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Endophytic *Bacillus* spp. isolated from *Archidendron pauciflorum*: Pharmacological property and their phytochemical constituents

Jepri Agung PRIYANTO[1] *, Muhammad Eka PRASTYA[2], Rika Indri ASTUTI [1],Minarti[2], Tjandrawati MOZEF [2]

[1] Division of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor, West Java, Indonesia

[2] Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research, and Innovation Agency (BRIN), Kawasan Sains dan Teknologi (KST) B.J Habibie (PUSPIPTEK) Serpong, South Tangerang, Banten, Indonesia

* Corresponding Author. E-mail: jepriyanto@apps.ipb.ac.id(J.A.P); Tel. +62-251-8622833.

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ABSTRACT: Endophytic *Bacillus* spp. associated with *Archidendron pauciflorum* could be the potential source of active compounds attributed to the pharmacological property. The present study aimed to determine the antioxidant, antibacterial, antibiofilm activities, and phytochemical profiles of the crude extracts derived from four endophytic *Bacillus* spp. isolated from *A. pauciflorum*. Among all extracts tested, extract from *Bacillus pseudomycoides* strain DJ8 displayed the strongest antioxidant activity against DPPH and ABTS with IC_{50} values of 24.87±1.81 and 9.18±0.10 µg/mL, respectively. As evaluated by the dual culture-agar diffusion and disc diffusion methods, three out of four *Bacillus* spp. extracts also exhibited broad spectra of antibacterial activity against four ATCC strains. Among other extracts, *B. pseudomycoides* strain DJ8 extract became the most active extract inhibiting *Staphylococcus aureus* strain ATCC 6538, indicated by the lowest MIC (31.25 µg/mL) and MBC (125 µg/mL), which is considered a highly active antibacterial agent. Three extracts attributed to broad spectra antibacterial activity were also capable to inhibit biofilm formation of four ATCC strains, up to 62.59% in 2 MIC of the extract concentration. Phytochemicals (flavonoid, alkaloid, and terpenoid) were present in four endophytic *Bacillus* extracts. Among other extracts, an extract derived from *B. pseudomycoides* strain DJ8 contains the highest flavonoid content (40.10±1.18 mg QE/g extract) and phenol (58.50±4.56 mg GAE/g extract). In conclusion, these four endophytic *Bacillus* spp. could be the source of pharmaceutically valuable compounds attributed to antioxidant, antibacterial, and antibiofilm properties.

KEYWORDS:Antibacterial; antibiofilm; antioxidant; *Bacillus*; endophyte.

1. INTRODUCTION

Endophytic bacteria are a facultative or obligate endosymbiotic group that inhabit plant tissues intercellularly or intracellularly without causing disease. The bacteria can enhance plant growth and protect the plant from biotic and abiotic stresses [1]. Among the endophytic bacterial group, *Bacillus* species is considered the prolific producer of active compounds, such as bacillomycin D [2], surfactin [3], fengycin[4], bacillibactin[5] and iturin[6]. Metabolites produced by endophytic *Bacillus* also showed various therapeutic potentials, such as antioxidant [7], antibacterial [8], antifungal [9], antiviral [10] and cytotoxic properties [11]. Therefore, this bacterial group is a precious source of medicinally important compounds.

The emergence of degenerative diseases induced by oxidative stresses and infectious diseases caused by antibiotic-resistant pathogens encourages researchers to explore new sources of antioxidant and antibacterial compounds. The main characteristic of antioxidant compounds is the capability to scavenge and stabilize free radicals and prevent or delay free radical production from cellular activities [12]. These properties reduce oxidative damage in human cells and tissues and become important in preventing degenerative diseases, such as cancer and coronary heart disease [13]. While, an antibacterial is characterized by the ability of certain compounds to kill or inhibit particular bacteria growth [14]. The use of avalaible antibiotics (levofloxacin, clarithromycin, andamoxicillin) to control infection can promote many side effects, especially increasing reactive oxygen species (ROS) in cellular level which can induce mytocondrial dysfunction and

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oxydative tissue damage in the human body [15]. Therefore, finding new antibacterial agents with antioxidant property is an urgent need to overcome infectious diseases.

Ethnobotanical knowledge provides a useful approach for selecting plant hosts to discover potential endophytic bacteria. *Archidendron pauciflorum*, a native Southeast Asia plant, was traditionally used to overcome dysentery[16]. Twenty-four endophytic bacteria associated with this plant have been isolated in the earlier study and four isolates identified as *Bacillus* spp. showed promising antibacterial and antibiofilm activities against multidrug-resistant strains[17]. To evaluate their antibacterial spectrum comprehensively, this study aimed to investigate their antibacterial activity against four ATCC (American Type Culture Collection) strains, such as *Pseudomonas aeruginosa* strain ATCC 15442, *Escherichia coli* strain ATCC 8739, *Staphylococcus aureus* strain ATCC 6538, and *Bacillus subtilis* strain ATCC 19659, along with the evaluation of their antibiofilm, and antioxidant activities, and phytochemical content of extracts derived from four endophytic *Bacillus* spp. associated with *A. pauciflorum*.

2. RESULTS

2.1. Antioxidant activity

All extracts derived from four *Bacillus* spp. endophytic bacteria displayed various antioxidant activities against DPPH and ABTS free radicals, as indicated by their IC_{50} values. *B. pseudomycoides* strain DJ8 exhibited the highest antioxidant activity towards DPPH and ABTS with IC_{50} values of 24.87±1.81 and 9.18±0.10 µg/mL, respectively. However, the antioxidant activity of four endophytic bacteria extracts is still lower than the positive control, ascorbic acid and trolox (Table 1).

Table 1. Antioxidant activity of endophytic *Bacillus* spp. derived extracts against DPPH and ABTS

Sample	Antioxidant activity*	
	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
B. megaterium strain AJ5	98.31±0.94^{d}	56.25±1.87^{d}
B. amyloliquefaciens strain DJ4	419.63±8.88^{f}	103.29±2.04^{e}
B. pseudomycoides strain DJ8	24.87±1.81^{c}	9.18±0.10^{c}
B. velezensis strain DJ9	217.70±9.35^{e}	106.05±5.67^{f}
Ascorbic acid	2.68±0.10^{b}	4.82±0.26^{b}
Trolox	1.77±0.12^{a}	3.17±0.13^{a}

*Note: The IC_{50} value are expressed as mean ± SEM. Different superscript letters in the same column indicate that the data were significantly different.

2.2. Antibacterial activity

Screening of antibacterial activity from four *Bacillus* spp. endophyte using dual culture agar diffusion test showed all isolates exhibited antibacterial activity against at least three ATCC strains (Table 2). A broad spectrum of antibacterial activity showed by *B. pseudomycoides* strain DJ8, *B. megaterium* strain AJ5, and *B. amyloquefaciens* strain DJ4 against *P. aeruginosa* strain ATCC 15442, *E. coli* strain ATCC 8739, *B. subtilis* strain ATCC 19659, and *S. aureus* strain ATCC 6538. Consistently, their antibacterial activity was also shown by their metabolite extracts in 1 mg/mL concentration (Figure 1). In general, extracts from *B. pseudomycoides* strain DJ8 showed stronger antibacterial activity than other extracts, with inhibition zone ranging from 8±0.8 to 18±0.8 mm against four ATCC strains (Table 3). Extract from the four endophytic bacteria were more active on the Gram-positive strains than on the Gram-negative strains. Tetracycline (200 µg/mL) as a positive control also exhibited antibacterial activity with inhibition zone diameter ranging from 15±0.8 to 29.3±0.9 mm.

The minimum inhibitory concentration (MIC) of all endophyte extracts was determined. The strong bacterial growth inhibitors are indicated by the lower MIC value. Commonly, all endophyte extracts varied in MIC value (31.25 – 1000 µg/mL) on four strains tested (Table 4). Extract from *B. pseudomycoides* strain DJ8 possessed the lowest MIC value (ranging from 31.25-500 µg/mL) against four ATCC strains. Tetracycline as positive control displayed MIC ranging from 1.95-62.5 µg/mL. The endophytic extracts also have different minimum bactericidal concentration (MBC) values. The most promising MBC value was also exhibited by *B. pseudomycoides* strain DJ8 extract (125-500 µg/mL) against four ATCC strains investigated.

Table 2. Antibacterial activity of *Bacillus* spp. endophyte from *A. pauciflorum* against ATCC strains by dual culture agar diffusion test

Endophytic isolates	İnhibition zone diameter (mm)*			
	E. coli ATCC 8739	*P. aeruginosa* ATCC 15442	*B. subtilis* ATCC 19659	*S. aureus* ATCC 6538
B. megaterium strain AJ5	+	+	++	+
B. amyloliquefaciens strain DJ4	+	+	+	++
B. pseudomycoides strain DJ8	+	+	++	++
B. velezensis strain DJ9	+	-	+	++

*Note: + = less active (clear zone diameter: ≤6 mm), ++ = moderately active (clear zone diameter: 7-10 mm), +++ = highly active (clear zone diameter: >10 mm), - =No activity

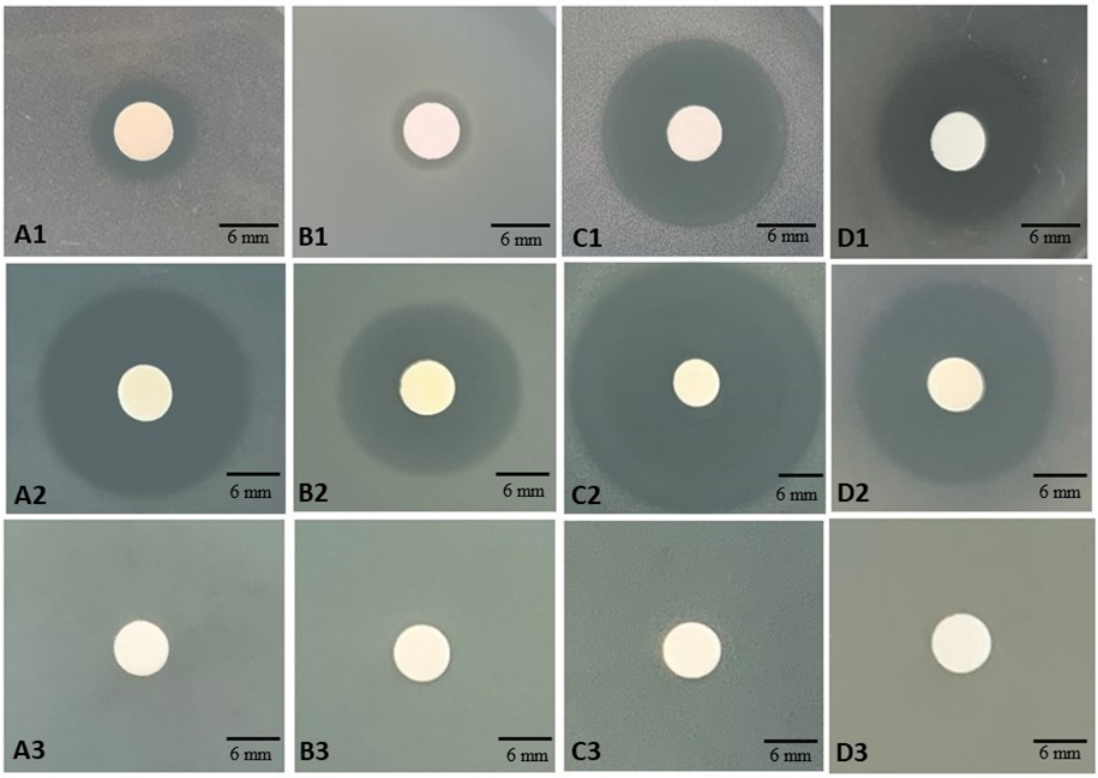

Figure 1. Antibacterial activity of *B. pseudomycoides* strain DJ8-derived extracts (1 mg/mL) against (A) *Escherichia coli* strain ATCC 8739, (B) *Pseudomonas aeruginosa* strain ATCC 15442, (C) *Bacillus subtilis* strain ATCC 19659, (D) *Staphylococcus aureus* strain ATCC 6538. (1) DJ8 extract, (2) Tetracycline (200 µg/ml), (3) DMSO (1% v/v).

Table 3. Antibacterial activity of endophytic *Bacillus* spp. derived extract against ATCC strains by disc diffusion method

Sample	İnhibition zone (mm)*			
	E. coli ATCC 8739	*P. aeruginosa* ATCC 15442	*B. subtilis* ATCC 19659	*S. aureus* ATCC 6538
B. megaterium strain AJ5	7.3±0.5^{b}	8±0^{b}	16±0.8^{b}	8.3±0.5^{c}
B. amyloliquefaciens strain DJ4	8±0.8^{c}	10±0^{d}	17.7±0.9^{c}	7.7±0.5^{b}
B. pseudomycoides strain DJ8	8.7±0.9^{c}	8±0.8^{c}	18±0.8^{d}	13±0.8^{d}
B. velezensis strain DJ9	9.6±0.5^{d}	NT	18±0.8^{d}	13±0.8^{d}
Tetracycline	28±0.8^{e}	15±0.8^{e}	29.3±0.9^{e}	23±0^{e}
DMSO	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}

*Note: NT: Not tested.

Table 4. The Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of endophytic *Bacillus* spp. derived extracts against ATCC strains

Sample	MIC and MBC (µg/mL) against ATCC strains							
	E. coli ATCC 8739		*P. aeruginosa* ATCC 15442		*B. subtilis* ATCC 19659		*S. aureus* ATCC 6538	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. megaterium strain AJ5	250	1000	1000	>1000	500	>1000	500	1000
B. amyloliquefaciens strain DJ4	1000	>1000	1000	>1000	500	>1000	125	500
B. pseudomycoides strain DJ8	125	500	62.25	250	125	500	31.25	125
B. velezensis strain DJ9	500	1000	NT*	NT*	62.50	250	62.50	250
Tetracycline	1.95	3.91	15.62	62.5	1.95	3.91	1.95	3.91

*Note: NT: Not tested. Endophytic extract, tetracycline, and DMSO are applied at 1 mg/mL, 200 µg/ml, and 1% (v/v), respectively. Different superscript letters in the same column indicate the data were significantly different

2.3. Antibiofilm activity

Three extracts with broad spectra of antibacterial activity were selected for antibiofilm analysis. Inhibition of biofilm formation was correlated to the extract concentration in doses dependent response (Figure 2). In general, all extracts in 2 MIC showed the highest inhibition of biofilm formation of the test strains ranging from 39.51-62.59%. *B. pseudomycoides* strain DJ8-derived extract in 2 MIC showed the strongest inhibition of biofilm formation (62.59%) against *B. subtilis* strain ATCC 19659. This extract also inhibited more than 50% biofilm formation of other ATCC strains in the concentration. However, biofilm formation of Gram-negative strains (*E. coli* strain ATCC 8739 and *P. aeruginosa* strain ATCC 15442) was more persistence in endophyte extracts, as indicated by the lower inhibition percentage compared to Gram-positive strains.

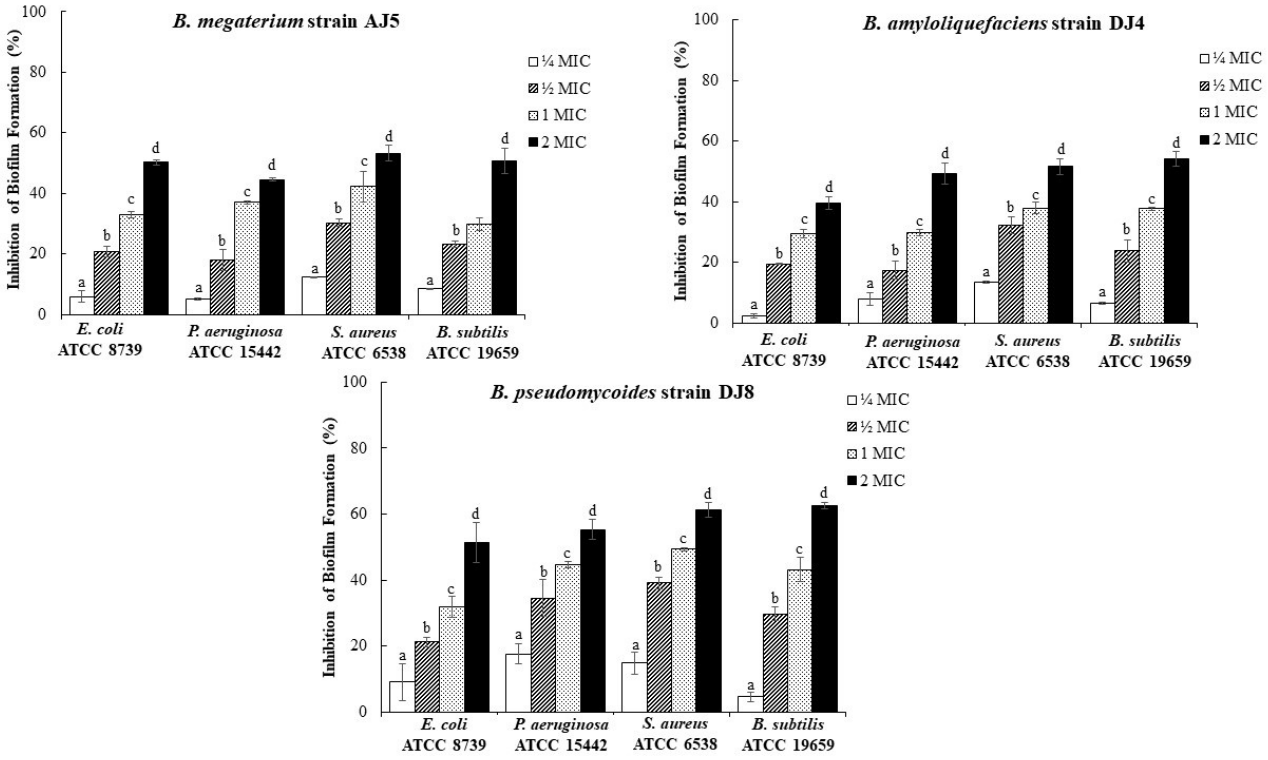

Figure 2. Inhibition of biofilm formation of endophytic *Bacillus* spp. associated with *A. pauciflorum* against ATCC strains

2.4. Phytochemical profile

Based on qualitative assay, all endophytic *Bacillus* spp. extract contain alkaloid, flavonoid, and terpenoid. However, tannin and saponin were absent in all endophyte extracts (Table 5).

Table 5. Phytochemical contents of *Bacillus* spp. endophytes from *A. pauciflorum*

Extract	Phytochemical Contents				
	Alkaloid	Flavonoid	Tannin	Saponin	Terpenoid
B. megaterium strain AJ5	+	+	-	-	+
B. amyloliquefaciens strain DJ4	+	+	-	-	+
B. pseudomycoides strain DJ8	+	+	-	-	+
B. velezensis strain DJ9	+	+	-	-	+

*Note: - : absent; +: present

2.5. Total flavonoid and phenolic compounds

Four endophyte extracts varied in the total flavonoid and phenolic content (Table 6). In general, the total phenolic content was higher than the total flavonoid in all extracts. The highest total flavonoid and phenolic compounds exhibited by *B. pseudomycoides* strain DJ8 extract which is 40.10±1.18 mg QE/g extract, and 58.50±4.56 mg GAE/g extract, respectively.

Table 6. Total phenolic and flavonoid of endophytic *Bacillus*-derived extracts

Extract	Total flavonoid (mg QE/g extract)	Total phenolic (mg GAE/g extract)
B. megaterium strain AJ5	32.37±0.50^{c}	41.18±2.86^{b}
B. amyloliquefaciens strain DJ4	23.96±0.53^{a}	50.34±1.34^{c}
B. pseudomycoides strain DJ8	40.10±1.18^{d}	58.50±4.56^{d}
B. velezensis strain DJ9	30.95±1.72^{b}	38.00±0.78^{a}

*Note: Different superscript letters in the same column indicate the data were significantly different

3. DISCUSSION

Medicinal plants provide a favorable environment for the colonization of endophytic microbes capable of producing many valuable natural products. The current study evaluated antioxidant, antibacterial, and antibiofilm properties of the crude extracts derived from four endophytic *Bacillus* spp. Antioxidant analysis showed that all extracts were active to two different free radicals, including DPPH and ABTS, with median inhibition concentration (IC_{50}) under 500 µg/mL. In this concentration, the antioxidant compounds could reduce 50% of free radicals. *B. pseudomycoides* strain DJ8 extract is considered highly active antioxidant activity due to its IC_{50} being less than 50 µg/mL, which is 24.87±1.81 µg/mL and 9.18±0.10 µg/mL against DPPH and ABTS, respectively. Extract from *B. megaterium* strain AJ5 classified into active antioxidant activity (IC_{50} range: 50-100 µg/mL), while extract derived from *B. velezensis* strain DJ9 classified to moderate (IC_{50} range: 101-250 µg/mL) and *B. amyloliquefaciens* strain DJ4 to weak antioxidant activity (IC_{50} range: 250-500 µg/mL). Compared to their host, methanolic *A. pauciflorum* fruit extract are still more active than endophyte extract, due to it has a lower value of IC_{50} (11.28 µg/mL) against DPPH [18]. However, growing and harvesting plant biomass takes more time than culturing bacteria. It takes 40-50 days after flowering to mature fruits. Therefore, the active compound extracted from its endophytes could be the new potential source of an antioxidant agent because culturing bacteria have a shorter culture time than the host plant.

Endophytic *Bacillus* spp. associated with *A. pauciflorum* also has antibacterial activity. Based on the dual culture agar diffusion test, these endophytic colonies showed an antagonism effect against ATCC strains (Table 2). Three isolates (*B. megaterium* strain AJ5, *B. amyloliquefaciens* strain DJ4, and *B. pseudomycoides* strain DJ8) displayed broad spectra of antibacterial activity, except *B. velezensis* strain DJ9 that was not active to *P. aeruginosa* strain ATCC 15442. In addition, secondary metabolites derived from each endophytic isolate were also tested on four test strains using the disc diffusion method. Endophytic *Bacillus* extracts were more active on the Gram-positive bacteria than on the Gram-negative bacteria. The difference in the antibacterial effects of endophytic extracts against a different group of bacteria may cause by the different external structures between Gram-positive and Gram-negative bacteria. Gram-negative bacteria have two layers of the cell membrane. Consequently, the Gram-negative bacteria group is commonly more resistant to antibacterial substances[19]. The potential of antibacterial activity observed in the current study suggests that endophytic *Bacillus* spp. may play a significant role in the protection of *A. pauciflorum* against phytopathogens. Although the host also reported having antibacterial activity against pathogens[20].

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of four endophytic extracts against ATCC strains were investigated. The bacterial growth was decreased by increasing endophytic extracts concentration. In general, all endophytic extracts have MIC ≤1000 µg/mL. In this

concentration, endophytic extract prevents the visible growth of ATCC strains under *in vitro* condition. *B. pseudomycoides* strain DJ8 extract was most effective in inhibiting *S. aureus* strain ATCC 6538, as indicated by the lowest MIC of 31.25 µg/mL, considered a highly active antibacterial agent [21]. Furthermore, all extracts also were varied in MBC value (except *B. velezensis* strain DJ9 was not to be tested to *P. aeruginosa* strain ATCC 15442). MBC value was indicated by the absence of bacterial growth on the agar plates after being treated with endophytic extracts. In this concentration the extract was completely killing the test strains. The lowest MBC value (125 µg/mL) was also shown by *B. pseudomycoides* strain DJ8 extract against *S. aureus* strain ATCC 6538.

Biofilm-forming bacteria are generally more resistant to antimicrobial agents, host defense systems, and other external factors compared to free-living bacteria [22]. Therefore, exploring an antibacterial agent that could inhibit biofilm formation is globally needed. In the present study, three endophytic extracts with broad spectra of antibacterial activity showed significant inhibitory effects against biofilm formation across four test strains. 2 MIC of each extract was found as the most effective concentration in inhibiting biofilm formation. In this concentration, the inhibitory activity ranges from 39.51 to 62.59%. Several mechanisms could possibly inhibit biofilm formations, such as quorum sensing blockage, inhibition of macromolecules synthesis and adhesion, inhibition of cell division and survival, and disruption of extracellular DNA, lipopolysaccharides, protein, and exopolysaccharides [23].

Investigation on the phytochemical profile of extract derived from four endophytic *Bacillus* associated with *A. pauciflorum* exhibited the presence of alkaloid, flavonoid, and terpenoid. The phytochemical profile of endophyte extract was also similar to the extract derived from their host. All these compounds are also found in the leaf and fruit peel extract of *A. pauciflorum* [20, 24]. Previous studies reported that phytochemicals were also commonly found in the crude extract of endophytic bacteria isolated from medicinal plants [25-26]. In some cases, flavonoid, alkaloid, and terpenoid are responsible for natural products' antioxidant and antibacterial properties [27-29]. The presence of these phytochemicals within endophyte extracts can be a potential source of active compounds for medicinal and biotechnological use.

Extract with the most potent antibacterial and antioxidant activities, *B. pseudomycoides* strain DJ8 extract, contains the higher phenol content (58.50±4.56 mg GAE/g extract) and flavonoid content (40.10±1.18 mg QE/g extract), than other endophyte extracts. The total phenol and flavonoid may correlate with their antibacterial and antioxidant activities. The phenolic content of endophyte extracts is still lower than their host fruit extract (97.50±2.45 mg GAE/g extract). However, all endophyte extracts contain higher flavonoid (ranging from 23.96±0.53 to 40.10±1.18 mg QE/g extract) than host fruit extract (6.67±0.38 mg QE/g extract) [18].

4. CONCLUSION

Four endophytic *Bacillus* spp. associated with *Archidendron pauciflorum* showed different antioxidant, antibacterial, and antibiofilm properties. Among four extracts tested, extract derived from *B. pseudomycoides* strain DJ8 markedly showed a highly active antioxidant effect against DPPH and ABTS. Extract derived from this isolate also showed promising antibacterial and antibiofilm activities, which were more active to *Bacillus subtilis* and *Staphylococcus aureus.* Phytochemicals (flavonoid, alkaloid, and terpenoid) were present in the extract. The extract also contained phenolic and flavonoid compounds.

5. MATERIALS AND METHODS

5.1. Source of bacterial strains

In the recent study, we used 4 *Bacillus* spp. endophytic strains, which isolated from the leaves and roots of *A. pauciflorum*[17]. Of note, the target bacterial strains utilized in this research were Gram-negative, including *P. aeruginosa* strain ATCC 15442 and *E. coli* strain ATCC 8739 along with Gram-positive strains such as *S. aureus* strain ATCC 6538 and *B. subtilis* strain ATCC 19659. All bacterial tested were kindly provided by the Laboratory of Microbiology, Department of Biology, IPB University. The reason of choosing these four strains for antibacterial testing is it due to their resistance to some antibiotics (clindamycin, oxacillin, lincomycin, bacitracin, quinupristin, andteicoplanin) [30-33].

5.2. Endophytic bacterial culture and secondary metabolite extraction

Bacterial culture was carried out in 1 L nutrient broth (NB) (Himedia) made in 3 L erlenmeyer flask. At the same time, each of endophytic bacteria were re-cultured in 100 mL NB for 1 days at 28 °C with 120 rpm shaking. Then, the bacterial culture was put into a 3 L NB flask, and incubated 3 days at 28 °C with shaking at 120 rpm. After incubation period is completed, ethyl acetate was added to the bacterial culture (1 :1 v/v) followed by ±15 min strong shaking. The upper layer was then collected and evaporated at 50 °C [34].

5.3. Analysis of 2, 2 di phenyl 1 picryl hydrazyl (DPPH) scavenging activity

A solution of 125 µM DPPH was prepared, and 100 µL of this radical was added to 96 well microplates followed by the addition of 100 µL sample. Further, this mixture was homogenized and placed for 30 min before being observed at 517 nm via spectrophotometer (Thermo Scientific Varioskan Flash-ThermoFischer Elisa reader). The inhibition value was measured using the formula: ((Abs. corrector — Abs. sample)/Abs. corrector) × 100%. Abs. Corrector represent absorbance of methanol + DPPH, while Abs. sample is the absorbance of samples + DPPH. Antioxidants activity was shown as capacity to reduce DPPH radicals by 50% (IC_{50}) [35]. Ascorbic acid and trolox were utilized as positive control. The use of ascorbic acid and trolox as a control in this study because the compounds were commonly known for having strong and stable antioxidant activity against DPPH and ABTS, and that is accepted as standard antioxidant compound in *in vitro* study[36-37].

5.4. Analysis of 2,20- azino-bis-(3- ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity

The ABTS radicals were made by mixing potassium persulfate (2.45 mM) and ABTS (7 mM) followed by incubation in the dark to produce a green-colored ABTS radicals solution with an absorbance at 745 nm of about 0.70±0.02. Next, 30 µL of the test sample was added with 270 µL of ABTS radical in 96-well microplate and incubated for 30 min. The percentage inhibition was determined using the formula: ((Abs. corrector — Abs. sample)/Abs. corrector) × 100%. Abs. corrector is the absorbance of ABTS radical + methanol, and Abs. sample is the absorbance of ABTS radical + samples. Trolox and ascorbic acid were used as a positive control. The antioxidant activity of extracts was reflected as IC_{50} value [35].

5.5. Antibacterial activity by dual-culture agar diffusion method

Four bacterial targets were cultured in 50 mL of NB in a shaker condition of 120 rpm for approximately 24 h at 28 °C. Further, 1% (v/v), equivalent to 1×10^8 CFU/mL of bacterial target was inoculated to molten NA media and poured into a sterile petri dish. After the NA media solid, bacterial endophyte colonies on the 24 h-old were streak on round shape into medium and incubated at ±37 °C for 24 h. After that, the clear zone diameter was determined and reflected in millimeters[38].

5.6. Antibacterial activity by disc diffusion assay

Initial screening for the antibacterial activity was analyzed by the disc diffusion assay. Shortly, the potent extract (1 mg/mL) was prepared in 1% dimethyl sulfoxide (DMSO). Further, 1% (v/v) of bacterial target strain (equivalent to 1×10^8 CFU/mL) was inoculated to molten NA media and poured into sterile petri dish. Then, a sterilized paper disc(diameter: 6 mm) was impregnated with 10 µL extract and put on the surface of NA media and incubated at 37 °C for 24 h. Discs treated with 1% DMSO and tetracycline (200 µg/mL) were applied as a negative and positive control, respectively. The inhibition zone to the nearest millimeter was reflected in the antibacterial activity of extracts. The use of tetracycline in this study because four test strains are susceptible to tetracycline [39].

5.7. Analysis of the minimum ınhibitory concentration (MIC) and the minimum bactericidal concentration (MBC)

In this assay, we used the serial microdilution method in 96 sterile wellplates. Briefly, each well was filled with 100 µL MHB. Further, the sample was to the wells in column A and followed by serial dilution from column A to H. After that, each well was added with 100 µL of target bacterial culture 24-h in a 0.5 McFarland (equivalent to 1×10^8 CFU/mL) and incubated at 37°C for 24 h with 200 rpm shaking. MIC was reflected as the lowest sample concentration at which bacteria showed no visible growth in wells. As for MBC value was obtained as the lowest extract concentration to kill 99.9% of the target bacteria. Positive and negative controls were conducted using tetracycline and 1% DMSO, respectively[40].

5.8. Analysis of antibiofilm activity

Three endophytic bacterial extracts at MIC value concentration (1/4; 1/2; 1; 2 MIC) were evaluated for their antibiofilm activity. Briefly, 100 µL of brain heart infusion (BHI) media was load onto 96-well sterile microplate. Subsequently, 100 µL of sample and controls was introduced into well A, followed by serial dilution up to well H. Next, 100 µL of bacterial culture in the standard of 0.5 McFarland's (equivalent to 1 × 10^8 CFU/mL) was pipetted to each well and incubated for 24 h at 37 C with 200 rpm shaking. Following incubation, each well were rinsed two times with phosphate buffer saline (PBS) to eliminate loose non-biofilm. Further, the plates were stained with 200 µL crystal violet (0.1%) and incubated at 37°C for 30 min. The plates were then rinsed with PBS and reconstituted with 200 µL of 99% DMSO before photometrically detection. Absorbance was determined using a microplate ELISA reader (Thermo Scientific Varioskan Flash-ThermoFischer Elisa reader) at 595 nm. The negative control was applied in the untreated conditions[40].

5.9. Phytochemical analysis

The endophytic bacterial extracts were used for phytochemical analysis for secondary metabolites identification using the qualitative phytochemical test, which was previously described[41]. Briefly, the presence orabsence of phytochemical compounds was determined by mixing an appropriate chemical reagent and sample. The absence or presence of targeted compounds, including alkaloids, terpenoids, flavonoids, tannins, and saponins classes, was qualitatively detected.

5.10. Analysis of total flavonoid and phenolic contents

The total phenolic content (TPC) and total flavonoid content (TFC) were assayed following the previous method[42]. As for TPC, distilled water (3.5 mL) was supplemented to the extract (500 µL, 1000 µg/mL). The Folin-Ciocalteu reagent (250 µL) and sodium carbonate (750 µL, 20%) were then added, followed by incubation at ±27°C for 2 hours. The absorbance was measured via a spectrophotometer (Thermo Scientific Varioskan Flash-Thermo Fischer Elisa reader) at 765 nm. On the other hand, estimation of TFC, in a 10 ml test tube, 500 µL of extracts, 2.2 mL of distilled water, 150 µL of NaNO2 (5%), and 150 µL of AlCl3 (10%) were mixed. After 8 min incubation, 2 mL of NaOH (1 M) was subjected, and the absorbance was measured at 506 nm. Of note, as for TPC, the same method was applied to the standard solutions of gallic acid and stated as mg Gallic Acid Equivalents (GAE)/g extract. As for TFC, the total flavonoids were exhibited as milligrams of quercetin (QE)/ g of extract.

5.11. Statistical analysis

Each test was determined in triplicate. The results are shown as mean values completed with standard deviation (±SD). The 95% and 99% confidence levels obtained from one-way analysis of variance (ANOVA) were utilized. Tukey's test was performed using a p-values<0.05, which was considered statistically significant in further statistical analysis.

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