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Antibacterial activity of phyllospheric bacteria isolated from *Rhizophora mucronata* against *Escherichia coli* and *Bacillus subtilis*

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Abstract. *Rizqoh D, Yolanda SD, Nuhraheni E, Sipriyadi, Ulyandari M, Wibowo RH, Oktoviani, Djatmiko EM, Putri AA. 2025. Antibacterial activity of phyllospheric bacteria isolated from* Rhizophora mucronata *against* Escherichia coli *and* Bacillus subtilis. *Biodiversitas 26: 199-210.* The treatment of bacterial infection often involves the administration of antibiotics. However, the increasing use of antibiotics has led to bacterial resistance. The black mangrove *Rhizophora mucronata* is a vital biological resource containing bioactive compounds with potential antibacterial properties. The objective of this study was to investigate the antibacterial potential of phyllospheric bacteria from *R. mucronata* leaves against *Escherichia coli* and *Bacillus subtilis*. The study was carried out using descriptive experimental research. In the initial stage, phylospheric bacteria were isolated from the leaves of *R. mucronata* using serial dilution method. The results showed that a total of 641 colonies were isolated from *R. mucronata*. Out of them, 53 dominant phyllospheric bacterial isolates were identified, which exhibit regular round shape with smooth edges colonies, flat elevation, moist texture and produce yellow color pigment. Gram staining revealed 42 Gram-positive and 11 Gram-negative bacterial isolates. The antibacterial activity test showed that 10 isolates had the ability to inhibit the growth of *E. coli*, 6 isolates inhibited the growth of *B. subtilis* and 3 isolates inhibited both bactera. Among 19 isolates, three isolates, namely BF1, BF4, and BF17 showed the best antimicrobial activity. The result of phytochemical tests revealed that all the crude extracts contained alkaloid, tannins, and saponins. Gas chromatography-mass spectrometry analysis showed potential metabolites, such as: [3,4-D]pyrimidine-5,7-dione, cyclo (L-Pro-L-Val), maculosin, 2,3,4-Trihydroxybenzaldehyde (TBA), phenethyl alcohol (PEA) tryptophol, benzene ethanol and benzeneacetic acid. The present study concluded that phyllospheric bacteria produce several active compounds that can inhibit bacterial growth.

Keywords: Antibacterial compounds, *Bacillus subtilis*, *Escherichia coli*, phyllosphere bacteria, *Rhizophora mucronata*

INTRODUCTION

Indonesia is a country that has a vast beach area on the coast and has overgrown various beach plant types. Mangroves contain bioactive compounds such as alkaloids, steroids, terpenoids, saponins, tannins, flavonoids, and quinones with various bioactivities, including antifungal, antimicrobial, antiviral, and antitumor (Dahibhate et al. 2019). Black mangroves (*Rhizophora mucronata* Lam.) are most abundant on Baai Island, Bengkulu City (Febriansyah et al. 2018). Some microbes, such as phyllosphere bacteria, produce bioactive compounds in plants. Phylospheric bacteria are the dominant microbes on leaf surfaces. The phyllosphere is considered to be an extreme and volatile environment resulting from exposure to ultraviolet radiation, temperature changes, and antimicrobial exposure from the external environment, thus causing the diversity of microbes in the phyllosphere to be relatively lower than that in the endosphere and rhizosphere (Dong et al. 2019). Phyllosphere bacteria can produce secondary metabolites similar to those of their hosts, creating a vast opportunity to isolate secondary metabolites by isolating only these bacteria (Sivakumar et al. 2020).

Bacterial infections can affect various human organ systems (Novard et al. 2019). In 2018, Riskesdas data from the Ministry of Health Republic Indonesia, showed the prevalence of infectious diseases based on the diagnoses of health workers and their symptoms in Indonesia, such as acute respiratory tract infection (ARTI) (9.3%), pneumonia (4.0%), diarrhea (8.0%), diarrhea in toddlers (12.3%). Bengkulu became the third province with a prevalence of diarrhea incidence of approximately 9%. *Escherichia coli* is a bacterium that often causes infections in the digestive tract, one of which is due to the consumption of contaminated food or water. *E. coli* infection can also cause food poisoning, diarrhea, urinary tract infections, sepsis, and meningitis in children. *E. coli* is an opportunistic bacterial that is widely found in the human colon as a microbiome. *Bacillus subtilis* is also one of the bacteria that cause infections; its large number in the intestine can cause

diarrhea transmitted through contaminated food (Haque et al. 2022). *B. subtilis* produces an extracellular toxin known as subtilisin. *B. subtilis* can cause diseases such as bacteremia, endocarditis, meningitis, urinary tract infections, and respiratory, urinary, and gastrointestinal tract infections (Utami et al. 2017).

Antibiotics are the primary treatment for bacteria-induced infectious diseases. Antibiotics can overcome and prevent communicable diseases and can cause overuse. The inappropriate use of antibiotics has led to the development of antibiotic-resistant bacteria (Widayati et al. 2012). In Indonesia, 30% of Methilin-resistance *Staphylococcus aureus* (MRSA) and 76% of *E. coli* are resistant to cephalosporin (World Health Organization 2023). The estimated number of deaths in 2050 as a consequence of antibiotic resistance could be 10 million, and 4.7 million of them are Asian population. The significant impact of antibiotic resistance is an increase in morbidity and mortality because resistant bacteria infection risks spread and costs more expensive treatment (CDC 2022).

This reflects the increasing importance of identifying new antibiotics from various natural sources that have potential as alternative antibiotics for treating infections caused by microbes. Phyllosphere bacteria can synthesize secondary metabolites in plant leaves. Through secondary metabolism, many bioactive compounds are used as essential drugs (Sivakumar et al. 2020). Rizqoh et al. (2016) reported that bacteria phyllosphere from Reundeu (*Staurogyne elongata*) can produce antimicrobial compounds and inhibit the growth of *E. coli*, *S. aureus*, *Candida tropicalis*, and *Candida albicans*.

It is crucial to carry out this research because there is still limited scientific information on the existence of *R. mucronata* phyllosphere bacteria on Baai Island, Bengkulu. Therefore, the objective of this study was to isolate and identify phyllosphere bacteria from *R. mucronata*, and to determine their benefits as agents that produce potential antibacterial compounds to inhibit the growth of *E. coli* and *B. subtilis* bacteria. The results of this study in the future can support the medical world in the invention of antibiotics.

MATERIALS AND METHODS

Type and design study

Samples were collected from Baai Island, Bengkulu City, Bengkulu, Indonesia, then phyllospheric bacteria were isolated at the Microbiology Laboratory of the Faculty of Medicine and Health Sciences at Universitas Bengkulu, Indonesia. The isolates used in the present study were obtained from three *R. mucronata* plants.

Isolation of phyllospheric bacteria

Phyllospheric bacteria were isolated from *R. mucronata* leaves. The leaf samples were cleaned with running water and then soaked in 10 mL of 0.85% NaCl solution for 10 minutes. Leaves were turned over so that the bacteria on the leaf surface were released and dissolved into the liquid. For dilution, five test tubes were filled with 9 mL of NaCl, 1 mL from the sample tube was taken with a micropipette and transferred to the first tube, then 1 mL from the first tube was taken and put into the second tube. This process was continued until the last concentration (10-4) formed. After dilution, 0.1 mL of the suspension was taken from each dilution and spread on King's A medium using a spreader. The plates were then incubated for 24 hours at room temperature.

Characterization of bacteria

Phyllosphere bacterial growth was assessed after 24 hours of incubation, and colony characteristics were evaluated according to Leboffe and Pierce (2016). Phyllospheric bacteria that grew were observed in the colony's characteristic features, such as shape, margin, elevation, texture, and color. Phyllospheric bacterial colonies were purified in King's A medium and incubated at room temperature for 18-24 hours. Gram staining was conducted to identify the shape of bacterial cells and differentiate between Gram-positive and Gram-negative bacteria.

Reculture of target bacteria

The target bacteria, *E. coli* FNCC-0091 and *B. subtilis* FNCC-0059 were collected from PT. Agritama Sinergi Inovasi and Study Center of Food and Nutrition, Universitas Gadjah Mada respectively. Bacteria were rejuvenated in the Nutrient Broth (NB) media and incubated at room temperature for 24 hours. The turbidity of each bacterial culture was measured by spectrophotometry at a wavelength of 600 nm (OD = 0.3, concentration 10^6 - 10^7 cells /mL) (Rizqoh et al. 2016).

Antagonist test of phyllospheric isolates against *E. coli* and *B. subtilis*

Antagonist test of phyllospheric bacterial isolates to inhibit the growth of target microbes was performed using a two-layer agar method consisting of semi-solid nutrient agar (NA) and solid NA media. The target bacteria in the NB medium were mixed with a semi-solid NA medium and poured onto a solid medium previously frozen on a plate. After freezing, phyllosphere bacterial isolates were dotted. The positive control (amoxicillin) and the negative control (sterile water) also dripped on the disc in the culture test plate. The culture was incubated for 3x24 hours at room temperature. The bacterial isolate positively inhibited target bacteria by forming clear zones around the bacterial colonies. The diameter of clear zone was measured, and the power of inhibitory activity was assessed based on the categories described previously by Rizqoh et al. (2024a), which explained the inhibition zone diameter category (Weak: ≤5 mm; Moderate: 5.1-10 mm; Strong: 10.1-20 mm; and Powerful: >20 mm).

Identification of phyllospheric bacteria

Bacterial DNA extraction: Bacterial isolates were cultured in NB media for 24 hours. A total of 1.5 mL of culture was centrifuged at 10,000 rpm for 10 minutes. Bacterial genomic DNA was extracted using the following bacterial DNA extraction kit, Wizard® Genomic DNA Purification Kit (Promega). 16S rRNA gene amplification by PCR: Gene amplification was carried out by mixing

12.5 PCR buffer GC II, 4 µL dNTPs (2.5 mM/dNTP), 1 µL primer 63F (CAGGCCTAACACATGC-AAGTC), 1 µL primer 1387R (GGGCGGWGTGTACAA-GGC), 4 µL template DNA, and 0.25 µL Taq DNA polymerase (GoTaq® Green Master Mix (Promega) and 2.25 µL ddH_2O. Amplification was carried out in a PCR machine for 30 cycles. The amplification stages were: pre-denaturation of 5 minutes and 1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of polymerization and 2 minutes of post PCR at 72°C. Visualization of the 16S rRNA amplicon was carried out through electrophoresis. The results of PCR gene amplification were then sequenced at a Macrogen's sequencing service company. 16S rRNA sequence analysis: Bacterial DNA sequences were analyzed to identify isolated bacteria. Species identification was carried out by sequence homology analysis using the Blast-N program from the NCBI website (http://www.ncbi.nlm.nih.gov/).

Ethyl acetate extraction of phyllospheric isolates

In this study, the best isolates (showed the highest inhibition zones) were selected and cultured into 250 mL of NB media and incubated on a shaker at 170 rpm (rotary per minute) at a temperature of 30°C for 72 hours so that the bacteria grow homogen. Next, the culture was taken, and 250 mL of ethyl acetate solvent was added, then incubated at 30°C for 24 hours and stirred for 20 minutes. After being left for 10 min in the separation funnel, the medium and extract solvent were separated. The top layer was taken and evaporated using a rotary evaporator HS-2005V vacuum at 40°C with a speed of 90 rpm. The crude extract was stored at 4°C for further use.

Phytochemical screening of crude extracts of mangrove phyllosphere bacteria

Crude extracts of phyllospheric bacteria were phytochemically screened for several bioactive compounds, such as flavonoids, alkaloids, saponins, and tannins, according to the method of Rizqoh et al. (2024a). The phytochemical test solution was made by mixing 150 µL of ethyl acetate extract of phyllospheric bacteria.

Gas chromatography-mass spectrometry (GC-MS) analysis

Phyllospheric bacterial crude extracts were analyzed using GC-MS to identify the components of metabolite compounds. The crude extracts of the phyllosphere bacterial mass spectrum were compared with the mass spectrum of a compound known as a comparator in database programmed into the GC-MS device.

RESULTS AND DISCUSSION

Isolation of phyllospheric bacteria from *R. mucronata* leaves

Result of isolation showed that a total of 641 colonies were isolated from black mangrove (*R. mucronata*) phyllosphere (Table 1).

Characterization of phyllospheric bacteria

Isolated colonies were evaluated on the basis of shape, margin, elevation, texture and pigmentation and were designated as BF code. A total of 53 dominant phyllospheric bacterial isolates were identified, which exhibited regular round shape with smooth edges colonies, flat elevation, moist texture and produce yellow color pigment (Table 2).

Results of Gram staining were performed to determine bacterial morphology by examining the shape and color of the bacteria. The result revealed that 24 isolates (45%) were coccus-shaped Gram-positive bacteria. 13 isolates (25%) were Gram-positive bacteria with bacillus form and 5 isolates (9%) were with coccobacilli form. 9 isolates (17%) were Gram-negative bacteria with cocci form and 2 isolates (4%) showed bacil form (Table 3). The total number of Gram-positive bacteria with 42 isolates (79%) was more than that of Gram-negative bacteria with 11 isolates (21%).

Antagonist test of phyllosppheric bacteria against *E. coli* and *B. subtilis*

The antagonist test results are presented in Table 4. The power test resistor showed that isolates BF1, BF2, BF4, BF16, BF17, BF25, BF29, BF 38, BF49, and BF52 inhibit the growth of *E. coli*, and isolates BF1, BF3, BF17, BF42, BF43, and BF49 could inhibit the growth of *B. subtilis* (Table 5) and BF1, BF17, and BF49 isolates were able to inhibit both bacteria. The antibacterial activity was determined by observing the clear zones formed around the bacterial isolates (Figure 1). Isolate BF1 showed the largest (2.4 ± 0.4 mm) inhibition zone against *E.coli*. At the same time, bacterial isolates BF1, BF3 and BF17 inhibited the growth of *B. subtilis*, indicating a strong category. Among the three, the largest diameter (13.7 ± 0.6 mm) was observed by the isolate BF17 (Table 5).

Table 1. Total isolated colony of phyllospheric bacteria from *R. mucronata*

Plant codes	Dilutions	Plates	Number of colonies
RM1	10^{-1}	1st	33
	10^{-2}	1st	10
		2nd	49
	10^{-3}	1st	4
		2nd	41
	10^{-4}	1st	3
		2nd	7
RM2	10^{-1}	1st	54
		2nd	221
	10^{-2}	1st	32
		2nd	46
	10^{-3}	1st	30
		2nd	32
	10^{-4}	1st	7
		2nd	3
RM3	10^{-2}	1st	28
		2nd	18
	10^{-3}	1st	5
		2nd	12
	10^{-4}	1st	1
		2nd	5
Total colonies			641

Identification of phyllospheric bacterial isolates

The results of nucleotide blast analysis showed that the BF1 isolate had a similarity with *Thiopseudomonas alkaliphila* strain C6819 (Acc No. CP012359.1) with a percent identity of 98.49%. The BF4 isolate was similar to *Proteus penneri* strain CPrp_RA24 (Acc No. MH788991.1) with a percent identity of 98.33, whereas, the BF17 isolate was similar to *Proteus hauseri* strain BPEM3 (Acc No. KX156166.1) with a percent identity of 99.02.

Extraction antimicrobial compound of *R. mucronata* phyllosphere bacteria

The extraction results of three *R. mucronata* phyllospheric bacterial isolates, namely BF1, BF4, and BF17 are presented in Table 6.

Phytochemical test of crude extract of phyllospheric bacteria

The results of phytochemical tests are shown in Table 7. The results showed that none of the crude extracts contained flavonoids. All crude extracts contained alkaloids. Crude extratcts of BF1 and BF4 showed the presence of tannins, except BF17 extract. Likewise, BF1 and BF17 crude extracts contained saponins, but BF4 did not contain saponin.

Gas chromatography-mass spectrometry (GC - MS) of crude extracts of phyllospheric bacteria

The result of GC-MS analysis showed that a total of 23 compounds were found in the crude extract of BF1 isolate (Table 8), 33 compounds in the BF4 extract (Table 9), and 31 compounds in the BF17 extract (Table 10). Table 11 shows the main 10 compounds with the highest peaks in each isolate with ethyl acetate (Figure 2).

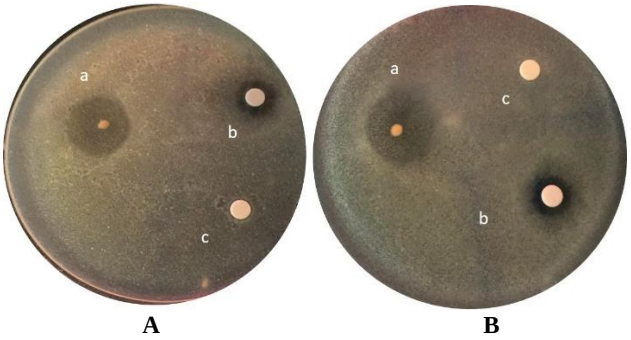
Table 2. Characteristics of *R. mucronata* phyllosphere bacterial isolate colonies

Colony characteristics					Isolates code	Total isolates
Shape	Margin	Elevation	Texture	Pigment		
Regular	Smooth regular	Flat	Moist	Glossy yellow	BF 1,BF 7, BF11, BF 14, BF 17, BF 22, BF 25, BF 32, BF 35, BF 38, BF 42, BF 43, BF 49	13
Regular	Smooth regular	Flat	Moist	Glossy white	BF 2, BF 3, BF4, BF 12, BF 13, BF 21, BF 30, BF 39, BF 51	9
Regular	Smooth regular	Convex	Moist	Shiny Yellow	BF 5, BF 28, BF47	3
Irregular	Irregular	Flat	Dry	Pale white	BF 6	1
Regular	Smooth regular	Flat	Dry	Glossy white	BF 8	1
Rhizoid	Rhizoid	Flat	Dry	Transparent white	BF 9	1
Irregular	Irregular	Flat	Dry	Pale yellow	BF 10	1
Regular	Smooth regular	Flat	Moist	Shiny red	BF 15	1
Regular	Smooth regular	Convex	Moist	Glossy white	BF16,	1
Irregular	Irregular	Flat	Dry	Transparent white	BF 18, BF48,	2
Regular	Smooth regular	Flat	Moist	Pale yellow	BF 19, BF 27, BF 37	3
Regular	Smooth regular	Flat	Moist	Pale white	BF 20, BF 24, BF 26, BF 29, BF 33, BF 36	6
Rhizoid	Rhizoid	Flat	Moist	Glossy yellow	BF 23	1
Regular	Smooth regular	Flat	Dry	White transparent	BF 31	1
Regular	Smooth regular	Convex	Moist	Pale yellow	BF 34	1
Regular	Smooth regular	Flat	Dry	Pale white	BF40, BF 52	2
Irregular	Irregular	Flat	Moist	Pale yellow	BF 41	1
Filamentous	Filamentous	Flat	Moist	Pale yellow	BF 44	1
Filamentous	Filamentous	Convex	Moist	Glossy white	BF46	1
Irregular	Irregular	Flat	Moist	Glossy yellow	BF 50	1
Regular	Smooth regular	Flat	Moist	Transparent yellow	BF45	1
Irregular	Irregular	Flat	Moist	Pale white	BF 53	1

Note: BF: Phyllospheric bacterial isolate codes

Table 3. Results of Gram staining of phyllospheric bacterial isolates

Forms	Isolates code	Gram's staining	Isolates amount
Coccus	BF 1, BF 2, BF 3, BF 4, BF 5, BF 7, BF 11, BF 12, BF 13, BF 14, BF 19, BF 20, BF 22, BF 26, BF 30, BF 31, BF 32, BF 37, BF 40, BF 41, BF 44, BF 46, BF 48, BF 53	Gram-positive	24
	BF 6, BF 24, BF 25, BF 27, BF 34, BF 35, BF 42, BF 43, BF 50	Gram-negative	9
Basil	BF 8, BF 10, BF 15, BF 16, BF 17, BF 18, BF 21, BF 28, BF 38, BF 45, BF 47, BF 49, BF 52,	Gram-positive	13
	BF23, BF33	Gram-negative	2
Coccobacilli	BF 9, BF 29, BF 36, BF 39, BF 51	Gram-positive	5



A **B**

Figure 1. Inhibition zone of isolates BF2 and BF 1. A. *E. coli*; B. *B. subtilis*. a: Sample isolate of phyllospheric bacteria, b: Positive control (amoxycilin), c: Negative control (sterile water)

Table 4. Inhibition test of phyllosphere bacterial isolates against pathogenic bacteria

Isolates code	*E. coli*	*B. subtilis*	Isolates code	*E. coli*	*B. subtilis*
BF1	+	+	BF28	-	-
BF2	+	-	BF29	+	-
BF3	-	+	BF30	-	-
BF4	+	-	BF31	-	-
BF5	-	-	BF32	-	-
BF6	-	-	BF33	-	-
BF7	-	-	BF34	-	-
BF8	-	-	BF35	-	-
BF9	-	-	BF36	-	-
BF8	-	-	BF37	-	-
BF11	-	-	BF38	+	-
BF12	-	-	BF39	-	-
BF13	-	-	BF40	-	-
BF14	-	-	BF41	-	-
BF15	-	-	BF42	-	+
BF16	+	-	BF43	-	+
BF17	+	+	BF44	-	-
BF18	-	-	BF45	-	-
BF19	-	-	BF46	-	-
BF20	-	-	BF47	-	-
BF21	-	-	BF48	-	-
BF22	-	-	BF49	+	+
BF23	-	-	BF50	-	-
BF24	-	-	BF51	-	-
BF25	+	-	BF52	+	-
BF26	-	-	BF53	-	-
BF27	-	-			

Notes: (+): Showed inhibition; (-): No inhibition

Table 5. Inhibitory test of phyllospheric bacterial isolates against *E. coli* and *B. subtilis*

Isolates code	Isolates diameter (mm)	Isolates diameter + inhibition zone (mm)	Inhibition zone diameter (mm)	Categories
E. coli				
Control +	5	9.6	4.6	Weak
Control -	-	-	-	-
BF1	3.3	15.7	12.4 ± 0.4	Strong
BF2	3.3	14.5	11.2 ± 0.3	Strong
BF4	3.4	15.6	12.2 ± 0.6	Strong
BF16	3.2	14.6	11.4 ± 0.5	Strong
BF17	3.2	14	10.8 ± 1.7	Moderate
BF25	4.4	14.3	9.9 ± 1.5	Moderate
BF29	4.2	14.3	10.1 ± 1.2	Moderate
BF38	4.5	15.3	10.8 ± 1.0	Moderate
BF49	3.7	13.9	10.2 ± 0.4	Moderate
BF52	3.9	13.9	10 ± 0.2	Moderate
B. subtilis				
Control +	5	9.6	4.6	Weak
Control -	-	-	-	-
BF1	3.3	15.5	12.2 ± 1.1	Strong
BF3	4	15	11 ± 1.5	Strong
BF17	3.7	17.4	13.7 ± 0.6	Strong
BF42	3.3	12.7	9.4 ± 0.2	Moderate
BF43	3.1	10.7	7.6 ± 1.1	Moderate
BF49	4.1	10.4	6.3 ± 0.5	Moderate

Table 6. Extraction results of phyllospheric bacteria with ethyl acetate solvent

Isolates	Extract volume
BF1	2.3 mL
BF4	0.4 mL
BF17	0.4 mL

Table 7. Qualitative phytochemical test of crude extracts of phyllospheric bacteria

Compounds	Isolates code		
	BF1	BF4	BF17
Flavonoid	-	-	-
Tannin	+	+	-
Alkaloid	+	+	+
Saponins	+	-	+

Notes: (+): Presence; (-): Absence

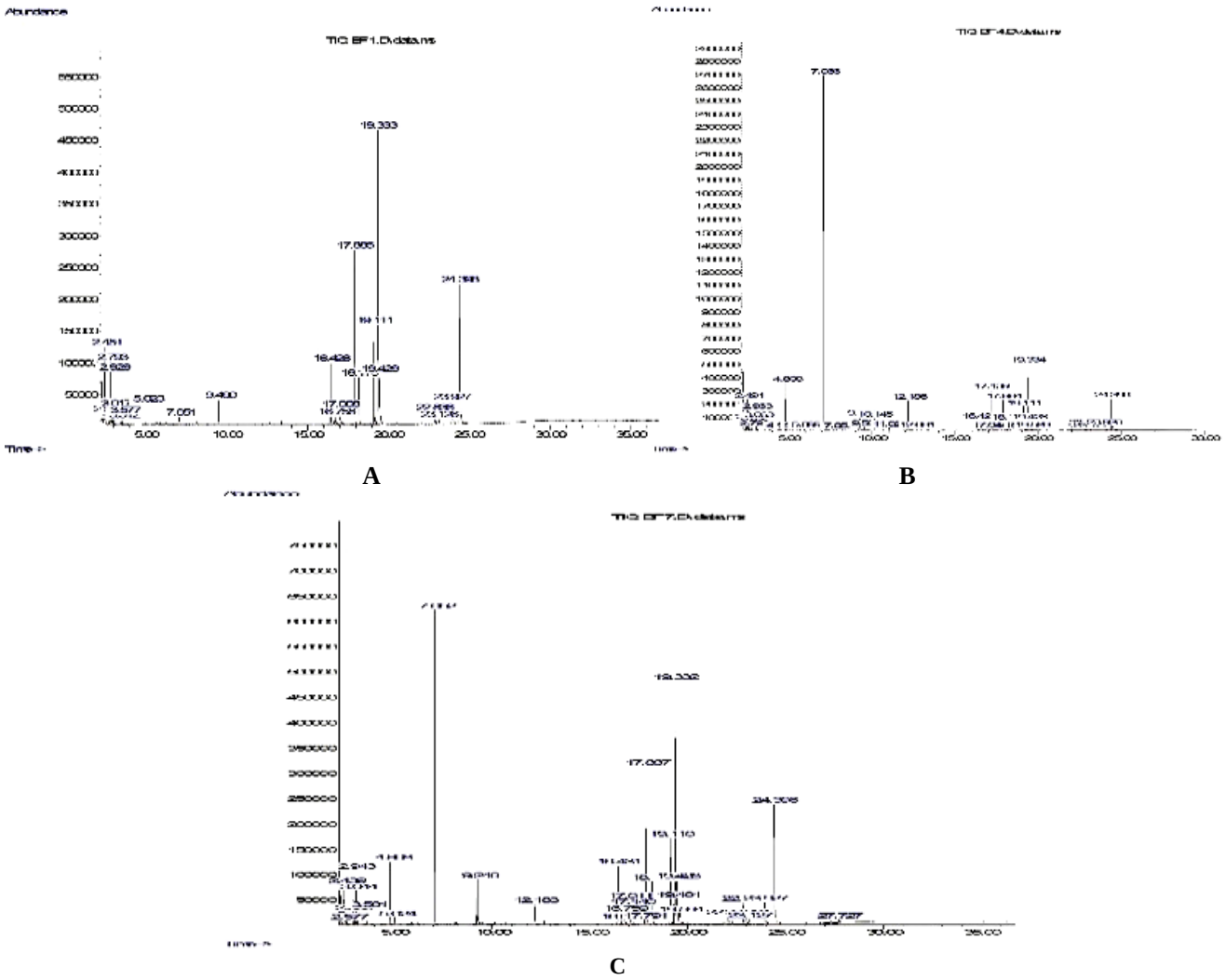


Figure 2. The results of GC-MS analysis of phyllospheric bacteria crude extract. A. BF1; B. BF4; C. BF17

Table 8. Analysis of metabolite compounds in the isolate BF1

RT	Area (%)	Compounds
2.451	4.30	Butanoic acid
2.572	0.41	2-Hexanol
2.793	2.72	Butyl acetate
2.929	4.01	Iso-Valeric Acid
3.017	0.87	Hexanoic acid
3.113	0.35	1,2-Ethanediol
3.512	0.22	Propanoic acid
3.577	0.46	Oxime-, methoxy-phenyl- Benzoic acid, 2-amino-4-methyl-
5.023	1.11	Heptane, 2,2,4,6,6-pentamethyl-
7.051	0.47	Benzeneethanol
9.490	1.58	Divalonic acid
16.428	4.75	1H-Pyrazole-1-carboxaldehyde, 4-ethyl-4,5-dihydro-5-propyl-
16.758	1.08	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione
17.008	1.85	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
17.885	13.56	Cyclo(-L-Pro-L-Val-)
18.179	4.55	Cyclo(prolylvalyl)
19.111	7.88	Diethyldithiophosphinic acid
19.333	24.22	Furazano[3,4-d]pyrimidine-5,7(4H,6
19.429	7.35	2,3,4-Trihydroxybenzaldehyde
22.836	1.10	L-Proline, N-pivaloyl-, ethyl ester
23.126	0.47	2-benzyl-3,6-dioxo-5-isopropylpiperazine
23.927	2.54	Ergotamine - GC Artefact I
24.398	14.15	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane

Table 9. Analysis of metabolite compounds in the isolate BF4

RT	Area (%)	Compounds
2.494	0.81	2,3-Butanediol
2.799	0.36	Acetic acid, butyl ester
2.963	3.66	Pentanoic acid
3.063	1.66	2-Methylbutanoic acid
4.396	0.20	3-Hexanol, 2,5-dimethyl-
4.808	4.55	1-Propanol, 3-(methylthio)-
5.026	0.14	Octamethylcyclotetrasiloxan
5.451	0.11	4-Heptanol, 2,6-dimethyl-
5.899	0.22	Benzeneacetaldehyde
7.066	42.27	Phenylethyl alcohol
7.857	0.15	Octanoic acid
9.254	2.61	Benzeneacetic acid
9.503	0.44	Acetamide, N-(2-phenylethyl)-
10.145	1.37	1H-Indole
11.021	0.28	Benzaldehyde, oxime
12.186	3.72	Benzeneethanol, 4-hydroxy-
12.661	0.18	Vanillyl alcohol
16.426	1.40	3-n-Pentylcyclohexanone
17.044	0.45	Tetradecanoic acid
17.139	5.46	1H-Indole-3-ethanol
17.884	4.26	Cyclo(-L-Pro-L-Val-)
18.177	1.58	3,6-Diisopropylpiperazin-2,5-dione
18.973	0.22	1H-Indole-3-ethanol, acetate
19.111	3.15	1,3-Cyclohexanedione, 2,5,5-trimethyl-
19.334	9.74	Furazano[3,4-d]pyrimidine-5,7(4H,6H)-dione
19.428	2.20	Phloroglucinol dimethyl ether
19.589	0.26	Hexadecanoic acid
22.836	0.66	3-(ethoxymethylen)-1-methyl-1-cyclobutanecarbaldehyd
22.983	0.26	3-Hydroxy-2-ethyl-5-methyl-4-pyron
23.127	0.34	2-[2'-(Hydroxycyclohex-1'-enyl)thio]benzoxazole
23.926	1.00	Ergotamine - GC Artefact I
24.396	5.85	3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane

Table 10. Analysis of metabolite compounds in the isolate BF17

RT	Area (%)	Compounds
2.439	0.40	Butanoic acid
2.577	0.13	(3-Methyl-oxiran-2-yl)-methanol
2.800	0.77	Acetic acid, butyl ester
2.943	3.83	Pentanoic acid
3.044	2.09	Butanoic acid, 2-methyl-
3.581	0.69	Oxime-, methoxy-phenyl-
4.808	2.90	1-Propanol, 3-(methylthio)-
5.024	0.33	Cyclotetrasiloxane, octamethyl-
7.052	15.91	Benzeneethanol
9.248	3.82	Benzeneacetic acid
12.183	1.23	Benzeneethanol, 4-hydroxy-
16.431	4.27	1H-Pyrazole-1-carboxaldehyde, 4-ethyl-4,5-dihydro-5-propyl-
16.626	0.29	dl-Alanyl-l-leucine
16.759	1.20	(3R,8aS)-3-Methyl-1,2,3,4,6,7,8,8a-octahydropyrrolo[1,2-a]pyrazine-1,4-dione
17.011	2.42	1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane
17.140	1.50	1H-Indole-3-ethanol
17.791	0.31	1H-Indole-3-carboxaldehyde
17.887	10.69	Cyclo(-L-Pro-L-Val-)
18.182	3.65	Cyclo(prolylvalyl)
19.110	5.58	2,3,4-Trihydroxybenzaldehyde
19.332	15.98	1,3-Cyclohexanedione
19.428	3.02	Phenol, 3,5-dimethoxy-
19.481	2.11	2,3,4-Trihydroxybenzaldehyde
19.594	1.14	Hexadecanoic acid
22.031	0.97	DL-Norleucine, N-allyloxycarbonyl-, allyl ester
22.837	1.70	3-(ethoxymethylen)-1-methyl-1-cyclobutanecarbaldehyd
22.984	0.80	1-Bromo-4-([1,3]dioxan-2-yl)benzen
23.127	0.50	Cyclo-(l-valyl-l-phenylalanyl)
23.927	2.12	Ergotamine - GC Artefact I
24.396	10.34	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-
27.727	0.13	Phenol, 2-amino-5-nitro-

Table 12. Results of GC-MS analysis of 10 main compounds in the crude extracts of phyllospheric bacteria

Extracts code	Compounds	RT	Chemical formula	Area %
BF1	Furozano [3,4- D]pyrimidine -5,7-dione	19.333	$C_6H_2N_2O_3$	24.22
	Cyclo (L-Pro-L-Val)	17.885	$C_{10}H_{16}N_2O_2$	13.56
	Maculosin	24.398	$C_{14}H_{16}N_2O_3$	14.15
	Diethyldithiophosphinic acid	19.111	$C_4H_{11}PS_2$	7.88
	Butanoic acid	2.451	$CH_3CH_2CH_2COOH$	7.35
	1H-Pyrazole-1-carboxaldehyde, 4-ethyl-4,5-dihydro-5-propyl-	16.428	$C_9H_{16}N_2O$	4.75
	2,3,4-Trihydroxybenzaldehyde	19.429	$C_7H_6O_4$	7.35
	Isovaleric acid	2.929	$C_5H_{10}O_2$	4.01
	L-leucine	19.429	$C_6H_{13}NO_2$	7.35
	Butyl acetate	2.793	$CH_3COO(CH_2)_3CH_3$	2.72
BF4	Phenethyl alcohol	7.066	$C_6H_5CH_2CH_2OH$	42.27
	Furozano [3,4- D]pyrimidine -5,7-dione	19.304	$C_6H_2N_2O_3$	9.74
	1-propanol	4.808	$CH_3CH_2CH_2OH$	4.55
	Tryptophan	17.139	$C_{10}H_{11}NO$	5.46
	Benzene ethanol	12.186	$C_6H_5CH_2CH_2OH$	3.72
	2,3-Butanediol	2.494	$C_4H_{10}O_2$	0.81
	cyclo (L-Pro-L-Val)	17.884	$C_{10}H_{16}N_2O_2$	4.26
	Pyrrolo [1,2- a]pyrazine -1,4-dione, hexahydro-3-(phenylmethyl)-	24.396	$C_{14}H_{16}N_2O_2$	5.85
	1,3-Cyclohexanedione	19.111	$C_6H_8O_2$	3.15
	Pentanoic acid	2.963	$CH_3(CH_2)_3COOH$	3.66
BF17	Benzene ethanol	7.052	$C_6H_5CH_2CH_2OH$	15.91
	2-Methyldimedone	19.332	$C_9H_{14}O_2$	15.98
	L-norvaline	17.887	$C_5H_{11}NO_2$	10.69
	Pyrrolo [1,2- a]pyrazine -1,4-dione, hexahydro-3-(phenylmethyl)-	24.396	$C_{14}H_{16}N_2O_2$	10.34
	2,3,4-Trihydroxybenzaldehyde	19.110	$C_7H_6O_4$	5.58
	1-propanol	4.808	$CH_3CH_2CH_2OH$	2.90
	1H-Pyrazole-1-carboxaldehyde, 4-ethyl-4,5-dihydro-5-propyl-	16.431	$C_9H_{16}N_2O$	4.27
	Pentanoic acid	2,943	$CH_3(CH_2)_3COOH$	3.83
	Benzeneacetic acid	9.248	$C_6H_5CH_2CO_2H$	3.82
	Cyclo (prolylvalyl)	18.182	$C_{10}H_{16}N_2O_2$	3.65

Notes: Area (%): The relative percentage of each component was obtained directly from the chromatographic peak area, considering the sum of all eluted peaks as 100 percent; RT (min): the time the component was found in the chromatographic peak

Discussion

Isolation and characterization of phyllosphere bacteria

Phyllosphere bacteria are communities that inhabit plant leaf surfaces, and are influenced by several factors that affect their growth. These factors, which contribute to the complexity of the phyllosphere, significantly impact the number of bacteria in the environment. In this study, 642 bacterial isolates were isolated from the phyllospheric of *R. mucronata*. A contrast result was obtained by Rizqoh et al. (2021), the study isolated 206 bacterial colonies from Andaliman plants (*Zancthoxylum acanthopodium*). Like those found in other natural settings, the majority of phyllosphere microorganisms cannot be cultivated in widely used medium and culture conditions (Müller and Ruppel 2014). They must adapt to living in environments with limited water and nutrients, UV radiation, abrupt changes in temperature, and reactive oxygen species (Lindow and Brandl 2003). According to Rastogi et al. (2013), just 0.1-8.4% of the entire population of bacteria could be grown.

Characterization of colonies and morphology of phyllospheric bacteria

Macroscopic isolates are characterized by their shape, margin, elevation, texture, and pigmentation (Leboffe and Pierce 2016). In the present study isolated phyllospheric bacterial colonies were regular with margin smooth regular, flat elevation, moist texture and pigment glossy yellow. This result differs from the study by Awidya (2024), who isolated phyllospheric bacteria from *Plumeria acuminata*. Results revealed that bacterial isolates had convex elevation, moist texture, regular round form, regular smooth edges, and brilliant yellow pigment. Several factors influence bacterial isolate characteristic variations, including bacterial shape, direction of bacterial division cells that affect the arrangement of isolates (pairs, clusters, chains, or filaments), and distinct properties, such as physiology of bacteria itself, including motility, pigments, enzymes, and bacterial capsules, which affect the appearance, color, and texture of the bacterial isolates (Pramono et al. 2019). Pigmentation may be a particular adaption strategy in the phyllosphere. The leaf surfaces are home to a large number of colored bacteria from the genera *Pantoea*, *Clavibacter*, *Xanthomonas*, etc. (Thapa and Prasanna 2018).

Bacterial isolates were also subjected to Gram staining to identify the morphology of bacterial shapes and types. The results exhibited that 24 Gram-positive isolates were cocci, 13 Gram-positive isolates were bacili, and 5 Gram-positive isolates showed coccobacilli form. In

comparison, 9 isolates of Gram-negative from cocci and 2 isolates of Gram-negative bacilli; the difference in the color of the bacteria after Gram-staining was due to the difference in the structure of the cell wall. The most dominant bacterial morphology obtained was Gram-positive cocci in as many as 42 isolates. Several similar studies related to the morphology and type identification of Gram-positive bacteria in the phyllosphere of other plants also found that the morphology of the bacteria that grew was dominated by coccus-shaped Gram-positive bacteria (Rizqoh et al. 2021).

Observing the morphological characteristics of bacterial colonies is important to facilitate the identification of different types of bacteria. The results showed that the phyllosphere bacteria were Gram-positive bacteria with a total of 42 isolates (79%) compared to Gram-negative bacteria, which only numbered 11 isolates (21%). This result is in line with the results of Awidya's study (2024), which showed that Gram-positive bacteria (86%) in the phyllosphere of white cambodia (*P. acuminata*) was higher than Gram-negative bacteria (14%). Research by Rizqoh et al. (2021) also showed the dominance of Gram-positive bacteria in the phyllosphere of *Z. acanthopodium*. The dominant phyla present in phyllosphere are Proteobacteria and Firmicutes. The genera present in majority of cases are *Bacillus, Pseudomonas, Pantoea, Erwinia, Sphingomonas, Acinetobacter, Xanthomonas,* and *Gluconobacter* (Thapa and Prasanna 2018).

Antagonist test of phyllosphere bacteria against E. coli *and* B. subtilis

A bacterial inhibition test was conducted to determine the inhibitory activity of phyllospheric bacterial isolates against the growth of *E. coli* and *B. subtilis*. The results of inhibitory test of bacterial isolates showed that 10 isolates had inhibitory power against *E. coli*, namely BF1, BF2, BF4, BF16, BF17, BF25, BF29, BF38, BF49, BF5 2. For comparison, six test isolates showed a positive inhibitory effect against *B. subtilis*, namely BF1, BF3, BF17, BF42, BF43, and BF49, while only three isolates BF1, BF17, and BF49 inhibited the growth of both bacteria. The formation of a clear zone around the colony of phyllospheric bacterial isolates indicated positive results. The clear zone is influenced by secondary metabolites released by phyllospheric bacteria into the environment as antibiotics that suppress pathogens. The formation of a clear zone indicated that phyllospheric bacteria can produce extracellular antibacterial compounds (Kusumawati et al. 2014).

Several studies reported that phyllospheric bacteria have antimicrobial activity against some pathogenic bacteria. Adeniyi et al. (2024) report that phyllospheric bacteria from *Futumia elastica* produce bioactive compounds against *Staphylococcus aureus, E. coli, C. albicans Klebsiella pneumoniae, Trycophyton rubrum*, and *Microsporum canis* (Adeniyi et al. 2024). Furthermore, phyllosphere bacteria from *P. acuminata* also could inhibit *Candida albicans* growth (Awidya 2024). Rizqoh et al. (2024b) also reported that phyllospheric bacteria from *Zanthoxylum acanthopodium* have potential to produce antimicrobial compounds against *E. coli, B. subtilis,* and *S. aureus* and inhibit their growth.

Molecular identification of potential phyllosphere bacteria

The result of molecular analysis of 16s rRNA sequence gene revealed that isolates BF1, BF4, and BF17 showed the highest similarities with *T. alkaliphila*, *Proteus penneri*, and *Proteus hauseri*, respectively. Another study identified the potential phyllospheric bacteria from *Z. acanthopodium* as *Pseudomonas* sp., *Brevundimonas* sp., and *Bacillus altitudinis* (Rizqoh et al. 2024b). Another study reported antimicrobial-producing phyllospheric bacteria were *Enterobacter hormaechei*, *Bacillus cereus*, *Pontoea dispersa*, and *Staphylococcus artalettae* (Adeniyi et al. 2024).

Isolate BF1 is similar to *T. alkaliphila* strain C6819, which is called *Oblitimonas alkaliphila* before, a bacterium of Pseudomonadaceae Family (Lauer et al. 2015). Using frozen defibrinated rabbit blood (Hemostat), the strains C6819 were cultivated on Heart Infusion Agar (HIA). *T. alkaliphila* are aerobic/microaerophilic, non-motile, halo-tolerant, alkali-tolerant, and Gram-negative.

The other two isolates, BF4 and BF17, is belong to genus *Proteus*, which similar to *Proteus penneri* strain CPrp_RA24 and *Proteus hauseri* strain BPEM3. *Proteus penneri* strain CPrp_RA24 has similarity of about 98.33% and has 7 different nucleotide base pair in line 494-553. Another study reported *P. penneri* strain CPrp_RA24 isolated from the rice-fish farming system cultivation area has antibacterial activity against *Aeromonas hydrophila* (Fitriadi et al. 2023). *Proteus hauseri* strain BPEM3 was isolated from elephant grass (*Arundo donax*), which produces succinate. *Proteus hauseri* is also known to has high potential application in bioremediation because it can reduce toxic sodium selenate in wastewater (Khalilian et al. 2015).

Phytochemistry test of crude extracts

Rhizophora mucronata possess secondary metabolites in the form of alkaloids, tannins, and flavonoids (Dahibhate et al. 2019). According to Egra et al. (2019), antibacterial activity found in plant *R. mucronata* contains active compounds triterpenoids, flavonoids, alkaloids, and tannins (Egra et al. 2019). Flavonoid compounds have the potential to act as antibiotics and antibacterials, and the mechanisms by which flavonoids prevent bacterial growth include damaging the permeability of bacterial cells, microsomes, and lysosomes. Due to the interaction between flavonoids and bacterial DNA, flavonoids are also able to release energy into the bacterial cytoplasm (Mile et al. 2021). Tannins also have targets on polypeptide wall bacterial cells so that the formation of bacterial cell walls could be disturbed. The mechanism of triterpenoid antibacterial action involves a reaction with porins (transmembrane proteins) on the outer membrane of the cell wall. Bacterial cells form a strongly bonded polymer, resulting in damage to the porin. Damage to the porin, which is the entry and exit point nutrient, causes compound inhibitors to lower the permeability of bacterial cell walls (Egra et al. 2019).

The presence of alkaloid compounds in phyllosphere bacteria inhibits cell wall synthesis. This alkaloid compound works through an inhibitory mechanism that disrupts the peptidoglycan-forming components in bacterial cells, resulting in layers of bacterial cell walls no longer forming properly,

and the cell dies. In addition, alkaloids can prevent protein synthesis, which can affect bacterial metabolism; therefore, the presence of these secondary metabolites can also prevent the development of Gram-negative and Gram-positive bacteria (Rizqoh et al. 2024a). The mechanism of antibacterial inhibition of bacterial growth is by damaging the bacterial cell wall and changing the permeability of the cytoplasmic membrane, thereby causing nutrients to escape through the cell wall, changing the properties of the cell wall, disrupting protein synthesis, and inhibiting enzyme activity (Septiani et al. 2017).

Metabolite compounds of phyllosphere bacteria

From the results of GC-MS analysis, several metabolite compounds were identified from the phyllosphere bacterial isolates. Several potential metabolites were detected in all three isolates. One of them was furozano [3,4-D]pyrimidine-5,7-dione, found in large amounts in the crude extracts of isolates BF1 and BF2. The compound is a pyrimidine derivative. Kumar et al. (2019) reported that pyrimidine derivative has potential as an anticancer and antiviral agent. Another potential compound detected in all three phyllospheric isolates was cyclo (L-Pro-L-Val). It is a piperazinone metabolite. Cyclo (L-Pro-L-Val), isolated from *Streptomyces* sp., has several antimicrobial targets that can inhibit antibiotic-resistant microbes and has potential as an antimicrobial agent against Methicillin-resistant *S. aureus* (MRSA) (Zin et al. 2020). These isolates also produced maculosins. Maculosin is a dipeptide, cyclic homodetic peptide, pyrrolopyrazine, and a phenol. Functionally, maculosin is associated with L-proline and L-tyrosine. Maculosin is known to have strong antioxidant activity and is nontoxic (Paudel et al. 2021). This compound has potential antimicrobial activity against several multidrug-resistant pathogenic bacteria (Driche et al. 2022). Butanoic acid and butyric acid are fatty acids with various biological functions. This compound acts as a *Mycoplasma genitalium* metabolite and a human urine metabolite. This compound also acts as an anticancer agent that inhibits several mammalian tumor cells (Prasad 1980).

1H-Pyrazole-1-carboxaldehyde and 4-ethyl-4,5-dihydro-5-propyl- were detected in the extracts of isolates BF1 and BF17. 1h-pyrazole-1- carboxaldehyde,4-ethyl-4,5-dihydro-5-propyl (9.78%) were the main phytochemicals in *M. officinalis* stem essential oils. In the present study, the compound showed potential insecticidal activity. The compounds 1-propanol, benzene ethanol, and pyrrolo [1,2-a]pyrazine -1,4-dione, hexahydro-3-(phenylmethyl)- were detected in both extracts of isolates BF4 and BF17. Propan-1-ol is the parent member of the propan-1-ol class, propane, in which a hydroxyl group replaces the hydrogen of one of the methyl groups. It acts as a protic solvent and metabolite. They are used in making cosmetics, skin and hair preparations, pharmaceuticals, perfumes, lacquer formulations, dye solutions, antifreeze, rubbing alcohols, soaps, window cleaners, acetone, and other chemicals and products (NCBI 2024a). Benzene ethanol's triazole compounds exhibit potential antibacterial and antifungal properties. When compared to standard medications (ciprofloxacin and itraconazole), the antimicrobial activities also showed that the compounds were potent antibacterial and antifungal agents; therefore, they could be promising novel lead molecules (Li et al. 2017). Pyrrolo [1,2-a]pyrazine -1,4-dione, hexahydro-3-(phenylmethyl)- is an organooxygen and organonitrogen compound. It is functionally related to alpha-amino acids. Prasad et al. (2021) reported that this compound exhibits antimicrobial activity against bacteria and fungi. Pentanoic acid or valeric acid is a straight-chain saturated fatty acid containing five carbon atoms. These compounds act as plant metabolites. It is a short-chain fatty acid and a straight-chain saturated fatty acid. It is a valerate-conjugated acid. This compound showed anticancer activity (Dutta et al. 2019).

The 2,3,4-Trihydroxybenzaldehyde is another bioactive compound present in the BF1 extract. 2,3,4-Trihydroxybenzaldehyde (TBA) is a phenolic compound. Friedman et al. (2003) reported that this compound exhibits antimicrobial activity against some bacteria. 2,3,4-Trihydroxybenzaldehyde was highly active, as evidenced by BA_{50} values ranging from 0.0037 to 0.20 for the four bacteria. The corresponding 2,3,4-trihydroxybenzoic acid was active against *Campylobacter jejuni* (BA_{50} = 0.046) and *S. enterica* (BA_{50} = 0.11) and slightly active against *E. coli* (BA_{50} = 0.66) (Friedman et al. 2003). Isovaleric acid is a saturated fatty acid containing a C5 branch. This acid acts as both a plant and mammalian metabolite. Isovaleric acid is a metabolite produced by *E. coli* (strain K12, MG1655) (NCBI 2024b). L-Leucine was also detected in the BF1 isolate extract. The L-enantiomer of leucine was referred to as l-leucine. It functions as a metabolite in plants, mice, humans, *Saccharomyces cerevisiae*, *E. coli*, and algae. It is an amino acid belonging to the pyruvate family, a proteinogenic amino acid, an l-alpha amino acid, and a leucine residue (NCBI 2024b).

High concentrations of phenethyl alcohol were detected in the extract of BF4 isolate extract. This compound, also called 2-phenylethanol, is a primary alcohol in which ethanol is substituted with a phenyl group at position 2. Phenethyl alcohol (PEA) was tested for its antibacterial properties against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecium*) and Gram-negative (*E. coli* and *Pseudomonas aeruginosa*) bacteria. In addition, we investigated PEA PEA's mode of action of PEA on bacterial cell membranes. The cell envelopes of Gram-negative bacteria were found to be permeabilized upon morphological analysis using transmission electron microscopy; Gram-positive bacteria displayed cytoplasmic membrane solubilization in *S. aureus*, whereas *E. faecium* showed minimal alterations (Corre et al. 1990). Tryptophol (indole-3-ethanol) was detected in the extract of isolate BF4. It is a metabolite that acts as a sponge in bacteria, fungi, and plants. The ability of tryptophol to operate as an autoantibiotic against *C. albicans*, which is known to be acquired nosocomially, is solely attributed to its function as a quorum-sensing molecule (QSM). Additionally, a study on the bactericidal activity of hydroxylated chemicals found in red wine revealed that tryptophol showed modest antibacterial activity against the food pathogen *C. jejuni* (Palmieri and Petrini 2019). Compound 2,3-butanediol was detected in the extract of isolate BF4. The chemical,

cosmetic, food, agricultural, and pharmaceutical sectors employ bio-based platform chemicals 2,3-butanediol (BDO) and acetoin in various ways. BDO derivatives, on the other hand, can be utilized as fuel additives, in creating polymers, and in manufacturing synthetic rubber (Maina et al. 2022).

The other compounds in the extract of isolate BF17 were 2-methyldimedone (or 1,3-Cyclohexanedione, and 2,5,5-trimethyl), L-norvaline, benzeneacetic, and cyclo (prolylvalyl). Various 1,3-cyclohexanedione derivatives are used in multiple applications, including herbicides, promoters, and antimalarials (Hassan et al. 2014). L-norvaline combined with DOX inhibits the proliferation of breast cancer cells (Zhu et al. 2022). l-Norvaline is a promising neuroprotective agent that can be tailored for the treatment of various neurodegenerative disorders, including Alzheimer's disease (Polis et al. 2019). Benzeneacetic acid is widely used in the pharmaceutical industry to manufacture antibiotics. It is the precursor (reactant) for penicillin G production. The compound cyclo (prolylvalyl) has antifungal activity, as shown by several studies (Choub et al. 2021).

In conclusion, a total of 641 phyllospheric bacterial colonies were obtained from *R. mucronata* leaves. Based on the characteristics, 53 isolates of bacteria were identified. Morphological observations from Gram staining revealed 42 isolates of Gram-positive bacteria and 11 isolates of Gram-negative bacteria. The inhibition test results showed that out of 53, 10 isolates had the potential to inhibit the growth of *E. coli*, 6 isolates could inhibit the growth of *B. subtilis* bacteria, and 3 isolates inhibited the growth of both bacteria. Phytochemical tests of the crude extract revealed the presence of alkaloids, tannins, and saponins. Several active metabolites were detected and identified from three isolates BF1, BF4, and BF17, which had antimicrobial activity. Therefore, further research should be conducted to investigate the antimicrobial activity of the identified compounds.

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