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Antibacterial Activity of *Bacillus* sp. SA11 from Mangrove Soil and its GC/MS Profile Against Selected Gram-Positive and Gram-Negative Bacteria

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Abstract

Background: Bacterial infections are a major global health concern, including in Indonesia. This has led to an increased effort to find new antibacterial compounds in natural resources. *Bacillus* sp. SA11, with reported antibacterial potential, was previously isolated from mangrove soil in Denpasar, Bali. This study further investigated its antibacterial activity. **Methods:** Secondary metabolites were extracted from *Bacillus* sp. SA11 liquid culture using ethyl acetate. The extract was then screened for antibacterial activity against *Escherichia coli* ATCC 25922, *Streptococcus mutans* FNCC 0405, *Staphylococcus aureus* ATCC 25923, and *Klebsiella pneumoniae* ATCC 70060 using the Kirby-Bauer method (three replicates). Chemical composition of the extract was analysed via GC/MS. **Results:** The ethyl acetate extract showed zones of inhibition (diameter ± SD) of 11.7±1.11 mm against *E. coli*, 10.22±1.5 mm against *S. mutans*, 8.7±0.94 mm against *S. aureus*, and 7.2±0.67 mm against *K. pneumoniae*. GC/MS analysis identified 165 compounds, twelve previously linked to antibacterial activities. The three most abundant of these were n-hexadecanoic acid (6.91%), 2-butoxyethyl acetate (6.3%), and Phthalic acid, di(2-propylpentyl) ester (4.45%). **Conclusion:** These findings provide preliminary evidence that *Bacillus* sp. SA11, isolated from the mangrove ecosystem, has the potential to produce significant antibacterial compounds.

Cite this Article

INTRODUCTION

Bacterial infections are a pressing worldwide health issue, particularly in Indonesia (1). Current clinical practice heavily relies on antibiotic therapy, yet the widespread misuse and overuse of these drugs have led to a serious crisis: the development of antibiotic-resistant bacteria. This resistance can cause severe health complications and treatment failures. For example, *Streptococcus pneumoniae* has demonstrated resistance to Penicillin G. (2), and other notable resistant strains include multi-resistant *Mycobacterium tuberculosis* and Penicillin-Resistant *Pneumococci (3).*

Infections caused by antibiotic-resistant bacteria pose a significant global challenge, leading to increased morbidity, higher treatment costs, and even fatalities (4). Previous studies in Indonesia have shown alarming rates of resistance, such as *Escherichia coli* exhibiting 90.27% resistance to ampicillin-sulbactam (5). Addressing antibiotic resistance requires a multi-faceted approach, including public education on responsible antibiotic use and, crucially, the exploration of novel antibacterial agents from natural sources. Continuous clinical and scientific innovation in drug development is essential to overcome this public health crisis (6).

Mangrove forest ecosystems, thrive in the intertidal zones of muddy coastlines (7), are rich biodiversity hotspots. These unique environments are increasingly recognized as promising sources for novel antimicrobial compounds. Prior research has identified various bacteria from mangrove ecosystems, such as *Bacillus amyloliquefaciens, Bacillus cereus, Enterobacter hormaechei, Klebsiella pneumoniae*, and *Enterococcus gallinarum*, exhibiting antibacterial activity against pathogens like *Vibrio alginolyticus* (8). Additionally, studies on endophytic bacteria isolated from mangroves have shown efficacy against *Staphylococcus aureus*, with clear zones of inhibition up to 13 mm (9).

In previous research, 22 out of 68 bacterial isolates that had antibacterial potential were found from the soil where the *Sonneratia alba* mangrove plant lives in the Ngurah Rai Mangrove Forest Park, Denpasar (10). Among the obtained isolates, one isolate of *Bacillus* sp. SA11 showed antibacterial activity of 3 mm against *Streptococcus mutans* using the agar block method, which served as an initial indication of the antibacterial potential of *Bacillus* sp. SA11. However, the reported information has not fully described the antibacterial activity of *Bacillus* sp. SA11 because the previous antibacterial screening did not include chemical solvent extraction. The use of chemical solvents for extraction is intended to allow for a more thorough analysis of the antibacterial potential of *Bacillus* sp. SA11.

Earlier investigation on *Bacillus* sp SA11 did not explore the potential secondary metabolite compounds, as chemical solvents were not employed in the previous study (10). The forthcoming research will concentrate on studying *Bacillus* sp. SA11, encompassing the extraction of active compounds using chemical solvent and screening for antibacterial activity. Therefore, the present study aimed to investigate whether *Bacillus* sp. SA11 could serve as a source for antibacterial compounds.

MATERIALS AND METHODS

Gram staining

The cell wall type and morphological features of *Bacillus* sp. SA11 were evaluated using the Gram staining technique (11). Subsequent microscopic observation of the bacterial cells was performed at 1000x magnification using a Leica DM750 light microscope.

Extraction of secondary metabolites from *Bacillus* sp. SA11

Briefly, cell colonies of *Bacillus* sp SA11 were grown in 100 mL of liquid ISP-2 media and incubated for 10 days on a shaker (150 rpm) at room temperature (12). Supernatant from pure culture isolates of *Bacillus* sp. SA11 was separated from the cell mass after 7 days. The cell-free supernatant was passed through a Whatman grade 1 filter paper. An equivalent volume of ethyl acetate pro-analysis was subsequently used for maceration of the filtrates (SMART-Lab, Indonesia). Finally, the liquid mixture was separated using a separating funnel. This extraction procedure was done twice. The mixture was evaporated at 40 °C to yield a thick extract, with its mass then determined using an analytical balance.

Antibacterial Activity Test

Antibacterial activity was evaluated using the disc diffusion assay, also known as the Kirby-Bauer method. The procedure involved inoculating an agar plate with the target bacteria and then placing a disc impregnated with the test substance onto the surface. For the experiment, separate suspensions of *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* FNCC 0405, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 were prepared by adding 200 µL of each into sterile Luria-Bertani (LB) broth. These suspensions were then uniformly spread across LB agar plates using sterile cotton swabs. Subsequently, three sterile 6 mm paper discs were impregnated with 20 µL of the *Bacillus* sp. SA11 ethyl acetate extract. The discs were positioned on the inoculated LB agar plates and left to incubate for 24 hours at 37°C. For comparison, Levofloxacin was used as a positive control, while ethyl acetate served as a negative control. After incubation, the diameter of the clear zones around the discs was measured with a digital caliper to determine the extract's inhibitory ability. The average diameter was then categorized into four levels of activity: weak (0-5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (> 20 mm) (13).

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was conducted to identify the secondary metabolites within the ethyl acetate extract of *Bacillus* sp. SA11. Ethyl acetate extracts were submitted to the laboratory of Forensic Polda Bali to run the analysis. The resulting chromatogram was then interpreted by comparing the data with existing literature. (11).

RESULTS AND DISCUSSIONS

Gram staining and observation

Morphological characteristics showed that the isolate is grouped as Gram-positive bacteria. Bacterial cells have a rod-shaped morphology as observed under a light microscope (Figure 1).

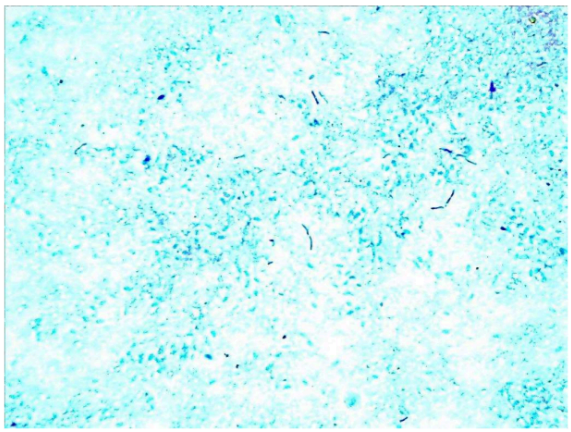

Figure 1. Gram Staining and Observation of *Bacillus sp.* SA11 under light microscopy with 1000x magnification

Antibacterial Activity Screening

No antibacterial activity was detected in the negative control against any of the bacterial strains tested. Meanwhile, the ethyl acetate extract of *Bacillus* sp. SA11 was able to inhibit the test bacteria *S. aureus* ATCC 25923 with an average inhibitory zone diameter of 8.7±0.94mm, 10.22±1.5 mm against *S. mutans* FNCC 0405, 7.2±0.67 mm against *K. pneumoniae* ATCC 70060, and 11.7±1.11 mm against *E. coli* ATCC 25922 (Table 1, Figure 2). However, when compared with the antibacterial activity using levofloxacin, the observed antibacterial activity showed an inhibitory diameter of approximately two to three times greater than the antibacterial activity formed by the *Bacillus* sp extract. SA11.

Table 1. Antibacterial Activity Test Results

Test Bacteria	Sample	Inhibition Zone Diameter (mm) ± SD	Interpretation
S. aureus ATCC 25923	*Bacillus* sp extract. SA11	8.7±0.94	Moderate
	Levofloxacin	21.7±0.90	Very strong
	ethyl Acetate	0±0	-
S. mutans FNCC 0405	*Bacillus* sp extract. SA11	10.22±1.5	Strong
	Levofloxacin	22±0.88	Very strong
	Ethyl Acetate	0±0	-
K. pneumoniae ATCC 70060	*Bacillus* sp extract. SA11	7.2±0.67	Moderate

	Levofloxacin	26.7±1.0	Very strong
	Ethyl Acetate	0±0	-
E. coli ATCC 25922	*Bacillus* sp extract. SA11	11.7±1.11	Strong
	Levofloxacin	27.8±1.9	Very strong
	Ethyl Acetate	0±0	-

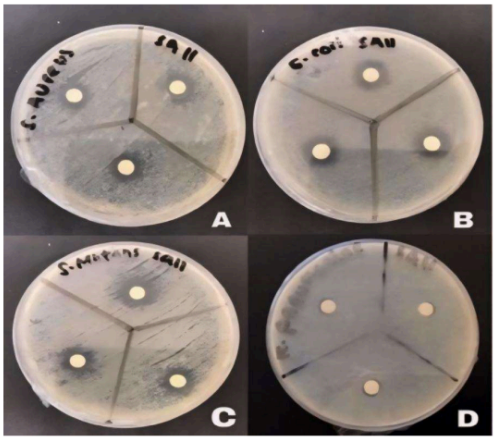

Figure 2. Antibacterial Activity Test of *Bacillus* sp SA11 against test bacteria A: *S. aureus* ATCC 25923, B: *E. coli* ATCC 25922, C: *S. mutans* FNCC 0405, D: *K. pneumoniae* ATCC 700603.

The ethyl acetate extract of *Bacillus* sp. SA11 demonstrated varying degrees of antibacterial activity against the tested pathogens. Specifically, it exhibited moderate antibacterial activity against *Staphylococcus aureus* ATCC 25923, with an average inhibition zone diameter of 8.7 ± 0.94 mm. Stronger activity was observed against *Streptococcus mutans* FNCC 0405, yielding an average inhibition zone of 10.22 ± 1.5 mm, and against *Escherichia coli* ATCC 25922, which showed the most potent inhibition with an average zone of 11.7 ± 1.11 mm. In contrast, *Klebsiella pneumoniae* ATCC 70060 exhibited moderate susceptibility, with an average inhibition zone of 7.2 ± 0.67 mm.

These findings indicate that the *Bacillus* sp. SA11 extract possesses broad-spectrum antibacterial activity, effectively inhibiting the growth of both Gram-positive (*S. aureus*, *S. mutans*) and Gram-negative (*E. coli*, *K. pneumoniae*) bacteria (14). This broad efficacy aligns with previous reports of bioactive compound production by endophytic bacteria from mangrove plants, including halogenases, terpenoids, coumarins, alkaloids, peptides, and polyketides. Genera such as *Burkholderia*, *Bacillus*, and *Azospirillum*, isolated from *Sonneratia alba* in mangrove environments, have been identified as producers of antibacterial compounds (15). The observed antibacterial activity of *Bacillus* sp. SA11 may be attributed to the production of bacteriocins, protein compounds known for their bactericidal effects on other microorganisms (16, 17). Bacteriocins produced by *Bacillus* species typically induce damage to bacterial cell membranes, disrupting the controlled influx and efflux of essential

cellular components and consequently impeding metabolic processes. This mechanism often involves the binding of bacteriocins to lipid components of the bacterial cell membrane, facilitated by their nonpolar nature (18). However, further research is required to confirm whether the antibacterial properties observed in *Bacillus* sp. SA11 is specifically associated with bacteriocins, as the precise quantity and nature of chemical compounds in the ethyl acetate extract were not quantified in this preliminary stage.

The differential antibacterial activity observed against the four test bacteria, with *E. coli* ATCC 25922 showing the highest susceptibility, could be explained by variations in bacterial defence mechanisms. The potential for *Bacillus* sp. SA11 to synthesize positively charged antibacterial compounds, such as bacteriocins, could be a mechanism for inhibiting Gram-negative bacteria. For example, the interaction of these compounds with the anionic outer membrane of *E. coli* could result in pronounced membrane disruption and suppress bacterial growth. Each bacterial species possesses unique cell wall compositions and resistance strategies, contributing to the varied responses to antibacterial agents.

GC-MS analysis

The GC-MS analysis detected 156 different peaks from the extract of *Bacillus sp.* SA11(Figure 3. Of these 156 compounds, 12 peaks corresponded with antibacterial activity based on literature studies (Table 2). The results of GC-MS analysis in Table 2 show that there are 12 groups of compounds that contain antibacterial, antioxidant, anticarcinogenic, and anticancer properties, as the compound benzeneacetamide, which has antibacterial, antioxidant, and anticancer activities. The three main compound components are compounds with large area percentages, namely n-hexadecanoic acid at 6.91%, 2-Butoxyethyl acetate at 6.3%, and Phthalic acid, di(2-propylpentyl) ester at 4.45%. Previous research on *Skimmia anquetilia* extract indicated that the presence of n-hexadecanoic acid has strong correlation with antibacterial activity (19). Similar findings on GC-MS analysis of Eucalyptus from Tunisia contained the component 2-butoxyethyl acetate, which was shown to have antimicrobial activity on *E. coli* and *Listeria monocytogenes* (20).

Table 2: GC-MS analysis results

Compound name	***Molecular formula***	***Activity***	***Peak areas***	***Retention time***	***References***
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Antibacterial	6.91	19.09	(21)
2-Butoxyethyl acetate	$C_8H_{16}O_3$	Antibacterial	6.03	7.29	(22)
Phthalic acid, di(2-propylpentyl) ester	$C_{26}H_{26}O_4$	Antibacterial	4.45	24.08	(23)
Benzene, 1,2,3-trimethyl-	C_9H_{12}	Antibacterial	3.76	4.61	(24)
Benzaldehyde	C_6H_5CHO	Antibacterial, Antioxidant	3.19	3.89	(25)
9-Octadecenoic acid, (E)- Oleic Acid	$C_{18}H_{34}O_2$	Antibacterial	3.17	20.71	(26)
trans-11-Tetradecenyl acetate	$C_{16}H_{30}O_2$	Antibacterial	2.74	8.88	(27)

5-(2-Thienyl)pentanoic acid	$C_9H_{12}O_2S$	Antibacterial	2.66	16.33	(28)
l-Leucine, N-cyclopropylcarbonyl-, dodecyl ester	$C_{10}H_{17}NO_3$	Antibacterial	2.63	18.83	(29)
Benzeneacetamide	$C_8H_7NO_2$	Antibacterial, antioxidant, anticancer	2.37	12.69	(30)
Dodecanoic acid	$C_{12}H_{24}O_2$	Antibacterial	2.11	14.81	(31)
2-Propenoic acid, 6-methylheptyl e ster	$C_{11}H_{20}O_2$	Antioxidant, anticarcinogenic	1.78	9.99	(32)

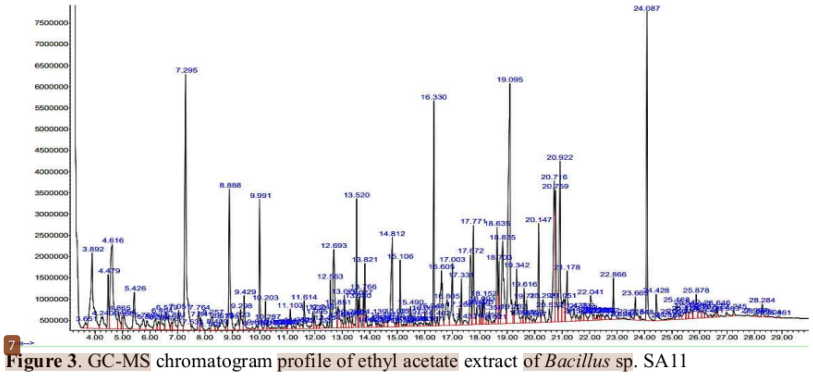

Figure 3. GC-MS chromatogram profile of ethyl acetate extract of *Bacillus* sp. SA11

CLINICAL IMPLICATION

This study provides preliminary yet significant clinical implications by demonstrating the broad-spectrum antibacterial activity of *Bacillus* sp. SA11 from mangrove soil against selected Gram-positive (*S. aureus*, *S. mutans*) and Gram-negative (*E. coli*, *K. pneumoniae*) pathogens. In an era of escalating antibiotic resistance, identifying novel sources of antibacterial compounds is critical. The GC/MS analysis, which pinpointed specific compounds like n-hexadecanoic acid, 2-butoxyethyl acetate, and Phthalic acid, di(2-propylpentyl) ester, offers a crucial starting point for further research. These findings suggest that *Bacillus* sp. SA11 could be a valuable natural resource for isolating and developing new therapeutic agents, potentially contributing to the pipeline of next-generation antibiotics to combat challenging bacterial infections in clinical settings.

LIMITATIONS

This study provides valuable preliminary findings, but it has several limitations. Primarily, the research utilized a crude ethyl acetate extract of *Bacillus* sp. SA11, meaning the specific active compounds identified by GC/MS were not isolated, purified, or individually tested for their antibacterial efficacy. Consequently, the exact contribution of each identified compound to the observed broad-spectrum activity remains unconfirmed. Furthermore, the study did not quantify the concentration of the active metabolites within the extract, which likely explains the smaller inhibition zones compared to the pure antibiotic positive control. Future research should focus on the isolation, purification, and individual characterization of these compounds, along with detailed studies on their mechanism of action and in vivo efficacy.

CONCLUSIONS

This study successfully demonstrated that the ethyl acetate extract of *Bacillus* sp. SA11, isolated from the Ngurah Rai Mangrove Forest, possesses broad-spectrum antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Streptococcus mutans*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*). Through GC/MS analysis, 165 different compounds were identified, including twelve previously associated with antibacterial properties, with n-hexadecanoic acid, 2-butoxyethyl acetate, and Phthalic acid, di(2-propylpentyl) ester being the most prominent compounds. These results provide strong preliminary evidence that *Bacillus* sp. SA11 from the mangrove ecosystem represents a promising natural resource for identifying and developing new antibacterial compounds

CONFLICT OF INTEREST

The authors declare there is no conflict of interest

AUTOR CONTRIBUTIONS

NKWDN contributed to the study's conception and design, analysis and interpretation of the data, and drafting of the article. AAGI contributed to the conception and design, provided materials and consumables, and was responsible for data analysis and interpretation, critical intellectual revisions, and final approval. AALP contributed to the study's conception, design, and article drafting, and to data collection and assembly. Lastly, DC provided assistance with data analysis and reviewed the manuscript.

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