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Isolation and Purification of Bioactive Compounds from Endophytic *Bacillus sp.* RD26 Associated with *Phyllanthus Amarus*

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ABSTRACT

This study investigated the bioactive properties of *Bacillus amyloliquefaciens* RD26, an endophyte isolated from *Phyllanthus amarus*. The methanol extract of *Bacillus* sp. RD26 showed significant antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA), with an inhibition zone of 25 ± 0.57 mm. The antibacterial compound, BR04, was isolated and identified as pyrimidine-2,4-dione (uracil), showing potent antimicrobial effects with minimum inhibitory concentrations (MICs) of 64 µg/mL against MRSA, 128 µg/mL against *Bacillus cereus*, and 512 µg/mL against *Escherichia coli*. In addition, BR04 showed antioxidant activity and scavenged up to 50% of DPPH free radicals at a concentration of 1800 µg/mL. These findings highlight the potential of BR04 as a bioactive compound for pharmaceutical and agricultural applications, particularly in combating infectious pathogens and providing a natural alternative to synthetic antioxidants.

Keywords: *Bacillus amyloliquefaciens*, Endophyte, *Bacillus* sp. RD26, MRSA, Antioxidant activity, Bioactive compounds

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Introduction

Endophytes, which include fungi, bacteria, and actinomycetes, are microorganisms that inhabit the tissues of healthy plants without causing harm to the host. These microorganisms are a rich source of natural products with a wide range of biological activities [1-4]. Typically located in the intercellular spaces of plant tissues, endophytes can be isolated from all parts of the plant, including its seeds [5]. Natural compounds derived from endophytes exhibit beneficial properties such as antibacterial, antifungal, and anticancer activities, making them valuable for applications in medicine, agriculture, and industry [2, 6, 7]. Furthermore, many endophytes produce bioactive metabolites that aid in the treatment of diseases in plants, animals, and humans, either directly or indirectly [8-10].

The *Bacillus* genus is particularly well-known for its ability to produce bioactive substances, such as antibiotics, proteins, enzyme inhibitors, and pharmacologically active components [11, 12]. These bacteria are prolific producers of various antibacterial and bioactive peptides with diverse chemical structures. Lipopeptides, in particular, synthesized by strains such as *Bacillus subtilis* and *Bacillus amyloliquefaciens*, are crucial in antifungal activity [13-16]. Tabbene *et al*. [17] demonstrated that purified bacillomycin from *Bacillus* exhibited anti-Candida effects. Likewise, research by Jeyanthi *et al*. [18] highlighted that phenolic compounds produced by *Bacillus amyloliquefaciens* MHB1 displayed anti-MRSA properties.

In our previous research, we successfully optimized the culture conditions for fermenting *Bacillus* sp. RD26 to target MRSA [19]. This current study aims to further isolate MRSA-resistant compounds from *Bacillus* sp. RD26 and evaluate their bioactive properties.

Materials and Methods

Microorganism cultivation

The *Bacillus* sp. RD26 strain, isolated from *Phyllanthus amarus* Schum. & Thonn. was grown in Tryptic Soy Broth (TSB). Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA) was provided by Nam Khoa Co. Ltd, Vietnam. Additional strains, *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922, were obtained from the Microorganism Laboratory at Ho Chi Minh City Open University, Vietnam, and cultured in Nutrient Broth (NB).

Bacillus sp. RD26 identification

The classification of *Bacillus* sp. RD26 as an endophytic bacterium was performed using Cowan and Steel's bacterial identification keys.

Bacillus sp. RD26 extraction

The *Bacillus* sp. RD26 strain was cultivated in a specialized medium containing 7.36 g/L peptone, 15 g/L glucose, 0.72 g/L $CaCO_3$, and 0.6 g/L $MgSO_4$. After fermentation, the culture was centrifuged at 10,000 rpm for 10 minutes to separate the supernatant. A 1:1 methanol solution was added to 100 mL of the supernatant, and the solvent was evaporated to obtain a crude extract.

Evaluation of anti-MRSA activity

To assess the anti-MRSA activity of the *Bacillus* sp. RD26 extract, the disk diffusion method was utilized. A suspension of MRSA (10^8 CFU/mL) was evenly spread on Mueller-Hinton Agar (MHA) plates. Filter paper discs (6 mm diameter) soaked in 10 µL of the extract were placed on the agar surface. The plates were incubated at 37 °C for 24 hours, and the zones of inhibition were measured. Dimethyl sulfoxide (DMSO) served as the negative control. The Minimum Inhibitory Concentration (MIC) values were determined using a dilution method as outlined in previous studies.

Purification of antibacterial compound from Bacillus sp. RD26

The extract of *Bacillus* sp. RD26 was combined with silica gel and loaded into a 50 × 3 cm column (230–400 mesh). Chromatographic separation used ethyl acetate: methanol (EA: Me) systems in ratios of 100:0, 90:10, 80:20, 70:30, 60:40, and 0:100. Fractions were collected, evaporated, and tested for anti-MRSA activity via thin-layer chromatography (TLC) with a chloroform: methanol solvent (10:1). Active fractions underwent further purification with EA: Me systems of increasing polarity and a silica gel column using 100% chloroform.

TLC analysis

TLC plates were visualized under UV light (254 nm), treated with MRSA suspension (1–2 × 10^6 CFU/mL), and incubated at 25 °C for 48 hours. Tetrazolium salt detected bacterial presence, with clear zones indicating antibacterial activity.

Compound identification

The anti-MRSA compound's structure was determined using NMR spectroscopy (^{1}H-NMR at 500 MHz; ^{13}C-NMR at 125 MHz) at the Vietnam Academy of Science and Technology.

Evaluation of bioactive activities

Anti-pathogenic bacterial activity

Bioautography: Compounds were spotted onto a TLC plate using a suitable solvent system. Separated compounds were visualized under UV light at 254 nm, and their Rf values were recorded. Agar plates were inoculated with pathogenic bacteria at 10^8 CFU/mL. The TLC plate was divided into sections corresponding to each compound spot and placed onto the agar plates. After incubation at 10 °C for 12 hours and then at 37 °C for 24 hours, zones of inhibition were measured.

Minimum Inhibitory Concentration (MIC): Antimicrobial properties were tested using the MIC method against strains of *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922.

Antioxidant activity

The antioxidant capacity was analyzed using the DPPH radical scavenging assay. The percentage of scavenging activity was calculated with the formula: scavenging activity = [(ODc - ODm)/ODc]×100. Where ODc and ODm represent control and sample optical densities, respectively [20]. The IC_{50} value, indicating the concentration at which 50% of radicals were scavenged, was calculated using a linear regression equation. A smaller IC_{50} value corresponded to stronger antioxidant activity.

Characterization of Bacillus sp. RD26

Biochemical assays conducted according to Cowan and Steel classification confirmed that *Bacillus* sp. RD26 shares an 88.89% similarity with *Bacillus amyloliquefaciens* **(Table 1)**.

Table 1. Biochemical profile of *Bacillus* sp. RD26

Assay	Result	Assay	Result	Assay	Result
Catalase	+	Galactose	d	Urease	-
Motility	+	Mannose	d	Indole	-
Growth at 50 °C	+	Melibiose	-	VP	+
Growth in 10% NaCl	+	Raffinose	+	Nitrate	+
Anaerobic growth	-	Salicin	+	Casein	+
Glucose	+	Xylose	+	Amylase	+
Cellobiose	+	Citrate	+	Oxidase	+

Conclusion: The organism was identified as *Bacillus amyloliquefaciens*. (*Note: Positive (+), Negative (-), Variable (d).*)

Assessment of anti-MRSA activity

The methanol extract of *Bacillus amyloliquefaciens* RD26 exhibited significant anti-MRSA activity, forming a 25 ± 0.57 mm inhibition zone **(Figure 1)**.

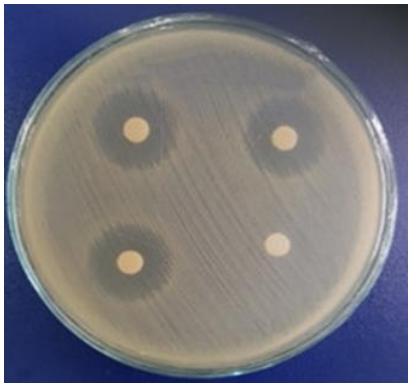


Figure 1. Disk Diffusion Test for Anti-MRSA Activity.

TLC analysis of BRM extract

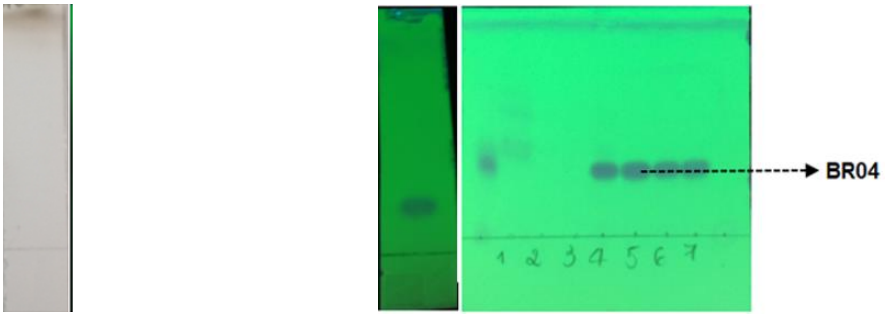
The BRM extract was subjected to thin-layer chromatography using chloroform: methanol (10:1) as the solvent system. The Rf values of the separated compounds were noted as 0.13, 0.31, 0.37, 0.52, 0.55, 0.68, 0.73, 0.78, and 0.90.

Isolation of antibacterial compounds from Bacillus sp. RD26

A total of 15.53 g of the BRM extract was subjected to column chromatography using an ethyl acetate-methanol (EA-Me) solvent system with increasing polarity. The solvent ratios were set at 100:0, 90:10, 80:20, 70:30, 60:40, and 0:100, resulting in five fractions, BRM1 to BRM5. These fractions were tested for anti-MRSA activity, and the BRM3 fraction showed the highest inhibition zone of 18.00 ± 0.00 mm.

For further purification, 4.6 g of the BRM3 fraction was reprocessed using an EA-Me solvent system of increasing polarity (80:1, 50:1, 30:1, 10:1, and 100% methanol). This yielded a single trace, labeled BRM31. The BRM31 (212.34 mg) was then purified using a normal-phase silica gel column with various chloroform: methanol ratios (50:1, 30:1, 10:1, and 1:1). TLC analysis grouped the traces into four distinct bands, BRM311 to BRM314. The

BRM313 fraction (38.31 mg) was further purified using normal-phase chromatography with 100% chloroform. TLC identified three sub-fractions within BRM313, named BRM3131 to BRM3133. The compound with Rf 0.31 was found to show UV light absorption at 254 nm but was non-reactive to 10% H2SO4/EtOH **(Figure 2)**. This compound was identified as BR04, isolated at 9.41 mg.

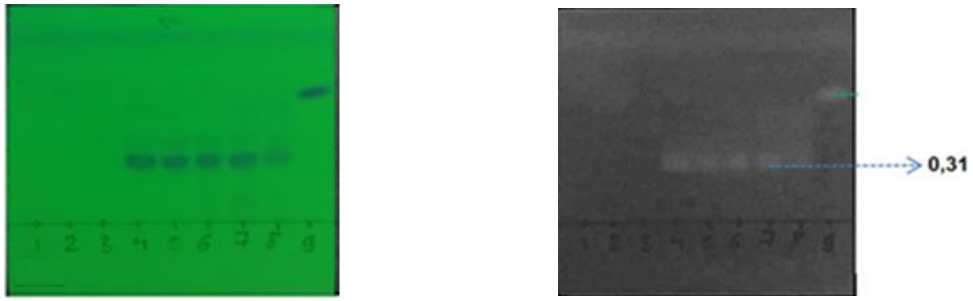


a) b)

Figure 2. TLC analysis of BR04 in chloroform: methanol (10:1); a) Not reactive to 10% H2SO4/EtOH, b) UV light absorption at 254 nm

Anti-MRSA activity in purified compounds

The BRM31 fraction was analyzed using bioautography to assess its anti-MRSA activity. The analysis revealed a white spot with an Rf value of 0.31, indicating its strong antibacterial effect **(Figure 3)**.



a) b)

Figure 3. a) TLC analysis of BRM31 fraction; b) Bioautography of BRM31 fraction showing inhibition against MRSA

Structural identification of the MRSA-resistant compound

The purified compound BR04 was obtained as a white powder. NMR spectroscopy (1H and 13C) was used to determine its structure. The 1H-NMR spectrum showed two amine protons at δH 10.98 (1H, s, 1-NH) and 10.79 (1H, s, 3-NH), and two cis-coupled olefin protons at δH 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 revealed two carbonyl carbons at δC 151.5 (C-2) and 164.3 (C-4) and two olefin carbons at δC 100.2 (C-5) and 142.1 (C-6). Based on the NMR data and literature comparison, BR04 was identified as pyrimidine-2,4-dione, also known as uracil **(Figure 4)**.

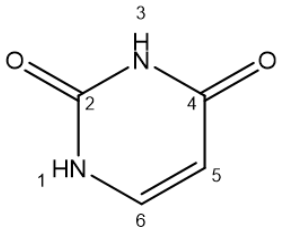


Figure 4. Pyrimidine-2,4-dione (Uracil).

Uracil derivatives, such as 5-fluorouracil (5-FU), have been widely used in the treatment of various cancers, including colon and breast cancer [21]. Pyrimidines, which include uracil, thymine, and cytosine, are essential for

the structure of DNA and RNA and have diverse therapeutic benefits. These include anticancer, anti-inflammatory, antibacterial, antiviral, anti-HIV, anti-malarial, and more [22, 23]. Several pyrimidine derivatives, such as 5-fluorouracil, idoxuridine, and trimethoprim, have been identified as bioactive compounds with significant pharmacological effects [23, 24]. Some pyrimidine derivatives also show antibiotic-like properties, with compounds such as bacimethrin and cytosine derivatives being effective against various bacteria [25].

Cieplik *et al*. [26] discovered pyrimidine compounds with antifungal and antibacterial activities, demonstrating potency against pathogens like Candida albicans, Escherichia coli, and Staphylococcus aureus, with minimum inhibitory concentrations (MIC) ranging from 4 to 32 mg/mL, outperforming erythromycin. Additionally, certain uracil derivatives exhibit antioxidant potential, as seen in their ability to scavenge DPPH free radicals. The antioxidant activity of 5-aminouracil, 5-amino-6-methyluracil, and 5-hydroxy-6-methyluracil was confirmed in DPPH assays, with reported IC50 values of 3 mg/mL, 5 mg/mL, and 15 mg/mL, respectively [27].

Evaluation of bioactive properties

Antibacterial activity against pathogens

The anti-pathogenic potential of BRM31 was assessed against various bacterial strains including MRSA, *Bacillus cereus* ATCC 14579, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922. Among the three fractions (Rf values = 0.13, 0.31, 0.37), the fraction with an Rf value of 0.31, identified as BR04, exhibited significant activity against *B. cereus*, *E. coli*, and *P. aeruginosa* **(Figure 5)**.

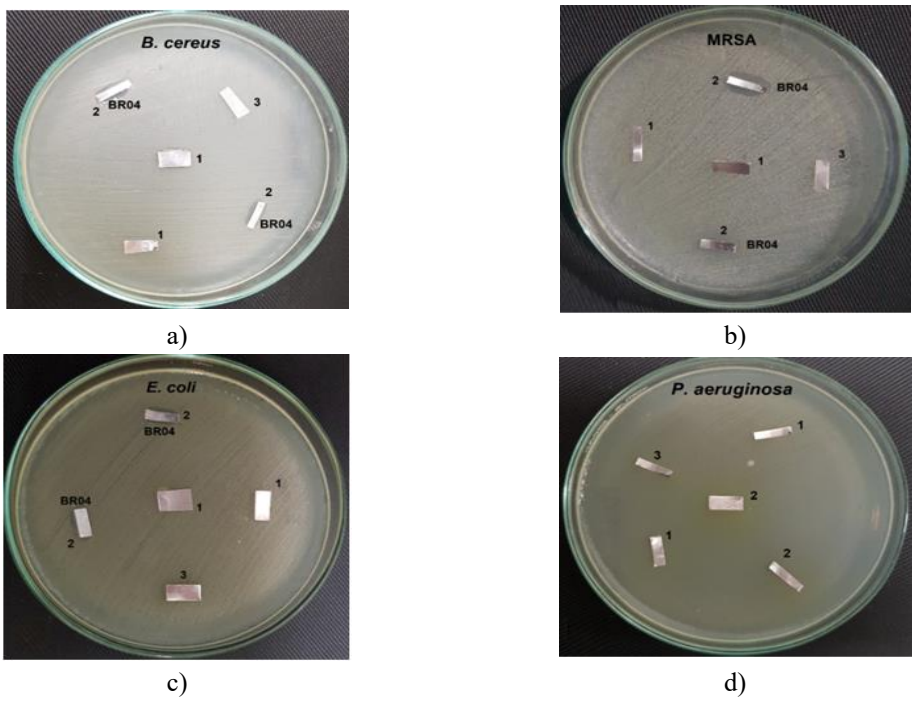


Figure 5. The anti-Gram negative and anti-Gram positive effect of BR04; a) *B. cereus* ATCC 14579, b) MRSA, c) *E. coli* ATCC 25922; and d) *P. aeruginosa* ATCC 27853

The minimum inhibitory concentration (MIC) values of BR04 against *MRSA*, *B. cereus*, and *E. coli* were determined to be 64 µg/mL, 128 µg/mL, and 512 µg/mL, respectively. These results suggest that BR04 possesses potent anti-Gram-positive activity. The distinction between Gram-positive and Gram-negative bacteria lies in their cell wall structure. Gram-positive bacteria have a thick peptidoglycan layer, whereas Gram-negative bacteria have a more intricate wall with a thinner peptidoglycan layer and an additional periplasmic membrane [28]. This structural difference likely contributes to the enhanced resistance of Gram-negative bacteria to antibacterial compounds. Notably, BR04 demonstrated a higher MIC value against MRSA compared to the methanol extract, which had an MIC of 128 µg/mL.

In a study by Jeyanthi *et al*. [18], phenolic compounds showed anti-MRSA activity, with a MIC of 62.5 µg/mL and an inhibition zone diameter of 17.66 ± 0.57 mm. Romero-Tabarez *et al*. [29] identified 7-O-Malonyl Macrolactin A from *B. subtilis*, which displayed anti-MRSA, MSSA, *Enterococcus faecalis*, *B. cepacia*, *C.*

parapsilosis, *C. krusei*, and *C. albicans* activities, with MIC values of 128 µg/mL or greater [29]. Another study by Kim *et al*. [30] reported that 7-O-succinyl macrolactin A from *Bacillus polyfermenticus* KJS-2 showed resistance against MRSA, MSSA, VRE, and *Enterococcus faecalis*, with MIC values of 2 to 16 µg/mL. These findings indicate that BR04 has significant antibacterial properties and potential for antibiotic development from medicinal plant endophytes.

However, research on the antibacterial and antifungal activities of Uracil derivatives remains scarce. Semenov *et al*. [31] investigated pyrimidinophane compounds, which replace Uracil rings in different positions, showing strong antibacterial effects. Compounds from the pyrimidinophane and acyclic pyrimidine groups (1, 4, 8, 9, 10) were found to be resistant to both Gram-negative bacteria (*P. aeruginosa*, *E. coli*) and Gram-positive bacteria (*S. aureus*, *B. subtilis*, *B. cereus*, *E. faecalis*), as well as fungal spores (*A. Niger*, *C. albicans*), with MIC values ranging from 0.2 to 500 µg/mL [31].

Antioxidant activity

BR04 demonstrated antioxidant potential by effectively scavenging DPPH free radicals, achieving approximately 50% scavenging at a high concentration of 1800 mg/mL. However, due to the scavenging activity being under 50% and an insufficient amount of sample, the IC50 value could not be established. In contrast, the positive control exhibited much higher efficiency, scavenging nearly 95% of DPPH free radicals at a concentration of 20 µg/mL. Numerous studies have investigated the antioxidant properties of endophyte-derived compounds. For instance, Ahmed *et al*. [32] evaluated phenolic compounds from *Bacillus firmicutes* at a concentration of 5300 µg/mL, reporting 60% scavenging of DPPH free radicals. In another study, Giri *et al*. [33] found that extracts from *Bacillus subtilis* VSG4 and *Bacillus licheniformis* VS16, when tested at 5000 µg/mL, exhibited scavenging activities of DPPH free radicals in the range of 63.3-73.5%.

When compared to these studies, BR04 exhibited a superior scavenging ability. Synthetic antioxidants, such as BHA, BHT, and TBHQ, are commonly used in various industries, including pharmaceuticals, cosmetics, and food preservation. However, these synthetic compounds are associated with adverse health effects, including liver toxicity, carcinogenicity, and overall toxicity in humans [34]. This has spurred interest in finding safer, natural alternatives to synthetic antioxidants. While much of the research has focused on plant-based antioxidants, microbial extracts—especially from bacteria—have received less attention for their antioxidant properties [35-38]. The findings from this study provide a foundation for future research into bacterial-derived antioxidants.

Conclusion

The methanol extract from *Bacillus amyloliquefaciens* RD26, obtained from *Phyllanthus amarus* endophytes, demonstrated anti-MRSA activity with an inhibition zone of 25 ± 0.57 mm. The purified compound, BR04, which was identified as pyrimidine-2,4-dion (Uracil), exhibited the strongest anti-MRSA activity, with MIC values of 64 µg/mL against MRSA, 128 µg/mL against *B. cereus* ATCC 14579, and 512 µg/mL against *E. coli* ATCC 25922. Furthermore, BR04 displayed antioxidant activity, scavenging almost 50% of DPPH free radicals at a concentration of 1800 mg/mL. These results suggest that BR04 holds promise for future use in various fields, particularly pharmaceuticals and agriculture.

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Conflict of Interest: None

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Ethics Statement: None

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