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Antibacterial Activity Screening of *Bacillus* sp. AM12 Associated with Mangrove Soil

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Abstract

The use of synthetic antibiotics to treat bacterial infections can trigger resistance of pathogenic bacteria to antibiotics. One effort to overcome this is to explore microorganisms that produce antibacterial compounds from nature. A previous study reported isolates of *Bacillus* sp. AM12 from mangrove soil displayed antibacterial potential based on preliminary screening of the perpendicular streak test. This study was designed to confirm the antibacterial potential of *Bacillus* sp. AM12 uses chemical solvent extraction. *Bacillus* sp. AM12 was fermented in 100 mL liquid ISP-2 sterile and shaken at 150 rpm for 7 days. The supernatant was filtered from the cell mass using Whatman paper and extracted using 100 mL of ethyl acetate solvent (1:1, v/v) twice. The filtrate was evaporated at 40ºC until a thick, clear yellowish colored extract was obtained. The thick extract was tested for antibacterial activity using the Kirby-Bauer method against two Gram positive and two Gram negative bacterial targets. Antibacterial screening showed moderate diameter zone of inhibition of 6.72 ± 0.21 mm, 6.82 ± 0.15 mm, and 6.62 ± 0.21 mm against *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* FNCC 0405, and *Klebsiella pneumoniae* ATCC 70060, respectively. However, no antibacterial activity was observed against *Escherichia coli* ATCC 25922. Analysis of the chemical composition of ethyl acetate crude extracts using GC-MS has detected 10 different compounds, in which the 3 most dominant antibacterial compounds were Benzene, 1,2,4-Trimethyl-, Benzene, 1-ethyl-2-methyl- and 2-butoxyethyl acetate. In general, these results provide an initial description of *Bacillus* sp. AM12 is a potential of antibacterial producer.

Keywords: antibacterial; bacterial infections; bioprospecting; natural products.

INTRODUCTION

Bacterial infection is one of the health problems in Indonesia and many regions in the world. Generally, bacterial infections are treated using synthetic antibiotics such as amoxicillin and tetracycline to inhibit growth or kill infectious bacteria (Simanjuntak et al., 2022; Susanto, 2020). However, the use of synthetic antibiotics often leads to negative effects such as nausea, vomiting and diarrhoea (Eveliani & Gunawan, 2021; Octavia et al., 2021; Parisa et al., 2022). In addition, misused and overused of antibiotics also trigger resistance to pathogenic bacteria which makes them more difficult to be treat (Amalia et al., 2017).

Bacterial resistance can occur as a result of administering antibiotics that are not in the right dose, not in the right diagnosis, and not against the correct target (Monica et al., 2018; Sugireng & Rosdarni, 2020; Afrilia & Alam, 2022). Despite of the importance of good education for society to consume antibiotics wisely, one important aspect is to explore microorganisms that produce secondary metabolite compounds with antibacterial activity from nature, which can later be developed as new antibiotic drugs with better effectiveness against bacterial infections.

Bacteria play a crucial role as the producers of a variety of secondary metabolites, including antibacterial activity. To date, bioprospecting on bacteria has mainly been focused on exploring terrestrial ecosystems, which consequently increases the chance of dereplication of the same compounds (Jose & Jha, 2017). Therefore, exploration in other ecosystems, especially marine and coastal environments, needs to be explored to obtain bacterial isolates that produce new and diverse antibacterial compounds.

The mangrove ecosystem is a meeting point between land and sea estuaries. This area experiences extreme high and low water conditions resulting in the integration of chemical, physical and biological elements of land and sea (Djamaluddin, 2018). The mangrove ecosystem shows high biodiversity, including a diversity of types of bacteria that can adapt to variations in high salinity, high temperatures, strong wind pressure, and the availability of water with low oxygen levels (Baderan & Rahim, 2017). This adaptability causes bacteria in mangrove sediments to produce secondary metabolite compounds,

which have the potential to be developed as alternative treatments for various diseases, such as antibiotics, antioxidants, anticancer and larvicides (Ambeng et al., 2019).

The Ngurah Rai Bali Forest Park is the largest mangrove ecosystem in Bali. A previous report described 68 bacterial isolates from the Ngurah Rai Mangrove forest, of which 22 of them displayed antibacterial activity (Indraningrat et al., 2021). One of the isolates, labelled as *Bacillus* sp. AM12 was isolated from the soil habitat of the *Avicennia marina* mangrove plant. Previous research did not employ chemical to investigate the antibacterial potential of *Bacillus* sp. AM 12 (Indraningrat et al., 2021). Therefore, this research will be focused to study the isolate *Bacillus* sp. AM12 by focusing on fermentation, chemical extraction, antibacterial screening and chemical profiling using gas chromatography and mass spectrometry (GC/MS). It is expected that the results of this research will provide a more comprehensive picture of the potential of *Bacillus* sp. AM12 is to be developed as a candidate for producing antibacterial compounds.

METHODS

Extraction of Secondary Metabolite Components of *Bacillus* sp. AM12

Stages of extraction of bacterial isolates *Bacillus* sp. AM12 begins by growing the isolate in 200 mL of sterile liquid ISP-2 medium (4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, 20 g/L bacto agar) and incubating at 28ºC. The isolate in liquid media was shaken using a shaker for 7x24 hours at a speed of 150 rpm. After 7 days, the supernatant of the pure culture was separated from the cell mass by filtration using Whatman paper no. 1.

The supernatant obtained was extracted using pro-analysis ethyl acetate solvent with a ratio of 1:1 (v/v) and repeated twice. The accumulation of macerate is then separated using a separating funnel. At this separation stage, the organic phase and liquid phase fractions formed were screened initially by taking 100 µL samples from each fraction and tested against the bacteria *S. aureus* ATCC 25923, *S. mutans* FNCC 0405, *E. coli* ATCC 25922, and *K. pneumoniae* ATCC 700603. Fractions that are proven to have an inhibition zone will be evaporated at a temperature of 40 ºC until a thick extract is obtained, which will then be weighed using an analytical balance.

Antibacterial Activity Test

The test was carried out using the Kirby-Bauer method aseptically in a biosafety cabinet (BSC). The first step is to place the disc of the substance to be tested on an agar media that already contains the test bacteria. A total of 200 µL each of the bacteria *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* FNCC 0405, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 were suspended in Luria Bertani (LB) media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L bacto agar) and spread with a sterile cotton swab. Each plate was divided into three quadrants and contains three discs, indicating that for each test on the extract, positive control and negative control for each test bacteria were carried out three times. Each sterile paper disc with a diameter of 6 mm was dripped with 20 µL of *Bacillus* sp. AM12 extract. Next, each paper disc was placed in LB media containing bacterial suspension and then incubated for 24 hours at a temperature of 37°C.

The negative control used was ethyl acetate, while the positive control used was the broad spectrum antibiotic levofloxacin. The diameter zone of inhibition was assessed by measuring the halo zone (clear) that formed around the paper disc after 1x24 hours using a calliper. Measurements of the diameter of the inhibition zone formed were carried out 4 times in vertical, horizontal and diagonal positions and then averaged. The zone of inhibition that forms would indicate that there is inhibition of bacterial growth due to the presence of antibacterial compounds. Interpretation of antibacterial test results will be grouped into 4 categories based on the diameter of the inhibition zone, namely weak (0-5 mm), medium (5-10 mm), strong (10 – 20 mm) and very strong (> 20 mm) (Davis & Stout, 1971).

Gram staining

Morphological characteristics and type of cell wall of *Bacillus* sp. AM12 were evaluated using Gram staining as previously described. Bacterial cells were observed under a light microscope (Leica DM750) with 100x magnification(Al-Enazi et al., 2022).

GC/MS analysis

Briefly, 0.1 grams of the crude extract was sent to the Forensic Laboratory Polda Bali for further analysis. The chromatogram results were checked with the literature to determine the type of content and function of the detected bioactive compounds.

RESULT

Fermentation and Extraction

Extraction of cell free supernatant of *Bacillus* sp. AM 12 using ethyl acetate resulted in two layers in the mixture, namely the clear organic phase fraction and the yellowish liquid phase (Figure 1). The organic phase contains secondary metabolite compounds from extracted bacterial isolates, while the liquid phase only contains primary metabolites and solvents. Antibacterial testing was carried out on each fraction phase with the aim of determining which phase had antibacterial activity.

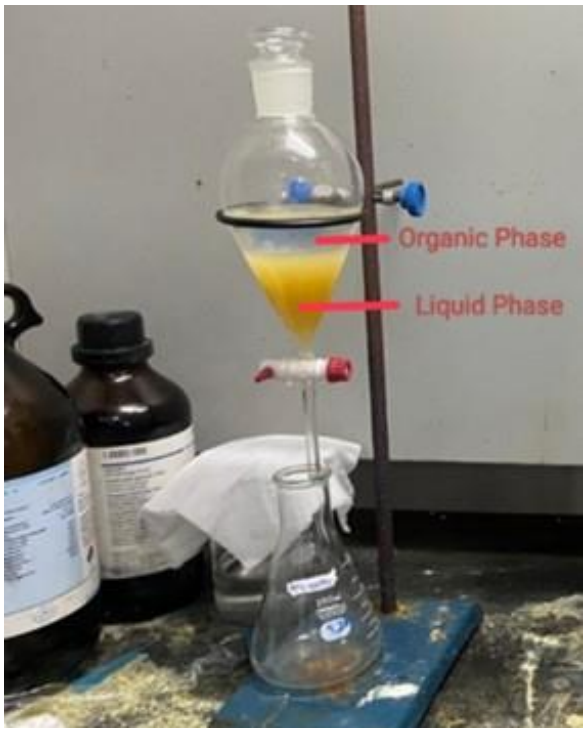

Figure 1. Extraction of cell free supernatant of Bacillus sp. AM 12 using ethyl acetate resulted in organic and liquid phases.

Based on the preliminary test results, it was found to be the inhibition zone was only present in the organic phase (Figure 2). After the organic phase was evaporated, a thick, clear yellowish-colored extract was obtained, then 1 mL of ethyl acetate was added to obtain a clear yellowish colored liquid extract weighing 1.40 grams (Figure 1).

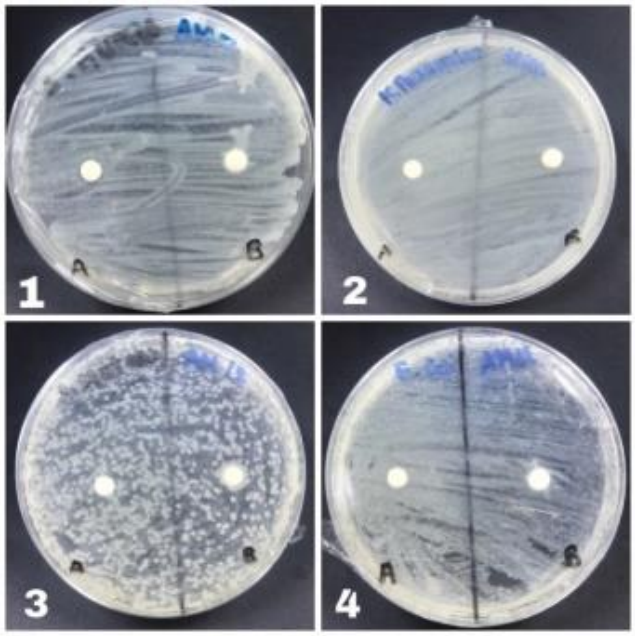

Figure 2. Pre-screening for Organic Phase (A) and Liquid Phase (B) of ethyl acetate *Bacillus* sp. AM12 against bacterial tests, namely 1: *S. aureus* ATCC 25923, 2: *K. pneumoniae* ATCC 700603, 3: *S. mutans* FNCC 0405, and 4: *E. coli* ATCC 25922**.**

Gram staining of bacterial isolate sp.AM12

The Gram staining test of the sp.AM12 isolate was Gram-positive. Morphological characteristics showed that the isolate has rod-shaped bacteria as shown under microscope observation (Figure 4).

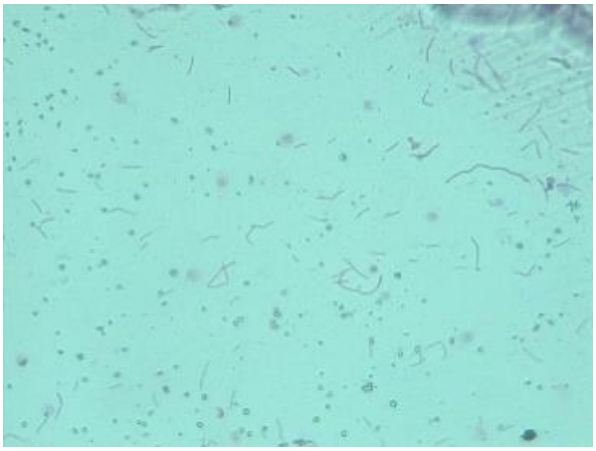

Figure 4. Gram Staining of *Bacillus* sp. AM12 under light microscope observation (1000x magnification)

Antibacterial Activity Test

Antibacterial screening showed that the extract of *Bacillus sp.* AM12 inhibited *S. aureus* ATCC 25923 with an average inhibition zone diameter of 6.72 ± 0.21 mm, *S. mutans* FNCC 0405 with an average of 6.82 ± 0.15 mm, and *K. pneumoniae* ATCC 700603 with an average of 6.62 ± 0.21 mm (Figure 5 and Table 1). Based on this result, the diameter zone of inhibition is classified as moderate activity. However, the antibacterial activity of *Bacillus sp*. AM12 was much smaller than levofloxacin, which is the positive control in this study. Levofloxacin even has antibacterial activity against *E. coli* ATCC 25922. Meanwhile, the extract of *Bacillus sp*. AM12 has no inhibitory effect on *E. coli* ATCC 25922.

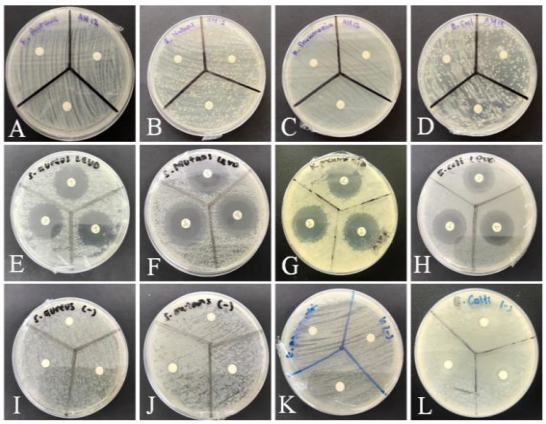

Figure 5. Antibacterial Activity Test Results of Bacillus sp extract. AM12 against A: *S. aureus*, B: *S. mutans*, C: *K. pneumoniae*, and D*: E. coli*. Results of Positive Control Antibacterial Activity Test (Levofloxacin) against E: *S. aureus*, F: *S. mutans*, G: *K. pneumoniae*, and H: *E. coli*. Results of Negative Control Antibacterial Activity Test *(Ethyl Acetate*) against I: *S. aureus*, J: *S. mutans*, K: *K. pneumoniae*, and L: *E. coli*.

Table 1. Results of antibacterial screening.

Test Bacteria	Sample	Mean Inhibition Zone Diameter (mm) ± SD	Interpretation
Staphylococcus aureus ATCC 25923	*Bacillus* sp extract. AM12	6.72 ± 0.21	Moderate
	Levofloxacin	17,26 ± 0,59	Strong
	Ethyl Acetate	0±0	-
Streptococcus mutans FNCC 0405	*Bacillus* sp extract. AM12	6,82 ± 0,15	Moderate
	Levofloxacin	18,80 ± 0,49	Strong
	Ethyl Acetate	0±0	-
Klebsiella pneumoniae ATCC 70060	*Bacillus* sp extract. AM12	6,62 ± 0,21	Moderate
	Levofloxacin	19,22 ± 0,90	Strong
	Ethyl Acetate	0±0	-
Escherichia coli ATCC 25922	*Bacillus* sp extract. AM12	0±0	-
	Levofloxacin	14,64 ± 1,58	Strong
	Ethyl Acetate	0±0	-

Analysis GC-MS

The GC/MS analysis results (Figure 6) showed that 156 peaks of compounds were detected. Of these many compounds, ten compounds corresponded to antibacterial activity (Table 2) present in the ethyl acetate crude extract of *Bacillus* AM 12. These ten compounds have been previously reported to play a role as antibacterial in other studies after literature search.

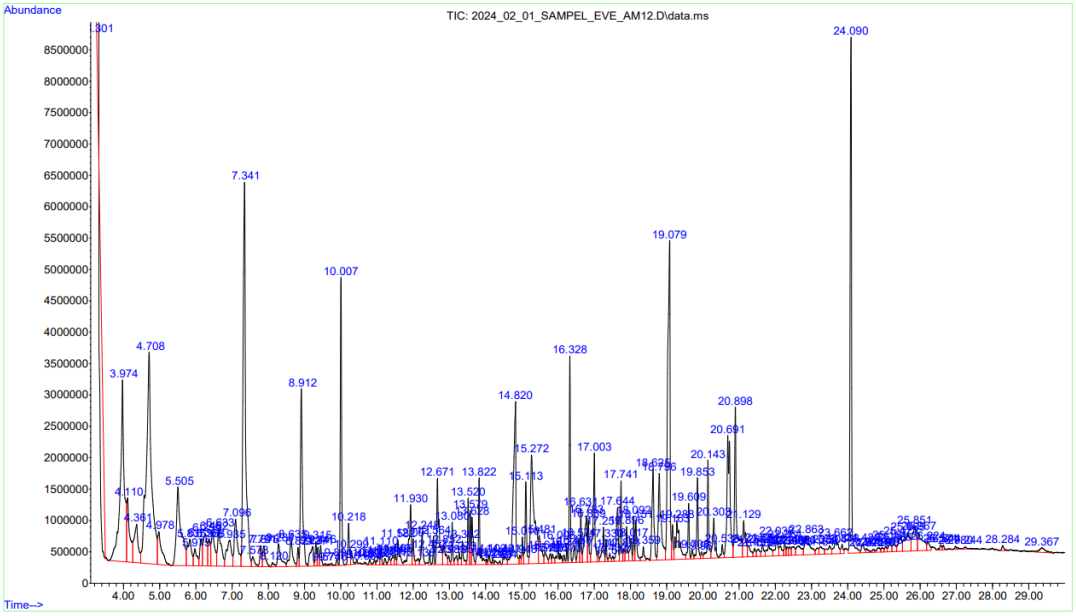

Figure 6. GC/MS chromatograms of ethyl acetate crude extracts of *Bacillus* sp. AM12.

Table 2. Results of GC-MS analysis of bacterial isolates *Bacillus* sp. AM12.

Compounds	*Molecular formula*	*Activity*	*Peak areas*	*Retention time*	*References*
Benzene, 1,2,4-trimethyl-	C9H12	Antibacterial	7.59	4.7	(Andila and Nugroho, 2022)
2-Butoxyethyl acetate	C8H16O3	Antibacterial	6.87	7.3	(Khedhri et al., 2023)
Bis(2-ethylhexyl) phthalate	C24H38O4	Antibacterial	5.20	24	(Javed et al., 2022)
n-Hexadecanoic acid	CH3(CH2)14COOH	Antibacterial	5.07	19	(Ganesan et al., 2022)
Benzylmonoxime	C12H11NO2	Antibacterial	4.92	3.9	(Abuskhuna et al., 2020)
Dodecanoic acid	C12H24O2	Antibacterial	2.81	14.8	(Shen et al., 2021)
Ethanol, 2-butoxy-	C6H14O2	Antibacterial	2.65	3.3	(Woiski et al., 2020)
Oleic Acid	C18H34O2	Antibacterial	2.59	20.7	(Dilika et al., 2000)
Mesitylene	C9H12	Antibacterial	1.32	4.11	(Savithri and Rajakumar, 2019)
Benzene, 1-ethyl-2-methyl-	C9H12	Antibacterial	1.08	4.36	(Safara et al., 2022)

DISCUSSION

Fermentation and Extraction

Fermentation of *Bacillus* sp AM12 was carried out in liquid ISP-2 media for 7 days so that the number of secondary metabolites produced by bacteria was maximized. Bacterial growth occurs for 5-7 days, referred to as the stationary phase, in which there is an increase in the amount of bacterial cell biomass so that more secondary metabolites are formed (Syarifuddin et al., 2022). However, the speed of bacterial growth in this phase is influenced by the bacterial growth media, such as nutrition, pH, temperature, and air humidity. Thus, the bacterial growth medium used is a liquid fermentation medium so that the composition and concentration of the medium are easier to regulate according to optimal environmental conditions for bacterial growth (Saida et al., 2022). Liquid ISP-2 media was chosen because it consists of simple carbon bases such as glucose, as well as organic nitrogen sources such as malt and yeast extract, which help growth, pigment formation, and the production of antibacterial substances (Al-Enazi et al., 2022). The bacterial fermentation media will change color to become cloudier as the bacteria grow in it. The more concentrated or turbid a medium is, the higher the number and mass of bacterial cells in it (Saida et al., 2022).

Extraction can be defined as the process of separating or pulling out a material from a mixture using a certain solvent until the concentration between the material and the solvent reaches equilibrium (Azis, 2020). In this research, the liquid-liquid extraction method uses a separating funnel with ethyl acetate as the solvent to obtain an organic phase and a liquid phase to separate compounds based on their relative solubility (Saida et al., 2022). Ethyl acetate was chosen as a solvent because it is semipolar, can attract polar and nonpolar solvents, and is volatile, non-toxic, and non-hygroscopic (Pratama et al., 2017). The organic phase has an inhibition zone because this phase contains secondary metabolite compounds from bacterial isolates that have been extracted. In contrast, the liquid phase only contains primary metabolites and solvents (Serrano et al., 2021). The organic phase extract was concentrated using a rotary evaporator to obtain a thick extract. After evaporation, a thick, clear yellowish-colored extract was obtained, and then 1 mL of ethyl acetate was added to obtain a clear yellowish-colored liquid extract weighing 1,401 grams.

Antibacterial Activity Test

The antibacterial activity test was carried out using the Kirby-Bauer method because the method is rather straightforward, affordable, and does not require complicated equipment (Rahayu et al., 2022). Results of antibacterial activity screening indicated that the crude extracts displayed moderate activity against three out of four bacterial tests (*S. aureus, S. mutans,* and *K. pneumoniae*). Generally, the antibacterial activity produced by *Bacillus* sp occurs through two mechanisms, namely directly and indirectly. The mechanism for inhibiting bacterial growth directly occurs through antibiosis and competition for nutrients (Rori *et al*., 2020). Meanwhile, the mechanism for inhibiting bacterial growth indirectly occurs due to the production of bioactive compounds that can inhibit bacterial growth (Rori et al., 2020). Endophytic bacteria isolated from mangrove plants have been reported to produce several bioactive compounds that act as antibacterials, such as halocarbon, terpenoids, coumarins, alkaloids, peptides, and polyketides (That et al., 2019; Khabthani et al., 2021).

In this study, the results of the antibacterial test showed that there was no antibacterial activity in the isolated extract of *Bacillus* sp. AM12 against *E. coli* ATCC 25922 so that there is no zone of inhibition around the test disc. This can happen because bacteria have different abilities to develop resistance to antimicrobial agents (Reygaert, 2018). The exact antibacterial action of *Bacillus* sp AM12 is unknown. Thus, it is likely that the mechanism of action is in line with the resistance pattern of *E. coli* bacteria. Some of the resistance mechanisms of *E. coli* are modifying antimicrobial targets, efflux pumps, porin loss, and producing β-lactamase and carbapenemase enzymes (Nurjanah et al., 2020).

Antibacterial test for *Bacillus* sp. AM12 showed that the average diameter of the resulting inhibition zone is smaller than the positive control. This could happen because the quantity and quality of secondary metabolites produced is less than optimal. Several aspects that can influence metabolite production are media optimization, fermentation time, and bacterial growth cycles (Mulyani et al., 2023). Therefore, each of these aspects needs to be optimized further in order to produce the ideal antibacterial compound.

The GC-MS results showed that there were 10 different compounds detected in the crude extracts, with the 3 most dominant compounds being Benzene, 1,2,4-trimethyl-, Benzene, 1-ethyl-2-methyl-, and 2-Butoxyethyl acetate. A study by Putra *et al.* (2024) confirmed that the compound 2- Butoxyethyl displays antibacterial activity against Gram-negative and Gram-positive bacteria. The hexadecanoic acid methyl ester was also detected in the crude extract, and the compound is one of the fatty acid groups with antibacterial properties. The mode of action of hexadecanoic acid is to destroy the structure of the bacterial cell wall and membrane (Arista et al., 2020). Hexadecanoic acid was also reported to display antioxidant activity by providing proton donors to DPPH, which turns into non-radical DPPH (Rismiyatun et al., 2024). Research by Vargas *et al.* (2021) stated that oleic acid is a compound that actively inhibits the growth of Gram-positive bacteria such as *S. aureus* and *S. pneumoniae* (Vargas *et al*., 2021).

CONCLUSION

Overall, this research showed that ethyl acetate extracts of *Bacillus* sp. AM12 displayed moderate antibacterial activity with an average inhibitory zone diameter of 6.72±0.21 mm, 6.82±0.15 mm, and 6.62±0.21 mm, respectively, against the bacteria *S. aureus* ATCC 25923, *S. mutans* FNCC 0405, and *K. pneumoniae* ATCC 70060. However, antibacterial activity was not observed against *E. coli* ATCC 25922. The presence of 10 different active molecules, which have been associated with antibacterial activities, provides an important clue regarding the potential of *Bacillus* sp. AM12 for synthetisizing antibacterial compounds. Future research should be focused on optimizing the growth of *Bacillus* sp. AM12 uses different growth media and chemical solvents and to screen extracts against multidrug-resistance bacteria strains.

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