Antioxidant, Antibacterial and Antibiofilm Activities of an Endophytic Fungi, *Penicillium* Sp. SAF6-EGY Strain AFL.2 and *Arthrinium* Sp. R22-1 Strain AFL.3 Isolated from *Aglaia Foveolata*

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Abstract. Endophytic fungi are widely underexplored in the discovery of novel bioactive compounds with promising pharmaceutical uses. *Aglaia foveolata*, which has important medicinal value, was investigated for its endophytic fungi to produce bioactive compounds by determining antioxidant, antibacterial, and antibiofilm activities. In this study, two endophytic fungi were collected from *A. foveolata* leaves and identified phenotypically along with genotypically by Internal Transcribed Spacer (ITS) gene sequence and known as *Penicillium* sp. SAF6-EGY strain AFL.2 and *Arthrinium* sp. R22-1 strain AFL.3. These fungal cultures were further grown on Yeast Malt (YM) medium and the culture was extracted by ethyl acetate. The ethyl acetate extract of AFL.2 and AFL.3 showed relatively similar in Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) ranging from 114.71 to 119.42 mg GAE/g extract and 74.09 until 76.31 mg QE/g extract for TPC and TFC, respectively. Further, AFL.3 showed the highest antioxidant activity in 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) scavenging at IC_{50} value of 123.41 and 256.95 μg/mL, respectively. As for antibacterial activity, AFL.2 exhibited the strongest activity against *Eschericia coli* (acrB JW0451-2) and *Staphylococcus aureus* (Newman) with the same MIC value of 250 μg/mL. Meanwhile, AFL.3 showed the best antibacterial activity against *Bacillus subtilis* (DSM10) and *S. aureus* with the same MIC value of 500 μg/mL. In addition, AFL.2 showed the best antibiofilm activity against *E. coli*, while AFL.3 against *B. subtilis* biofilm with the inhibition value of 42 and 35%, respectively. Liquid Chromatography Quadrupole-Mass Spectrometry Analysis (LC-MS/MS) revealed that both AFL.2 and AFL.3 extract contained several putative antibacterial compounds which are correspond to its promising activity.

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1 Introduction

Endophytic microorganisms are popular as a group of microbes including bacteria, fungi, yeasts, or viruses that live in mutualistic symbiosis inside plant tissues. These microorganisms benefit by obtaining a place to live and nutrients, while plants are gained for some aspects including the production of phytohormones or active compounds from microorganisms that can induce plant defense mechanisms against phytopathogens or environmental stresses [1]. One of the types of endophytic microorganisms reported as the main source of bioactive compounds is fungi. Historically, the discovery of drugs shows a phenomenal insight in the discovery of anticancer drugs, namely Taxol compounds isolated from the *Taxus brevifolia* plant, as well as from its the endophytic fungi *Taxomyces andreanae*. Simultaneously, various types of compounds from endophytic fungi from numerous plants were reported to have several bioactivities such as fumigaclavine C from the fungus *Aspergilus* sp. (antimicrobial), solamargine from *A. flavus* (cytotoxic), aspernidine from *Emericella* sp. (antivirus H_1N_1), extract from *Talaromyces* sp. (anti-inflammatory), and epoxycytochalasin H from *Diaporthe* sp. (antimalarial) [2].

Aglaia foveolata is one of the endemic medicinal plants from Kalimantan which belongs to the Meliaceae family. This plant grows wild in the Kerangas forest area of Kalimantan and has been used for generations by the community as building materials, food, and traditional medicines such as healing wounds, headaches, and fragrances [3]. *A. foveolata* is known to produce various active compounds that have various pharmacological functions. One of popular compounds is Silvesterol which belongs to the rocaglamide or flavaglines and was reported to have various activities such as anticancer, bioinsecticide, and antiviral [4]. Even though, it has potential bioactivities, the use of Silvestrol or other class of compounds isolated from *A. foveolata* has several obstacles such as the small ratio of the number of active compounds and the limitation of raw materials. Of note, if overexploited, it will threat the sustainability of the ecosystem. Therefore, other strategies need to be carried out, one of which is using the endophytic fungi. Based on this background, this study was designed to investigate the potential antioxidant, antibacterial and antibiofilm activities derived from active compounds produced by 2 endophytic fungi isolated from *A. foveolata* leaves.

2 Materials and Method

2.1 Endophytic Fungal Collection and Identification

The endophytic fungi used in this study encode AFL.3 and AFL.3 were obtained from previous studies isolated from the leaves of *A. foveolata* (taken from the Kerangas forest, East Kalimantan, Indonesia; GPS location: 1.017039, 114.113809) (Unpublished data). Both AFL.2 and AFL.3 were then re-cultured in Yeast Malt Extract (YM) media, then identified macroscopically, microscopically using a 400x magnification microscope

(Olympus) along with molecular identification. Briefly, genomic DNA of two isolates was extracted using Invisorb Spin Plant Mini Kit (USA). Universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3', forward) and ITS4 (5' TCC TCC GCT TAT TGA TAT GC-3', reverse) were used for amplification of the fungal ITS region [5]. The PCR products were sequenced and aligned to the database of National Center for Biotechnology Information (NCBI) using the BlastX program (https://blast.ncbi.nlm.nih.gov/).

2.2 Fermentation and Extraction of Endophytic Fungal Bioactive Compounds

Endophytic fungi were cultured in 500-ml Erlenmeyer flasks containing 100 mL of YM broth. In short, three agar plugs of 6-mm diameter from a 7-day YM plate were used as inoculum. Flask fermentations were incubated at 27 °C under orbital agitation of 120 rpm for 28 days. After the end of the culture period, the mycelium and liquid cultures were mixed for further extraction procedures. Extraction was conducted using liquid: liquid extraction method with ethyl acetate as the solvent. Briefly, samples were placed onto liquid solvent with the ratio of 1:1 (samples: solvent): 100 mL sample on 100 mL ethyl acetate and shaken at high speed (250 rpm) at the room temperature for 2 h. Subsequently, solvent was separated and evaporated to obtain crude ethyl acetate extracts.

2.3 Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and Antioxidant Assays

Quantitatively TPC and TFC were examined according to the Folin-Ciocalteu colorimetric and aluminum chloride colorimetric techniques following the corresponding methods as described elsewhere [6]. Furthermore, as for antioxidant activity was conducted using two types of radicals namely DPPH and ABTS following previous method [7]. The absorbance of both DPPH and ABTS assays was examined using Thermo Scientific Varioskan Flash (ThermoFischer) at 515 nm and 734 nm, respectively. The inhibition values were calculated using the formula of % inhibition: $((A1 - A2)/A1) \times 100\%$, which A_1 represent the absorbance of DPPH/ABTS blank (without samples) and $A_2 =$ the absorbance of samples. The results are further reflected as inhibitory concentration of 50% (IC_{50}).

2.4 Antibacterial Activity by Disc Diffusion Method

The preliminary screening of antibacterial activity of the endophytic fungal extracts was determined using standard the agar-disc diffusion method. Two gram-negative bacterial strains: *Escherichia coli* (BW25113), and *Pseudomonas aeruginosa* (PA114), along with and two gram-positive bacterial strains: *Staphylococcus aureus* (Newman) and *Bacillus subtilis* (DSM10) were used as bacterial indicator. The dried extracts of each fungal isolate were dissolved in 1% dimethyl-sulphoxide (DMSO). The bacterial cultures were spread evenly using sterile cotton buds for three times. Disc of 6 mm diameter were impregnated on the plates after 10 μl of 4 mg/mL of fungal extract was added to the

disc. 1% DMSO was used as negative control and Tetracycline (200 μg/mL) (Sigma) was used as a positive control. The plates were incubated at 30 ± 2 °C overnight. The zones of inhibition were observed and measured.

2.5 Determination of MIC and MBC Values

MIC value was examined using a standard micro-dilution assay with slight modifications [8]. Briefly, each of 96 sterile well plate was filled with Muller Hinton Broth (MHB). Further, 4 mg/ml stock solution of fungal extract in 1% DMSO was serially diluted. Each concentration of extract was mixed with bacterial culture in equal volume to make a total volume of 200 μL. Each bacterial cells were set up in 0.85% NaCl sterile and adjusted to McFarland standard 0.5 which is equivalent to 1×10^8 CFU/mL. Tetracycline and 1% DMSO were used for positive and negative control, respectively. All treatments were incubated at 30 °C overnight. The MIC of fungal extracts was determined by observing the clarity of the well. As for MBC value was determined to be the lowest concentration of the extract that could suppress 100% the bacterial growth observing on plate medium.

2.6 Antibiofilm and Eradication of Bacterial Biofilm

Antibiofilm assay was performed using the standard method on sterile 96 well plates with slight modifications [8]. The test was carried out by preparing the test bacteria culture at a concentration of 0.5 Mc Farland. The extract was prepared by dissolving in 1% DMSO at 4 different concentrations, including 1/4, 1/2, 1x and 2x fold of MIC value. Then each well was further filled with Brain Heart Infusion (BHI) medium and the extract was added according to the desired concentration. Then 100 μL of bacterial culture was added to a final volume of 200 μL and incubated for 24 h at 37 °C. Each well was then added with 200 μL of 0.1% crystal violet and then incubated for 30 min at 37 °C. Absorbance indicating biofilm formation was measured at a wavelength of 595 nm using Thermo Scientific Varioskan Flash (ThermoFischer) Elisa reader. In addition, the biofilm cell eradication assay was carried out using the same method as the biofilm formation test above as described elsewhere [8].

2.7 Liquid Chromatography Quadrupole-Mass Spectrometry (LC-MS/MS) Analysis

Analysis of the compounds profile contained in the endophytic extract was carried out using LC-MS/MS analysis. We utilized Xevo G2-XS QTof (Quadrupole Time-of-Flight) mass spectrometry instrument (Waters, USA) via an electron spray interface (ESI). Further, chromatographic separation was conducted using an LC system in the form of Ultra Performance Liquid Chromatography (UPLC)/Q-Tof MS analytical system (Waters). As for separation of compounds was set by stepwise gradients from 95% A (0.1% formic acid + distilled water) and 5% B (acetonitrile + 0.1% formic acid) to 5% A and 95% B for 16 min. The accurate mass and composition for the precursor ions and fragment ions were identified using the UNIFI software library and the natural products atlas services (https://www.npatlas.org/).

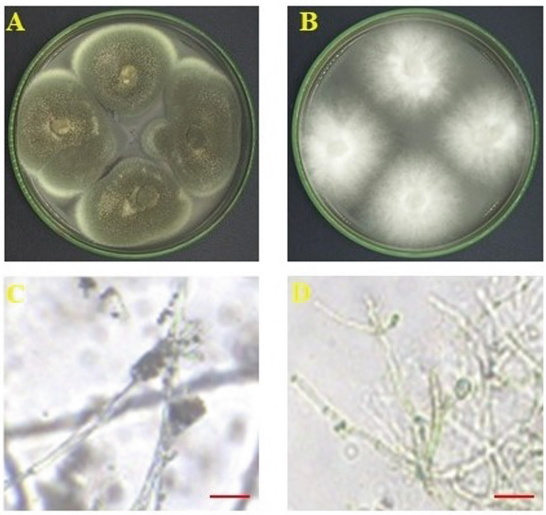

Fig. 1. Macroscopic and microscopic of endophytic fungi AFL.2 and AFL.3 on the YM agar plate after 7 days of incubation at 28 °C; A (macroscopic of AFL.2); B (macroscopic of AFL.3); C (microscopic of AFL.2); D (microscopic of AFL.3); magnification 400x; Bars represents 5 μm.

2.8 Statistical Analysis

All data were obtained as mean ± SEMs (3 replicates). Further analysis was determined using one-way Analysis of Variance (ANOVA) followed by multiple Duncan test range. A *p*-value of less than 0.05 was considered statistically difference.

3 Results

3.1 Identification of Endophytic Fungi

From a well-known medicinal plant *Aglaia foveolata*, we have collected 2 endophytic fungal from leaves encoding AFL.2 and AFL.3. The endophytes were identified in the genus level based on macroscopic, microscopy and molecular approach. The morphology of the endophytes was characterized based on taxonomically relevant features including colony morphology, pigmentation, growth pattern and sporulation structure, and colony color. From the observation, the endophytes were classified in genus Penicillium and Arthrinium for AFL.2 and AFL.3, respectively. All endophytes contained heavily intertwining hyphae. Cultures from genus Penicillium appeared olive green in colour, while genus Arthrinium appeared as white mycelium after 7 days in YM agar (Fig. 1).

The phylogenetic tree of fungal isolates is shown in Fig. 2. Both AFL.2 and AFL.3 were grouped into 2 genera: *Penicillium* sp. and *Arthrinium* sp. Of note, both AFL.2 and AFL.3 show the highest similarity for 100% with the genus of *Penicillium* sp. SAF6-EGY and *Arthrinium* sp. R22-1, respectively.

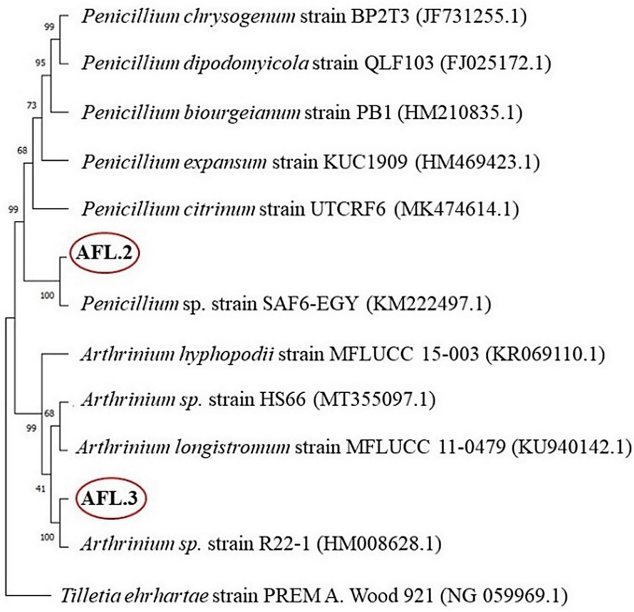

Fig. 2. Phylogenetic tree of AFL.2 and AFL.3 based on ITS1-4 rDNA genes sequences analysis.

Table 1. Total phenol, flavonoid, and antioxidant properties of AFL.2 and AFL.3 extracts.

Code	Total phenolic (mg GAE/g extract)	Total flavonoid (mg QE/g extract)	Antioxidant	
			DPPH IC_{50} (ug/mL)	ABTS IC_{50} (ug/mL)
AFL.2	119.42 ± 0.22	76.31 ± 0.79	685.12 ± 6.91	634.75 ± 10.75
AFL.3	114.71 ± 0.47	74.09 ± 6.01	123.41 ± 1.95	256.95 ± 3.72
Ascorbic acid	-	-	5.27 ± 0.71	18.93 ± 0.79

3.2 TPC, TFC and Antioxidant Activities of AFL.2 and AFL.3 Extracts

Furthermore, we determined the TPC and TFC. The results showed that both AFL.2 and AFL.3 relatively had similar in TPC and TFC contents ranging from 114.71 to 119.42 mg GAE/g extract and 74.09 to 76.31 QE/g extract, respectively. Moreover, as for antioxidant the highest antioxidant activity was shown by the crude extract of AFL.3 with an IC_{50} value of 123.41 and 256.95 μg/mL for DPPH and ABTS, respectively (Table 1).

3.3 Antibacterial Properties of AFL.2 and AFL.3 Extracts

AFL.2 extract exhibited the best activity against *E. coli* and *S. aureus* as indicated by clear zone formation with diameter of 24.26 mm and 18.37 mm, respectively. On the

Table 2. Antibacterial activity of AFL.2 and AFL.3 extracts

Code	Bacterial tested			
	E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
	Diameter of clear zone (mm) ± SD/MIC/MBC (μg/mL)			
AFL.2	24.26 ± 1.87/250/500	13.69 ± 2.73/500/750	15.11 ± 1.82/500/750	18.37 ± 2.74/250/500
AFL.3	15.89 ± 3.85/500/750	8.27 ± 3.85/1000/ > 1000	20.15 ± 3.17/500/750	18.15 ± 4.12/500/750
Tetracycline	25.36 ± 1.4/3.9/3.9	19.21 ± 2.79/3.9/7.8	26.83 ± 4.16/3.9/3.9	21.47 ± 2.19/3.9/3.9

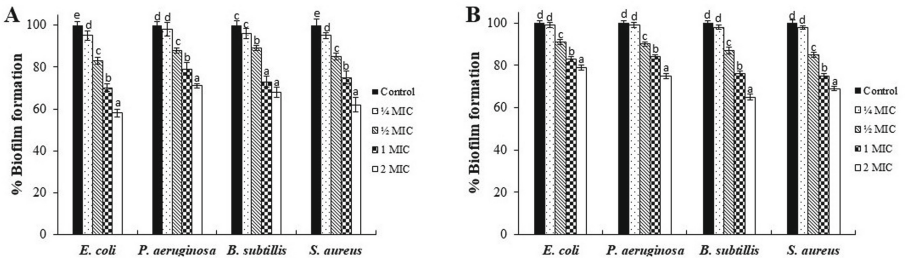

Fig. 3. Antibiofilm activity of AFL.2 and AFL.3 extracts against 4 microbial tested. Values with the same superscript letter are not significantly difference ($p < 0.05$)

other hand, AFL.3 isolate showed the highest activity against *B. subtilis* and *S. aureus* with clear zone diameter of 20.15 and 18.15 mm, respectively (Table 2). In addition, AFL.2 crude extract had the lowest similar MIC and MBC values towards *E. coli* and *S. aureus*. As for AFL.3 has the best MIC and MBC values against *E. coli*, *B. subtilis* and *S. aureus*. In general, AFL.2 has lower MIC and MBC values compared than AFL.3 crude extract focusing on *E. coli* and *S. aureus* with the MIC/MBC value of 250/500 μg/mL (Table 2).

3.4 Antibiofilm and Eradication of Bacterial Biofilm Growth Cells

The effect of endophytic fungal extract on inhibition of biofilm formation are shown in Fig. 3. AFL.2 extract exhibited the best antibiofilm activity against *E. coli* in two times of their MIC value. In this concentration, more than 40% of *E. coli* biofilm have been inhibited. As for AFL.3 extract showed the highest biofilm inhibition towards *B. subtilis* in two times of twice than MIC value with a biofilm inhibition of more than 30%.

In addition, both extracts exhibited the eradication activity with the same pattern as that seen for antibiofilm activity. The lowest percentage of viability of bacterial biofilm-grown cells was identified in 2 times of MIC value. Two times MIC value of AFL.2 and AFL.3 were effective in eradication against *E. coli* and *B. subtilis* cells-biofilm with the inhibition of 35 and 34%, respectively (Fig. 4).

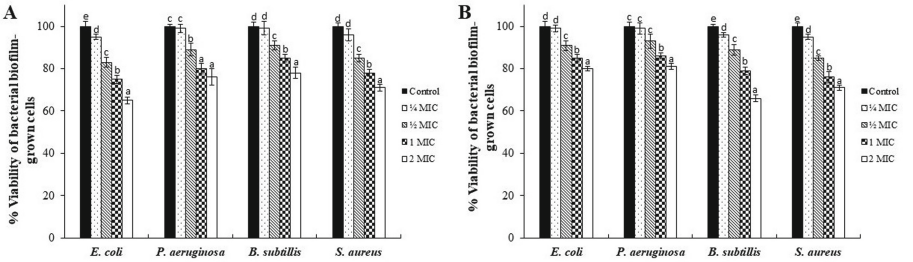

Fig. 4. Eradication of bacterial biofilm growth cells towards 4 microbial tested. Values with the same superscript letter are not significantly difference ($p < 0.05$)

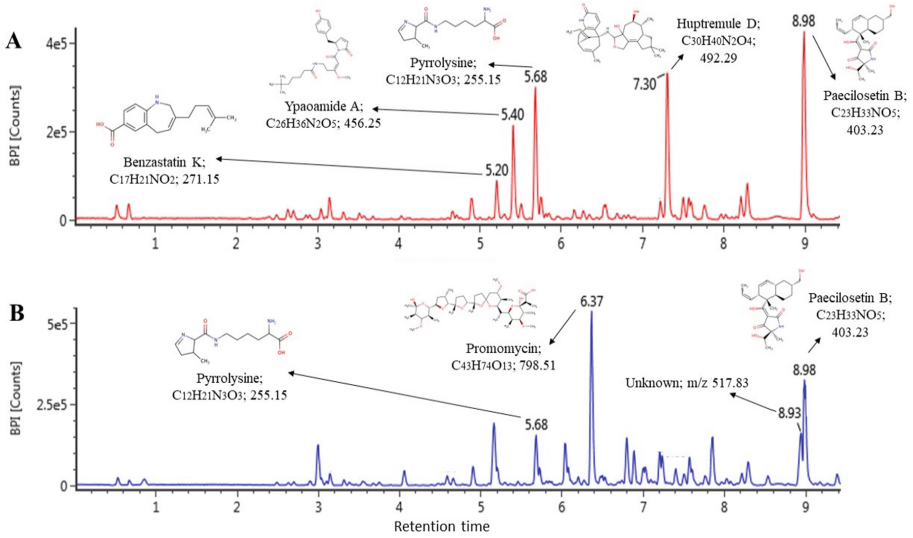

Fig. 5. LC-MS/MS profile of AFL.2 (A) and AFL.3 (B) extracts.

3.5 Chemical Constituents of AFL.2 and AFL.3 Extracts

We identified 8 predicted compounds from both extract which were dominant and clearly identified as shown in Fig. 5(A) and 5(B). Some putative compounds were identified in AFL.2 including benzastatin k, ypaoamide a, pyrrolusine, huptremule d and paecilosetin b. While pronomycin, pyrrolysine, paecilosetin b and unknown compounds were detected in AFL.3 extract (Fig. 5).

4 Discussion

The value of combining morphological characters and phylogenetics has been demonstrated in this current study to confirm the identification of an endophytic fungi strain both AFL.2 and AFL.3. The endophytic fungi AFL.2 and AFL.3 was found to be a homolog of type culture *Penicillium* sp. strain SAF6-EGY (KM.222497.1) and *Arthrinium* sp. strain

R22-1 (HM008628.1), respectively. Of note, the genus *Penicillium* sp. strain SAF6-EGY has been isolated from an agricultural soil in Egypt and reportedly as having potential cellulase/xylanase multifunctional enzyme for bio-finishing of textiles [9]. As for, *Arthrinium* sp. strain R22-1 has been isolated as endophyte from wild rice (*Oryza granulate*) in China, which has not been reported to have numerous potential bioactivities [10]. Therefore, in this study we are interested to investigate the potential pharmaceutical properties of bioactive compounds produced by endophytic fungi AFL.2 and AFL.3 in several patterns including antioxidant, antibacterial and antibiofilm.

DPPH and ABTS assay are a widely used method to evaluate the antioxidant activity of extracts. It measures the ability of samples to act as hydrogen donors or free radical scavengers and as a proton donor, respectively. These antioxidant compounds have important health effects including reducing heart disease, the risk of cancer, and neuro-degenerative disorders [11]. Our results showed that AFL.3 (*Arthrinium* sp. R22-1) extract had higher antioxidant activity than AFL.2 extract. Previous study reported that ethyl acetate extract of endophytic fungi *Arthrinium rasikravindrae* showed DPPH antioxidant activity with an IC_{50} value of 556.91 μg/mL [12]. Thus, our potential extract AFL.3 has antioxidant activity with an IC_{50} value belonging to a moderate level compared to other genus *Arthrinium* sp.

We further carried out an antibacterial assay followed by an antibiofilm and eradication of the cell biofilm. The results showed that AFL.2 (*Penicillium sp.*) had the strongest all those activities against gram-negative bacteria *E. coli*, while the extract of AFL.3 (*Arthrinium sp.*) has the best activity against gram-positive bacteria *B. subtilis*. Of note, gram-negative bacteria are mostly more resistant than gram-positive bacteria, therefore more difficult to eradicate, because of their outer membrane protects the peptidoglycan cell wall [13]. The results obtained in our study revealed the similar results from some previous studies derived from genus *Penicillium* sp. which also showed better activity against gram-negative bacteria. *Penicillium* sp., the endophytic fungi *Garcinia nobilis* which is a rich source of novel antibacterial compounds, especially in Gram-negative bacteria [14]. On the contrarily, based on previous references, genus *Arthrinium* sp. which related to AFL.3 isolates had antibacterial activity also against gram-negative bacteria such as *Arthrinium* sp. MFLUCC16-1053 which isolated from *Zingiber cassumanar* [15]. Thus, the results obtained in our study reveal the opposite, and this can be explained by the fact that the mode of action of the active molecules produced by AFL.3 isolate is not on the cell wall but might possesses a different mode of action.

Several compounds on AFL.2 extract, primarily pyrrolysine and paecilosetin b, were reported as having strong antibacterial activity [16, 17]. As for AFL.3 extract, some compounds including pyrrolysine, paecilosetin b, and promomycin also exhibited strong antibacterial activity [16–18]. These results suggested that these putative compounds in both AFL.2 and AFL.3 proven with strong antibacterial activity might affect to the antibiofilm and eradication of cells biofilm formation.

5 Conclusion

In conclusion both AFL.2 and AFL.3 endophytic fungal extracts can scavenge free radicals, promote antibacterial activity, and eradicate biofilm and cells biofilm formation.

These results would be essential for the development of the bioactive compounds derived from endophytic fungi, particularly as pharmaceutical uses. However, further research should be conducted to elucidate each of those potential compounds and retest the corresponding effect in the bacterial targeted, since reports of the activity from those compounds in relation to antibiofilm and eradication of cells biofilm are still limited.

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