conidiophore with conidia)

annealing temperature of fungal DNA is varied and depended on the species, primers and PCR instrument. Other research successfully amplified fungal DNA with annealing temperature of 40, 47 and 54 °C (Choo *et al.* 2015; Kramer *et al.* 2016; Krishnan *et al.* 2016).

According to the homology comparison, fungus RS 1A was closed to several strains of *Aspergillus versicolor*. This fungus had 99% nucleotide similarity to *A. versicolor* strain NR 131277.1 which was done by Haugland *et al.* (2016). Phylogenetic relationship of this fungus is shown in Figure 6. The result of microscopic morphological characterization of fungus RS 1A is shown in Figure 7.

Based on the microscopic morphology characterization, fungus RS 1A had smooth conidiophore without any branches, biseriate phiallides and produced round conidia. This fungus produced unique *Peniciliium*-like conidiophore (Fig.7c). *A. versicolor* is known to produce *Penicillium*-like conidiophores. This conidiophore had phialides which attached to stipe. These conidiophores are vegetative hyphae with very short stipe. The production of conidia using *Penicillium* like-head was faster than the production of conidia by *Aspergillus*-head (Klich 1993; Ama 2016). The production of this unique conidiophore usually causes misidentification for morphological characterization. Ama (2016) successfully showed the production of *Penicillium*-like head started in the first day of incubation.

A. versicolor has been reported as an endophytic and associatic fungi in plants and animals (Zhuang *et al.* 2011; Hawas *et al.* 2012). Hawas *et al.* (2012) isolated new metabolite name *Isorhodoptilometrin-1-methyl ether* from endophytic *A. versicolor* with antibacterial activity against *Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus*. In 2013, a new alkaloid named *Asperverin* was isolated from an algicolous *A. versicolor*. In addition, *A. versicolor* has been reported as endophytic fungi in *Paris polyphylla* var. *yunnanensis* and produced new *butyrolactones versicolactones E-F (*Zhou *et al.* 2016). Yan *et al.* (2016) reported this fungus was isolated from the mud of deep water in South China Sea and had antioxidant property. Wang *et al.* (2017) discovered a new antimicrobial compound from deep sea sediment which was *A. versicolor* named *2-(dimethoxymethyl)-*

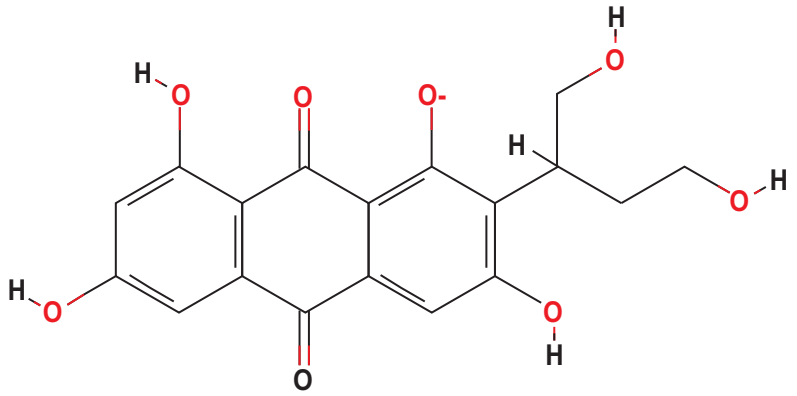

Figure 8 Chemical structure of *versiconol* produced by *A. versicolor* (Source: NCBI 2009)

1-hydroxyanthracene-9,10-dione. Several studies proved that *A. versicolor* produced pigments (Jurjevic *et al.* 2012; Ama 2016). Red pigment from *A. versicolor* has been discussed since 1960s. In 1967, Hamasaki *et al.* (1967) reported three anthraquinoid pigments with red color. Three years later, Hatsuda *et al.* (1969) isolated a new orange red pigment named *versiconol* (Fig. 8).

CONCLUSIONS

This research successfully isolated seven endophytic fungi from epiphytic plant *H. formicarum*, from which only fungus RS 1A produced the red pigment. The yield of red pigment was 8.8657% (w/w). Red pigment had antibacterial activity against clinical MDR gram-negative bacteria especially *E. coli*. According to morphological and molecular identification, fungus RS 1A was identified as *Aspergillus versicolor*.

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