



PURIFICATION OF BIOACTIVE COMPOUND FROM ENDOPHYTES *Bacillus* sp. RD26 of *Phyllanthus amarus* Schum. et Thonn

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ABSTRACT

The strain of *Bacillus* sp. RD26, isolated from *Phyllanthus amarus* Schum. et Thonn. exhibited antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). In the current study, *Bacillus* sp. RD26 was identified as *Bacillus amyloliquefaciens* according to Cowan and Steel classification keys. The methanol extract (BRM) from *Bacillus amyloliquefaciens* RD26 was resistant to MRSA bacteria with an inhibition zone of 25 ± 0.57 mm. From total BRM, the high-efficient column chromatographic extraction was conducted with solvent system EA:Me (the ration of EA: Me were 100:0; 90:10; 80:20; 70:30; 60:40; and 0:100) to collect total of five fractions (BRM1 - BRM5). As the result, all fractions had antibacterial activity against MRSA. Among them, the fraction of BRM3 had the highest anti-MRSA ability with the zone of inhibition reached to 18 mm. Combining the method of column chromatographic extraction and thin-layer chromatographic extraction, the compound of BR04, pyrimidine-2,4-dion (Uracil), which had the anti-MRSA ability, was isolated. BR04 was identified to be resistant to many Gram-positive and Gram-negative bacteria such as MRSA, *Bacillus cereus*, *Escherichia coli* by evaluation of MIC. The values of MIC were 64 $\mu\text{g/mL}$, 128 $\mu\text{g/mL}$, and 512 $\mu\text{g/mL}$ for MRSA, *Bacillus cereus*, and *Escherichia coli*, respectively. Additionally, BR04 had antioxidant activity at the concentration of 1800 $\mu\text{g/mL}$. In conclusion, the current study reported bioactive compounds from *Bacillus amyloliquefaciens* RD26 had the potential to be further applied against infectious microorganisms.

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Introduction

Endophytes are microorganisms (mainly fungi, bacteria, or actinomycetes) that dwell within healthy plant tissues without causing harm to the host plant. They have been proven to be a source of natural products with many biological activities [1-4]. These endophytes are usually present in the space zone between cells, thus, they could be isolated from all parts of host plants, including seeds [5]. The natural products with antibacterial, antifungal, anti-cancer, etc. activities are collected from endophytes and can be applied in the field of medicine, agriculture, or industry [2, 6, 7]. Many endophytes have the ability to synthesize different bioactive metabolisms that could be directly or indirectly used as rectification of many diseases in plants, animals, and humans [8-10].

Bacillus has been reported to be rich in bioactive compounds, such as antibiotics, proteins, enzyme inhibitors, and pharmacologically active ingredients [11, 12]. They produce a large number of antibacterial and biological peptides with different chemical structures. Among antifungal compounds, lipopeptides, which could be synthesized by some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*, are considered to play the main roles in the function of inhibition [13-16]. According to Tabbene *et al.* (2011), purified bacillomycin from the genus of *Bacillus* had the anti-Candida ability [17]. Jeyanthi *et al.* (2016) identified that the phenolic compounds, produced from *Bacillus amyloliquefaciens* MHB1, were anti-MRSA [18].

In our previous study, we successfully optimized the culture medium for the fermentation of *bacillus* sp. RD26 against MRSA [19]. Continuously, we conducted the current study to purify the MRSA-resistant compounds and evaluate their different bioactivities.

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Materials and Methods

Preparation of Microorganisms

The *Bacillus* sp. RD26 strain was isolated from *Phyllanthus amarus* Schum. et Thonn and was cultured from the medium of Tryptic Soy Broth (TSB).

Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) was provided by Nam Khoa Co. Ltd, Vietnam. *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were provided by the Laboratory of Microorganism, Ho Chi Minh City Open University, Vietnam. All of these microorganisms were cultured on Nutrient Broth (NB).

Nomenclature Identification of Bacillus sp. RD26

Endophyte of *Bacillus* sp. RD26 was classified according to the keys of Cowan and Steel [20].

Extraction of Bacillus sp. RD26

The strain of *Bacillus* sp. RD26 was fermented on the optimal medium, included 7.36 g/L peptone, 15 g/L glucose, 0.72 g/L CaCO₃, and 0.6 g/L MgSO₄ [19]. Then, the supernatant was collected by centrifugation at 10000 rpm for 10 minutes. 100 mL of supernatant was supplemented by methanol with a ratio of 1:1. Finally, the crude methanolic extract was obtained by evaporation.

Evaluation of the Anti-MRSA Activity

The assay of anti-MRSA of *Bacillus* sp. RD26 extraction was performed by using the disk diffusion method [18]. The MRSA of 10⁸ CFU/ml was spread in the MHA disc. The filter paper discs (about 6 mm in diameter, Nam Khoa Co. Ltd, Vietnam), containing 10 µL extract, were placed on pre-inoculated agar. The Petri dishes were incubated under suitable conditions of 37°C/24 hours. Then, the zone of inhibition was determined by measuring the diameter of the clear zone. DMSO was used as a control. Additionally, the values of minimum inhibitory concentration (MIC) were determined by the method of dilution in the culture medium [21].

Purification of the Antibacterial Compound Synthesized by Bacillus sp. RD26

The *Bacillus* sp. RD26 extract was mixed with silica gel and loaded into a column (50 cm × 3 cm, 230-400 mesh, Hi-Media Laboratories, Mumbai, India). The column chromatographic extraction was conducted with following solvent systems ethyl acetate: methanol (EA:Me) in the following ratio: 100:0, 90:10, 80:20, 70:30, 60:40, and 0:100. The lysing through the column was collected and extracted by evaporation. The fractions were spotted on a TLC plate, chromatography using chloroform: methanol (ratio: 10:1) then, evaluated the ability of anti-MRSA [18]. Fractions within the anti-MRSA effect were subjected to column chromatography with an EA-Me solvent system of increasing polarity (80:1; 50:1; 30:1; 10:1, and 100% methanol). The active fractions were purified through the normal phase of the silica gel column (60 cm × 2 cm, 230-400 mesh, Hi-Media Laboratories, Mumbai, India) with a 100% chloroform solvent system.

Thin-Layer Chromatography (TLC) Analysis of the Purified Anti-MRSA Compound

The fraction was obtained by thin-layer chromatography (TLC) using chloroform: methanol (ratio: 10:1). TLC plates were observed under UV light at 254 nm. The lysed chromatographic plate was dried and dipped in the MRSA (1-2 × 10⁶ CFU/mL) and incubated at 25°C for 48 hours. The presence of bacteria was detected by tetrazolium salt, which converted the dehydrogenase of living microorganisms to formazan [22]. Then, it was incubated at 25°C for 24 hours or 37°C for 3-4 hours. The white areas on the violet background on the TLC plate indicated the antibacterial activities of the sample [23].

Identification of MRSA Resistant Compound

The Nuclear Magnetic Resonance Spectroscopy (Bruker Avance) with 500 MHz for ¹H-NMR and 125 MHz for ¹³C-NMR was performed at the Institute of Chemistry - Vietnam Academy of Science and Technology, Ha Noi, Vietnam, were applied to identify the structure of the anti-MRSA compound.

Evaluation of Bioactive Abilities

Anti-Pathogenic Bacteria Activity

Bioautography method: Dot the compound on the TLC plate with a suitable solvent system. The separation of different compounds was detected by UV spectroscopy at 254 nm. Then, the number of separated traces and R_f value were determined. The pathogenic bacteria were spread onto the agar at the concentration of 10⁸ CFU/mL. TLC plate was cut into small pieces corresponding to each spot in the TLC plate, then, placed on the petri dish covered with the pathogenic bacteria and incubated at 10°C for 12 hours, followed by incubation at 37°C for 24 hours. Finally, the zone of inhibition was recorded.

Minimum inhibitory concentration (MIC): The antimicrobial activity was tested based on the MIC method with pathogenic bacteria strains: *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922.

Antioxidant Activity

The antioxidant activity was performed by DPPH (2,2-diphenyl - 1 picrylhydrazyl) free radical scavenging. The percentage of antioxidant activity was determined according to the following formula: % DPPH free radical scavenging activity = $[(ODc - ODm)/ODc] \times 100$ [24]. From the percentage of DPPH free radical scavenging activity, the IC₅₀ value was calculated based on a linear correlation equation. The lower value of IC₅₀ indicated the higher antioxidant activity.

Results and Discussion

Identification of *Bacillus sp. RD26*

The results of the biochemical assay, which was performed according to Cowan and Steel classification keys, indicated that *Bacillus sp. RD26* was similar to *Bacillus amyloliquefaciens* (Similarity = 88.89%) (**Table 1**).

Table 1. The biochemical identification of *Bacillus sp. RD26*

Catalase	+	Galactose	d	Urease	-
Motility	+	Mannose	d	Indol	-
50°C	+	Melibiose	-	VP	+
10% NaCl	+	Raffinose	+	Nitrate	+
Anaerobic	-	Salicin	+	Casein	+
Glucose	+	Xylose	+	Amylase	+
Cellobiose	+	Citrate	+	Oxidase	+
Conclusion: <i>Bacillus amyloliquefaciens</i>					

Note: -: negative, +: positive, d: positive/negative

The MRSA-resistant ability of *Bacillus sp. RD26* extract

As the result, the inhibition zone of 25 ± 0.57 mm indicated that the methanol extract of *Bacillus amyloliquefaciens* RD26 had the anti-MRSA ability (**Figure 1**).



Figure 1. Anti-MRSA of *Bacillus sp. RD26* extract by disk diffusion method

The BRM extract was diluted with methanol to conduct spotting on a TLC plate, chromatography was conducted using chloroform: methanol (ratio: 10:1) as mobile phase. The values of R_f of each separated trace were recorded. As the results, the R_f values of each separated trace were 0.13, 0.31, 0.37, 0.52, 0.55, 0.68, 0.73, 0.78, and 0.90.

Purification of the Antibacterial Compounds Synthesized by *Bacillus sp. RD26*

15.53 g BRM extract subjected to column chromatography with an EA-Me solvent system of increasing polarity (ratio of EA-Me were 100:0, 90:10, 80:20, 70:30, 60:40, 0:100). As the results, five fractions noted as BRM1-BRM5 were collected and subjected to evaluate the ability of anti-MRSA.

The anti-MRSA ability of the BRM3 fraction was the highest (18.00 ± 0.00 mm). 4.6 g BRM3 was subjected to column chromatography with an EA-Me solvent system of increasing polarity (80:1; 50:1; 30:1; 10:1 and 100% methanol). As the result, only a clear trace of BRM31 was observed. BRM31 (212.34 mg) was subjected to the normal phase of silica gel column with chloroform:methanol within the increasing polarity (ratio: 50:1, 30:1, 10:1, and 1:1).

Based on thin-layer chromatography, the same traces were grouped into four traces: BRM311-BRM314. BRM313 (38.31 mg) was subjected to normal-phase silica gel column chromatography with 100% chloroform. Based on thin layer chromatography, the same traces were grouped into three segments: BRM3131-BRM3133. BRM3132 showed the UV light at 254 nm, and was not traceable by 10% H₂SO₄/EtOH (**Figure 2**). The purified compound was named as BR04 (9.41 mg).

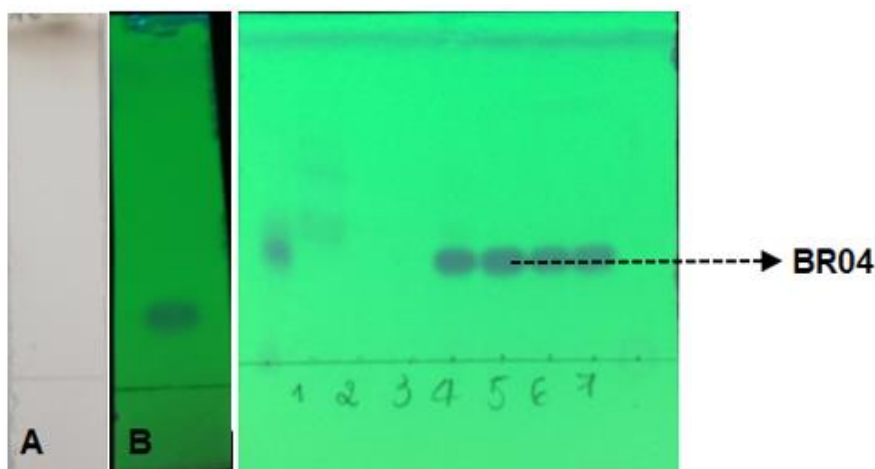


Figure 2. The thin-layer chromatography of BR04 with chloroform:methanol (10:1)
a. Undetectable by 10% H₂SO₄/EtOH. b. under UV light

Thin-layer Chromatography Analysis of the Purified Anti-MRSA Compound

The TLC of the BRM31 fraction showed anti-MRSA activity by the bioautography method. The results showed a white fraction with the R_f of 0.31 (**Figure 3**).

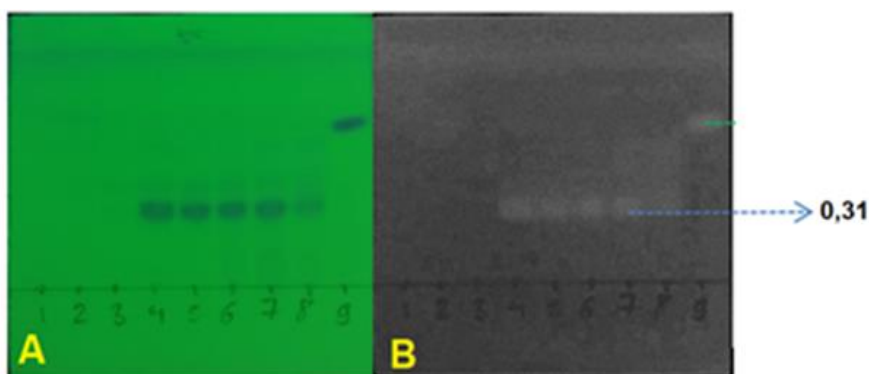


Figure 3. (a) TLC analysis of BRM31 fraction (b). Bioautography of BRM31 fraction anti-MRSA activity on TLC plate

Identification of the Structure of MRSA Resistance Compound

Compound BR04 was obtained as a white powder. The ¹H-NMR spectrum (DMSO-*d*₆, 500 MHz) of BR04 gave a signal of protons consisting of 2 primary amine protons at δ_{H} 10.98 (1H, s, 1-NH) and 10.79 (1H, s, 3-NH), and the 2 olefine protons *cis*-couple together at δ_{H} 5.44 (1H, *d*, *J* = 7.5 Hz, H-5) and 7.37 (1H, *d*, *J* = 7.5 Hz, H-6). The ¹³C-NMR spectrum (DMSO-*d*₆, 125 MHz) of BR04 showed that there were 4 carbons including 2 carbonyl carbons of the amide group at δ_{C} 151.5 (C-2) and 164.3 (C-4), 2 olefine carbons at δ_{C} 100.2 (C-5) and 142.1 (C-6). It indicated that BR04 belongs to the pyrimidine-2,4-dione heterocyclic group. From ¹H-NMR, ¹³C-NMR spectral data and comparison with literature, BR04 structure was identified as pyrimidine-2,4-dione (uracil) (**Figure 4**).

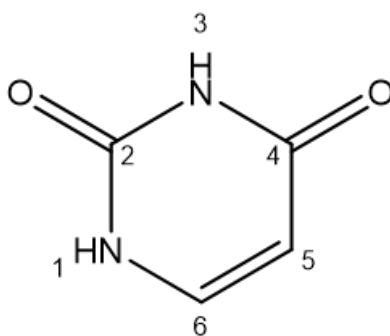


Figure 4. Pyrimidine-2,4-dione (Uracil)

Uracil is a common pyrimidine-based compound and its 5-fluoro derivative is 5-Fluorouracil (5-FU). It has been reported as a drug for the treatment of cancers, such as colon cancer and breast cancer [25]. The uracil derivatives have been reported to be used as anticancer therapies [26]. Pyrimidines, represented by uracil, thymine, and cytosine, are biologically very important heterocycles that are present in a nucleic acid (DNA and RNA) [27]. Pyrimidine has been reported to have many biological potentials, including anti-inflammatory, antibacterial, anti-cancer, antiviral, anti-HIV, anti-malarial, antihypertensive, sedative, antiallergic, and anticonvulsant activities [28, 29]. Several bioactive compounds have been identified, such as 5-fluorouracil (anti-cancer); idoxuridine and trifluridine (anti-virus); zidovudine and stavudine (anti-HIV); trimethoprim, sulfamethazine, sulphadiazine (antibacteria), etc. [29, 30]. Pyrimidine derivatives have immunomodulatory, antitumor, anti-inflammatory, membrane-stabilizing, and anti-radiation properties [29, 31]. Additionally, pyrimidine derivatives have been identified to have properties, which were similar to those of antibiotics, such as: bacimethrin (5-hydroxymethyl-2-methoxypyrimidine-4-amine) derivative is effective against some infections caused by staphylococci. Cytosine derivatives has been reported to be resistant against mycobacteria and some Gram-positive and Gram-negative bacteria [32].

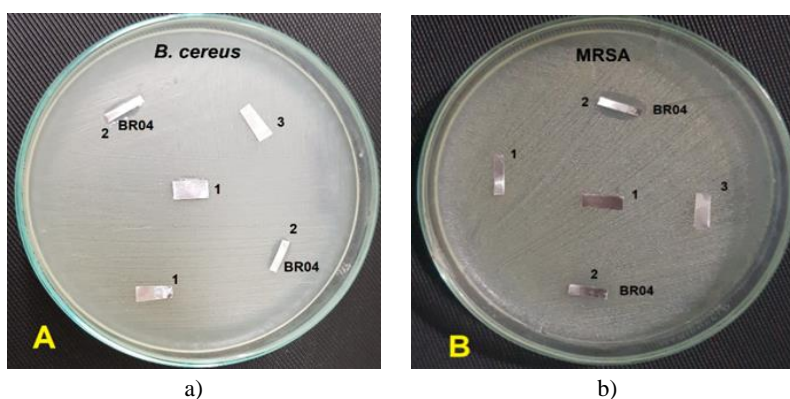
Cieplik *et al.* (2015) isolated three derivatives: [Tetrasulfane-1,4-diylbis-(6-methyl-2-phenylpyrimidine-4,5-diyl)] dimethanol, Diethyl 4,4'-disulfanediybis[6-(methoxycarbothioyl)-2-phenylpyrimidine-5-carboxylate] and 5-[[4-Ethoxyphenyl]amino]methyl-N-(3-trifluoromethyl)phenyl-6-methyl-2-phenylpyrimidine-4-amine. These derivatives were reported to have anti-fungi and anti-bacteria activities against *Candida albicans*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* within the MIC from 4 to 32 mg/mL, which was higher than Erythromycin [33].

Additionally, uracil derivatives also had the anti-oxidant ability based on the assay of DPPH free radical scavenging (5-aminouracil (IC₅₀: 3 mg/mL), 5-amino-6-methyluracil (IC₅₀: 5 mg/mL), and 5-hydroxy-6-methyluracil (IC₅₀: 15 mg/mL) [34].

Evaluation of Bioactive Abilities

Anti-Pathogenic Bacteria Activity

BRM31 was subjected to evaluate its anti-pathogenic effects against MRSA, *B. cereus* ATCC 14579, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922. Among three fractions (R_f = 0.13, 0.31, 0.37), the fraction of 0.31 (BR04) was identified as having anti-*B. cereus*, anti-*E. coli*., and anti- *P. aeruginosa* activities (**Figure 5**).



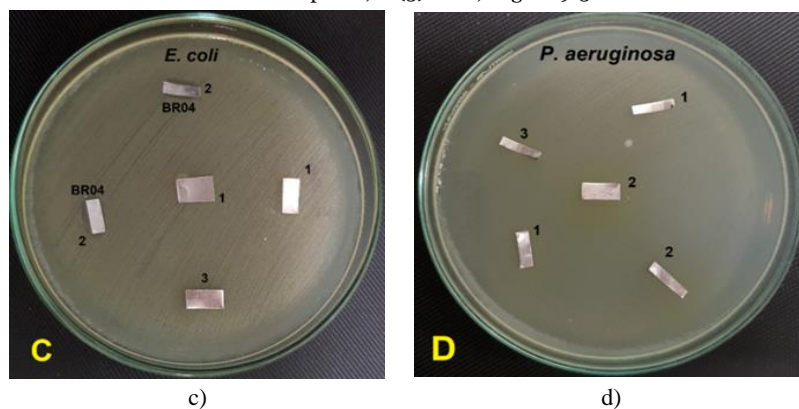


Figure 5. The anti-Gram negative and anti-Gram positive effect of BR04

a) *B. cereus* ATCC 14579; b) MRSA

c) *E. coli* ATCC 25922; d) *P. aeruginosa* ATCC 27853

The MIC values of antibacterial BR04 had antibacterial effects against MRSA, *B. cereus*, and *E. coli* (MIC of anti-MRSA: 64 $\mu\text{g/mL}$, MIC of *B. cereus*: 128 $\mu\text{g/mL}$, MIC of anti-*E. coli*: 512 $\mu\text{g/mL}$). It was concluded that BR04 had anti-Gram-positive activity. It could be explained that the cell walls of gram-positive and gram-negative bacteria are different. The Gram-positive cell wall consists of a thick layer of peptidoglycan. Meanwhile, the Gram-negative cell wall is more complex with a thin peptidoglycan layer, separated by a periplasmic membrane [35]. Due to this peptidoglycan layer, it protected Gram-negative bacteria from anti-bacterial compounds. The purified compound BR04 had a higher MIC against MRSA than the methanol extract with a MIC of 128 $\mu\text{g/mL}$.

According to the study of Jeyanthi *et al.* (2016), phenolic compounds had anti-MRSA activity (MIC = 62.5 $\mu\text{g/mL}$, the diameter of the inhibition zone was 17.66 ± 0.57 mm) [18].

In the study of Romero-Tabarez *et al.* (2006), a compound, 7-O-Malonyl Macrolactin A, isolated and purified from *B. subtilis*, was reported to have anti-MRSA, -MSSA, -*Enterococcus faecalis*, -*B. cepacia*, -*C. parapsilosi*, -*C. krusei*, and -*C. albicans* activities (MIC \geq 128 $\mu\text{g/mL}$) [36]. Kim *et al.* (2010) reported that 7-O-succinyl macrolactin A from *Bacillus polyfermenticus* KJS-2 was resistant to MRSA, MSSA, VRE, and *Enterococcus faecalis* (MIC = 2; 2; 16 and 16 $\mu\text{g/mL}$, respectively) [37]. Thus, it was indicated that BR04 had high antibacterial activities, and had the potential for antibiotic production from endophytes of medicinal plants.

In particular, there are still limited studies on the antibacterial and antifungal abilities of compounds belonging to the Uracil group. The only study was performed by Semenov *et al.* (2011) who evaluated the antibacterial and antifungal abilities of pyrimidinophanes groups when replacing Uracil rings at different positions and showed high antibacterial activity. Compounds belonging to the group of pyrimidinophanes 1, 4 and acyclic pyrimidines 8, 9, 10 were resistant to gram-negative bacteria: *P. aeruginosa*, *E. coli*, gram-positive bacteria: *S. aureus*, *B. subtilis*, *B. cereus*, *E. faecalis*, and fungal spores: *Aspergillus niger*, *C. albicans* (MIC varied from 0.2 to 500 $\mu\text{g/mL}$) [38].

Antioxidant Activity

The antioxidant activity of BR04 had the ability to scavenge DPPH free radicals at a high concentration of 1800 $\mu\text{g/mL}$ reaching nearly 50%. However, IC_{50} could not be determined because the percentage of DPPH free radical scavenging was lower than 50% and the sample amount was not enough. The results showed that the positive control had a better percentage of DPPH free radicals, at a concentration of 20 $\mu\text{g/mL}$, reaching nearly 95%.

Up to date, many studies related to the antioxidant property of compounds isolated from endophytes have been performed. Ahmed *et al.* (2018) evaluated the antioxidant property of phenolics isolated and purified from *Bacillus firmicutes*, at a concentration of 5300 $\mu\text{g/mL}$, and reported a percentage of free radical scavenging activity DPPH of 60% [39]. In the study of Giri *et al.* (2019), extracts from *Bacillus subtilis* VSG4 and *Bacillus licheniformis* VS16 at the concentration of 5000 $\mu\text{g/mL}$ had the percentage of free radical scavenging activity DPPH varied between 69.1-73.5% and 63.3-69.8%, respectively [40].

In summary, BR04 was identified as having the antioxidant property within a higher percentage of DPPH free radical scavenging, compared to previous studies. Currently, synthetic antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary hydroquinone (TBHQ) are commonly used as preservatives by many pharmaceutical, cosmetic, and food companies. However, these compounds were reported to cause liver damage, carcinogenicity, and toxicity in humans [41]. Therefore, there is an increasing demand to replace synthetic antioxidants with safer natural compounds. Some studies have focused on plant compounds [42, 43]. However, only a few reports have been studies on the antioxidant capacity of microbial extracts [44, 45]. Therefore, the results of the study are the premise to further studies on the antioxidant activity of purified compounds from bacterial extracts.

Conclusion

Methanol extract from *Bacillus amyloliquefaciens* RD26 endophytes *Phyllanthus amarus* Schum. et Thonn had the anti-MRSA ability with an inhibition zone of 25 ± 0.57 mm. BR04, purified from *Bacillus amyloliquefaciens* RD26 extract, had the highest anti-MRSA ability. BR04 was identified as pyrimidine-2,4-dion (Uracil). BR04 was anti-MRSA (MIC: 64 $\mu\text{g/mL}$), *-B.cereus ATCC 14579* (MIC: 128 $\mu\text{g/mL}$), and *-E. coli ATCC 25922* (MIC: 512 $\mu\text{g/mL}$). Additionally, BR04 was identified as the antioxidant compound at a concentration of 1800 mg/mL reaching to nearby 50%. The results of this study indicated that BR04 can be a promising compound in the future in many fields, such as pharmacy and agriculture.

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