Biomedical & Pharmacology Journal, December 2024. Vol. **17**(4), p. 2341-2354

Antibacterial Potential of *Pseudomonas aeruginosa* ISP1RL4 Isolated from Seaweed *Eucheuma cottonii* against Multidrug-resistant Bacteria

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https://dx.doi.org/10.13005/bpj/3029

(Received: 14 August 2024; accepted: 28 November 2024)

E*ucheuma cottonii* is a type of seaweed that are commonly found in Indonesia. As many other marine organisms, *E. cottonii* builds a strong bonding with its endophytic bacteria. These bacteria are well known to synthesize various of bioactive compounds including antibacterial compounds to protect its host from bacterial infections and pathogenic bacteria. Previous study has successfully isolated bacterial encoded as ISP1RL4 with antibacterial potential against nonresistant Gram-positive and Gram-negative bacterial target. This research aimed to identify the ISP1RL4 isolate based on DNA sequencing, to evaluate antibacterial activity of the crude extract of ISP1RL4 isolate against multidrug-resistant bacterial target and to analysis chemical profiling of the extracts. Briefly, the cell mass of ISP1RL4 bacterial isolate was fermented for 2 weeks in 100 mL sterile liquid ISP-2 medium and then filtered. Extraction was carried out using ethyl acetate with an extraction ratio of 1:1 twice. Ethyl acetate extracts of ISP1RL4 were tested against multidrug-resistant bacteria Methicilin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* ESBL, *Klebsiella pneumoniae* ESBL, and *Acinetobacter baumanii*. Our findings revealed that the ISP1RL4 phylogenetically related to *Pseudomonas aeruginosa* strain M4 with 100% of sequence similarity. The crude extract of P. aeruginosa ISP1RL4 showed diameter zone of inhibition of 9.0±1.0 mm, 10.3±2.0 mm and 9.4±0.1 mm against MRSA, *E. coli* ESBL, and *K. pneumoniae* ESBL respectively. No antibacterial activity of the crude extract was observed against A. baumanii. The liquid chromatography-high resolution mass spectrometry (LC-HRMS) analysis detected 381 compounds with 2-Amino-1,3,4-octadecanetriol (11.2%) identified as the major antibacterial compound present in ethyl acetate extracts of *P. aeruginosa* ISP1RL4. In addition, gas chromatography/mass spectrometry (GC/MS) analysis identified 39 compounds and 11 of them have been associated as antibacterial molecules. Among these 11 molecules, four prominent antibacterial compounds (> 8%) were 2-hexanol, 3-hexanol, 3-Pentanol, 2-methyl- and 2-hexanone. Overall, the ability of P. aeruginosa ISP1RL4 crude extract to inhibit selected

multidrug-resistant bacterial target and the presence antibacterial compounds in the extract provided an important insight. This change to avoid redundancy of using the word promising that the isolate could potentially be a promising antibacterial producer.

Keywords: Anti-bacterial; Bioprospecting; Marine; Multidrug resistant bacteria; Secondary metabolites; *Pseudomonas aeruginosa*.

Bacteria that are resistant to multiple drugs (MDR) have become a significant public health issue due to their increasing prevalence[1]. The excessive and improper use of antibiotics has driven the development of resistance in various bacteria, leading to the accumulation of multiple resistance gene[2]. A number of Gram-positive and negative bacterial species have transformed into resistance strains against different types of antibiotics such as β-lactamase classes and vancomycin[3]. Furthermore, the emergence of broad-spectrum beta-lactamase (ESBL) enzymes in Enterobacteriaceae has worsened the global antibiotic resistance crisis[4]. Although raising public awareness to use antibiotic rationally remains essential, the urgent need for new and potent antibiotic producers to combat MDR bacteria cannot be overstated.

For decades, terrestrial microorganisms particularly bacteria and fungi have been the primary target discovery for novel antibacterial compounds[5]. Nevertheless, many of these antibiotic compounds have been previously isolated and reported, leading to a reduction in the novelty rate of these compounds[6]. Conversely, marine habitats offer variety of bioactive molecules, including antibiotics that hold significant pharmaceutical importance[7]. Various marine species have been proven to produce arrays of bioactive molecules, with pharmaceutical potential including that of antibiotic compounds[8]. However, to perform clinical test and to synthesize specific bioactive molecules require a large number of biomasses to acquire sufficient extracts[9]. Furthermore, ecological concerns prevent the direct cultivation of such substantial biomass in nature. As a result, the focus has shifted towards bioprospecting marine bacteria, particularly those associated with marine organisms, as they offer faster and relatively easier cultivability under laboratory conditions, making them suitable for synthesizing compounds of interest[10,11].

Marine microorganisms, particularly bacteria, have emerged as significant sources of novel antibacterial compounds. This is largely due to their unique evolutionary adaptations to the marine environment, which have equipped them with diverse biochemical pathways for producing bioactive metabolites[12–14]. Among these microorganisms, members of the genus Bacillus are particularly noteworthy. They are known to synthesize a variety of antimicrobial substances, including polyketides, lipopeptides, and bacteriocins, which exhibit broad-spectrum antimicrobial activity against various pathogens[15–17]. Other marine bacterial species such as actinobacteria are among the prominent producers of antibacterial compounds, showcasing their potential in drug discovery and development[18]. Overall, marine bacteria represent a rich reservoir of antibacterial compounds with diverse mechanisms of action.

Eucheuma cottonii, a seaweed species commonly found in Indonesia, is rich in nutrients and valuable compounds like carrageenan, flavonoids, and tannins[19,20]. Like other seaweeds, it forms a beneficial partnership with bacteria, which help it grows, develops, and defends against threats by producing antibacterial substances[21]. However, research on *E. cottonii*-associated bacteria is rather scarce[22,23]. In a previous study, *Aeromonas* bacteria isolated from *E. cottonii*, actively inhibited *Staphylococcus aureus* and *Escherichia coli*[22]. Additionally, twenty-three bacterial isolates were discovered on *E. cottonii* in a recent study conducted in the coastal waters of Buleleng, Bali, with six of these isolates exhibiting antibacterial activity against *S. aureus*, *Streptococcus mutans*, *E. coli*, and *Klebsiella pneumoniae*[23].

Among six reported potential isolates, a bacterial isolate encoded as ISP1RL4 specifically displayed the strongest antibacterial activity[23]. However, it is remained unclear to what species

this isolate is assigned. Therefore, it requires further molecular identification and characterization. In addition, it is intriguing to evaluate antibacterial activity of the isolate crude extract against multidrug-resistant bacteria to confirm its previous reported antibacterial potential based on agar block method[23]. Furthermore, chemical profiling on the bacterial extract was performed to provide a clear insight on the possible promising antibacterial compounds.

MATERIALS AND METHODS

Materials

The ISP1RL4 bacterium was isolated from the seaweed *E. cottonii* from Patas village, Buleleng Regency, Bali, Indonesia[23]. The pure culture was grown in slant agar and stored at 4°C until further used. Gram staining and Ziehls-Neelsen staining kits were purchased from local suppliers. The test pathogenic bacteria used were Methicilin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* Extended-spectrum beta-lactamases (ESBL), *Klebsiella pneumoniae* ESBL and ESBL *Acinetobacter baumanii* were used as previously described[24]. All other analytical grade chemicals such as aqua distillation, ethyl acetate was purchased from local suppliers.

DNA isolation

Cell mass of ISP1RL4 pure culture was grown in 1.5 mL sterile International *Streptomyces* Project-2 (ISP-2) broth medium and was incubated at 28°C for 7 days. Bacterial DNA was extracted using a bacteria DNA preparation kit following the protocol (Jena Bioscience, Germany). DNA concentration was determined by a Nanodrop with a 260/280 nm ratio[25].

16SrDNA gene amplification and phylogenetic analysis

Molecular identification was performed by PCR amplifying 16S rRNA gene using two primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5'-CGGTTACCTTGTTACGACTT-3') based on the previously described protocol[24]. PCR product was sent for Sanger sequencing to PT Genetika Science, Tangerang, Indonesia (https://ptgenetika.com/). The quality of raw sequence was checked using sequence scanner V.2.0 and low-quality sequences were trimmed using ChromasPro 2.1.10. Finally, sequences were assembled using software DNAMAN Ver.9.0. The nucleotide sequence was compared against a database of known sequences using the n-BLAST method on the NCBI BLAST platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Following the BLAST search, the ten most relevant sequences were chosen and their base information was used to construct a phylogenetic tree. The Neighbor-joining method with the Kimura-2 parameter model was implemented in MEGA X software (https://www.megasoftware.net)[24]. The reliability of the tree was assessed using bootstrap analysis with 1000 repetitions.

Scanning Electron Microscope (SEM) preparation

The SEM observation method was adapted from a previous study[26]. Cell mass of ISP1RL4 isolate were obtained from overnight culture on ISP-2 medium. Cell biomass was washed by Phosphate Buffer Saline (Merck, Germany) 1x. The cell biomass was soaked with 2% glutaraldehyde in PBS for 2 h at room temperature. The cell biomass was separated from solution was centrifugation method (13,000 rpm for 5 min). The cell biomass was rinsed with PBS twice. The sodium tetraoxide (1% in PBS) was added in cell biomass and was soaked for 2 h in room temperature. Sodium tetraoxide was removed by centrifugation method. Before coating process, the cell biomass was dehydrated with serial ethanolic solution (70% for 10 min, 96% for 10 min, absolute ethanol for 10 min) and followed by centrifugation method to obtain ethanolic free cell biomass. The cell biomass was placed on carbon tape and subsequently applying a gold coating through a sputtering process. The scanning electron microscope was set to a high vacuum, 5 kV accelerating voltage, 30% spot intensity, and magnifications of 2,000x, 5,000x, and 10,000x (Hitachi SU3500, Japan)[26].

Fermentation and Extraction Condition

Ten mL of ISP1RL4 pre-cultured in ISP-2 media was added aseptically into 90 mL sterile ISP-2 broth and the mixture was fermented in 14 days at 150 rpm[27]. After reaching the fermentation period, the supernatant was separated from cell mass using Whatman paper no 1. Subsequently, it was extracted using 100 mL of ethyl acetate pro Analisa (Merck, Germany). A separatory funnel

was used to separate the organic layer from liquid layer. The extraction and separation process were repeated two times. Finally, the pool of organic layers was evaporated in a vacuum evaporator to achieve the final extract for further antimicrobial screening[28].

Antibacterial Assay of Against Selected Multidrug-Resistant Bacteria

Screening of the antibacterial activity of ISP1RL4 isolate crude extract was performed following disc diffusion assays. Thirty microliters of ISP1RL4 crude extract were applied to sterile 6-millimeter diameter papers disc. These discs were then placed in triplicates on LB agar plates containing various multidrug-resistant bacteria, including MRSA, extended-spectrum beta-lactamase (ESBL) *Escherichia coli* and ESBL *Klebsiella pneumoniae*, and ESBL *Acinetobacter baumannii*. These plates were stored at a 37°C incubator for one day. The diameter of inhibition zone formed against each bacterial target was quantify using a digital caliper.

Thin Layer Chromatography and Antibacterial Assay

The extract was separated and purified using thin layer chromatography (TLC). Briefly, TLC of the substance was applied on the GF-254 silica gel and developed in a solvent system containing n-hexane and ethyl acetate in a ratio of 4:6. Distinct components were identified by viewing them under ultraviolet light (254 nm), subsequently their retention factors (Rf values) were determined. Each visible spot on the TLC plate was then collected, dissolved in the same solvent mixture, and tested for antibacterial activity against the MDR bacterial test using the Kirby-Bauer method as previously described.

Gas Chromatography/Mass Spectrometry preparation and analysis

Volatile compounds that constitute ethyl acetate crude extract of ISP1RL4. Briefly, 0.1 gram of the crude extract was prepared and sent to the Forensic Laboratory Polda Bali, Indonesia

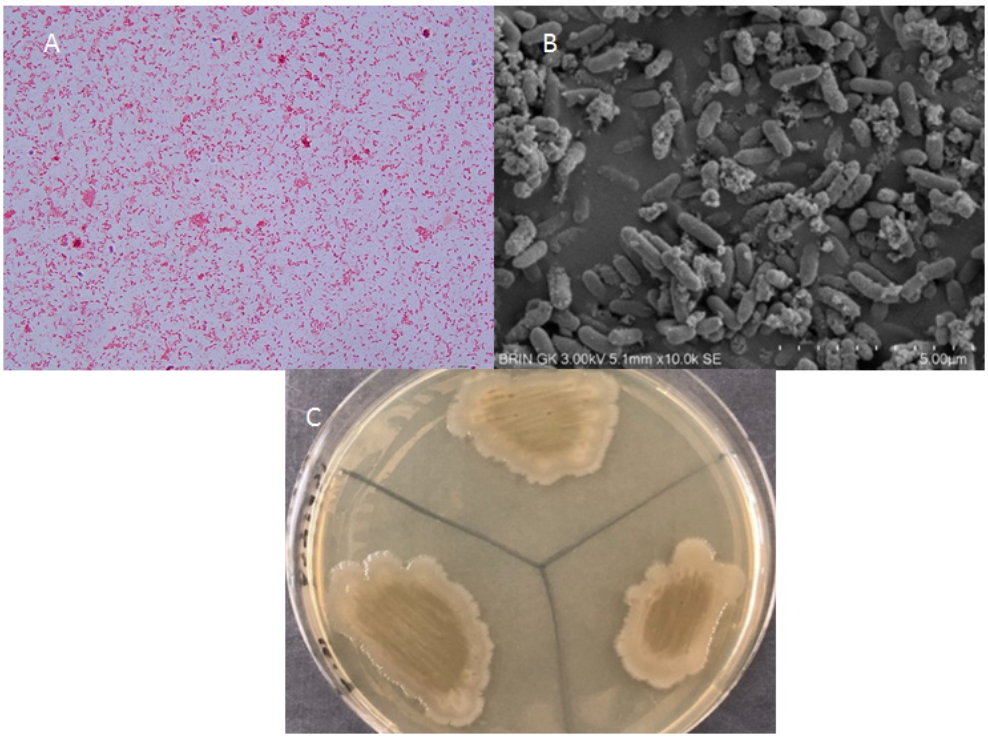

Fig. 1. Microscopic view of ISP1RL4 isolate bacterial cells (A) by Gram staining using 1000x magnification and (B) by Scanning electron microscopy using 10,000x magnification and (C) macroscopic view of ISP1RL4 pure isolate on agar plate

for further analysis. The extract was injected to the GC/MS instrument (Agilent Technologies 7890B/Agilent Technologies 5977B) based on the following setting: HP-5ms ultra inert column 30 m x250 µm x 0.25 µm, oven temperature (-60°C to 325°C), mode (splitless), pressure (25.523 psi), total flow (20.9 ml/min), average velocity (62.662 cm/sec), purge flow to split vent (15 mL/min at 0.75 min), and gas (He). Chromatograms were analyzed by matching the compound fragments from each chromatogram peak with literature to determine the type of content and to understand the biological properties of the discovered compounds.

Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) preparation and analysis

Metabolomic analysis of ISP1RL4 ethyl acetate was performed in Ultra-High-Performance Liquid Chromatography coupled to untargeted High-Resolution Mass Spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC paired with Thermo Scientific Q Extractive (Thermo Fisher Scientific, Massachusetts, USA). The machine was run by following previously described procedure[26].

RESULTS AND DISCUSSION

Morphological Observation

The cell morphology of the ISP1RL4 bacterial isolate, as observed under a microscope after Gram staining, categorizes the isolate as a Gram-negative bacterium with bacilli-shaped cells. (Figure 1A). This observation was confirmed by examining bacterial cells of the ISP1RL4 isolate under a scanning electron microscope, which provided a clearer appearance with smooth surface, approximately 5 µm in length, and exhibiting attachment to each other (Figure 1B). On agar plate, the ISP1RL4 pure isolate had colonies with irregular surface shapes, firmly attached to the media, with a powdery consistency, and rough textured and dull surfaces, and had a grayish-yellow pigmentation with the reverse of colony color with light yellow green pigmentation on ISP-2 agar at 11 days of age (Figure 1C). Based on the

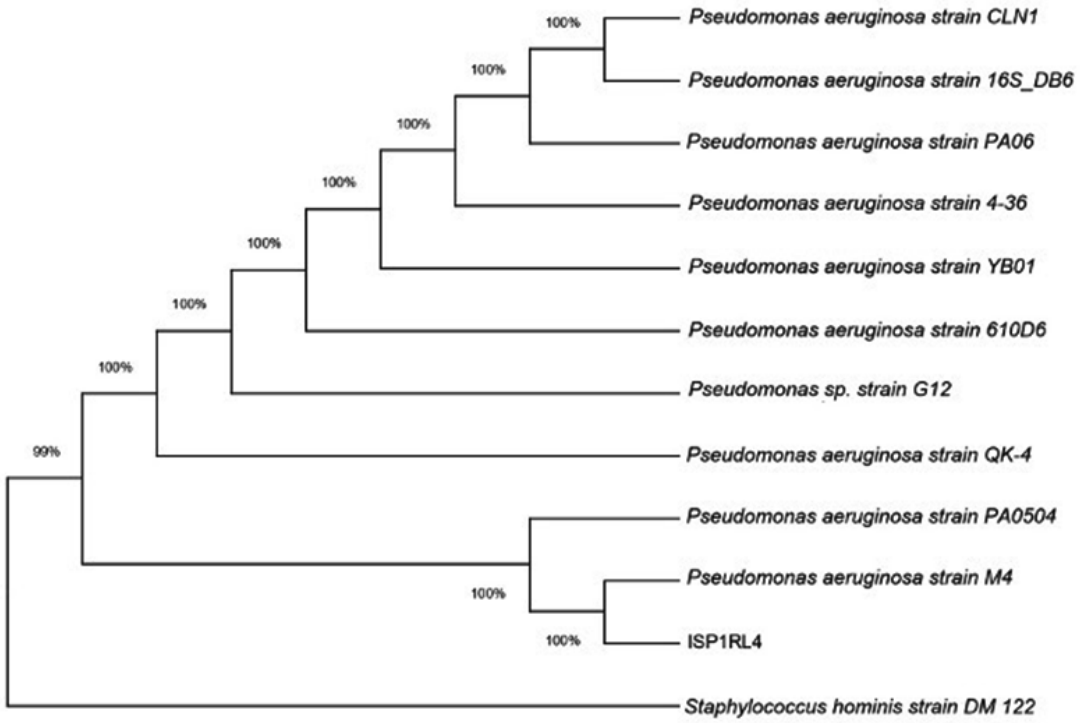

Fig. 2. ISP1RL4 isolate phylogenetic tree which describes the phylogenetic position of ISPIRL4 isolate with other bacteria in one clade or another clade (*Staphylococcus hominis* strain DM 122 as the outer group).

results of Ziehl-Neelsen's acid-resistant staining, ISP1RL4 isolate was a non-acid fast bacterium because it could not retain the red dye from carbolic fuchsin after being dripped with alcoholic acid.

Molecular Identification

DNA isolation of ISP1RL4 exhibited concentration of 296 ng/µL with a DNA purity level of 1.78 nm at a ratio of A260/280 nm. Based on the result of the alignment of ISP1RL4 isolate, it was revealed that ISP1RL4 isolate had DNA sequences with 100% homology (percentage identity) with *Pseudomonas aeruginosa* strain M4. The PCR results of the 16S rRNA product of ISP1RL4 has a DNA sequence of less than 1500 bp, which is 1411 bp. Percentage identity or what is known as the homology value is a percentage that indicates how well the input DNA sequence matches the target DNA sequence. The results of the phylogenetic tree construction as shown in Figure 2 showed that the ISP1RL4 isolate was located in the same clade and branch, and shared the same node with *Pseudomonas aeruginosa* strain M4 on the phylogenetic tree. According to Figure 3, the phylogenetic tree of ISP1RL4 isolate formed was not a paraphyletic group of phylogenetic trees, but a monophyletic. Based on molecular identification, ISP1RL4 isolate can be identified as

Table 1. Antibacterial activity of the crude extract of ISP1RL4 isolate against multi-drug resistant bacteria

Multidrug resistant bacteria	Zone of Inhibition (mm)
MRSA	9,0 ± 1,0
E. coli ESBL	10,3 ± 2,0
K. pneumoniae ESBL	9,4 ± 0,1
A. baumanii	0± 0,1

Notes:
Average diameter of the inhibition zone for each isolate was measured from three replications.

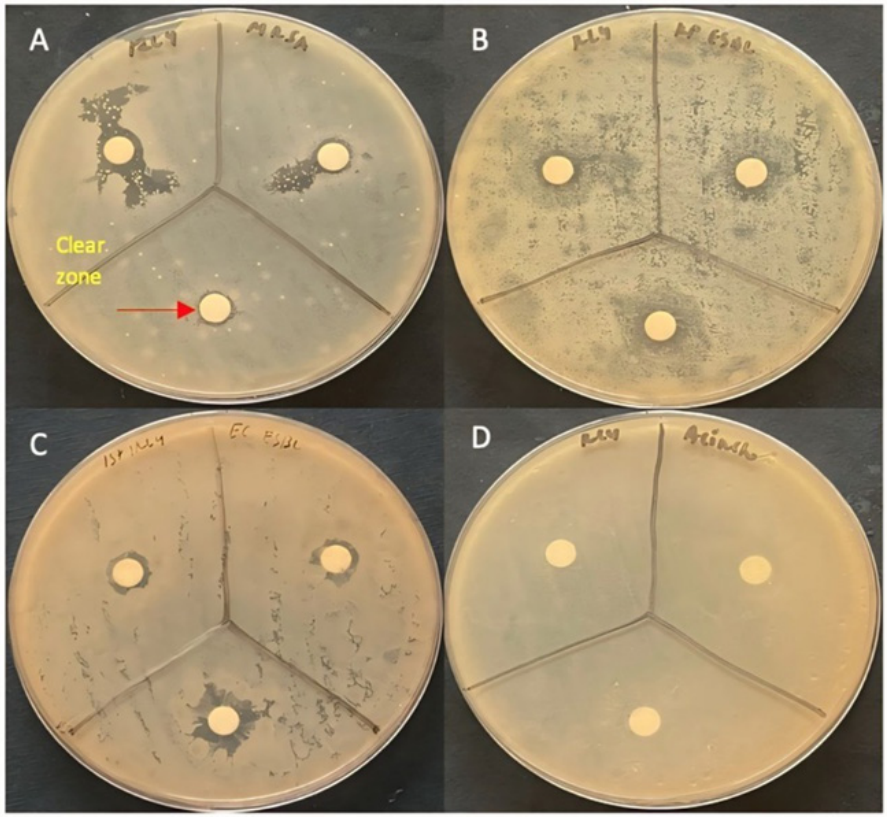

Fig. 3. Antibacterial activity of ISP1RL4 crude extract against multidrug-resistant bacteria (A) Methicillin-resistant *Staphylococcus aureus* (MRSA), (B) *Klebsiella pneumoniae* ESBL, (C) *Escherichia coli* ESBL and (D) *Acinetobacter baumanii*. Red arrow showed a clear zone as the inhibition zone

Pseudomonas aeruginosa sp. and phylogenetically related to *Pseudomonas aeruginosa* strain M4.

Evaluation of Antibacterial Activity of ISP1RL4 Crude Extract Against Multidrug-resistant Bacteria

The results showed that ISP1RL4 isolate could inhibit the growth of three multidrug-resistant bacteria, except for *A. baumanii* (Table 1). ISP1RL4 extract had moderate category of antibacterial activity with an average inhibition zone of >9 mm. The highest antibacterial activity of ISP1RL4 extract was shown in multidrug resistant *E. coli* ESBL bacteria with an inhibition zone diameter of 10.3 ± 3.0 mm. ISP1RL4 extract had the lowest ability to inhibit the activity of MRSA bacteria with diameter zone of inhibition of 9.0 ± 1.0 mm.

Table 2. Antibacterial activity of the active compound fraction of ISP1RL4 isolate on the test bacteria

Fraction	R*f* value	Zone of Inhibition (mm)			
		MRSA	*E. coli* ESBL	*K. pneumoniae* ESBL	*A. baumanii* ESBL
1	0,06	18±4	0	7,4±0,2	0
K+ (*Nalidixic acid* 30 µg)		9±0,3	9,8±0,2	9,2±0,2	9,5±0,3
K- (Ethyl acetate)		0	0	0	0

Notes:
K+: Positive control; K-: Negative control. The average diameter of the inhibition zone for each isolate was measured from three replications

Ethyl acetate extract of ISP1RL4 isolate was able to inhibit the growth of the Gram-positive and Gram-negative multidrug resistant bacteria as shown in Figure 3. Ethyl acetate crude extract of

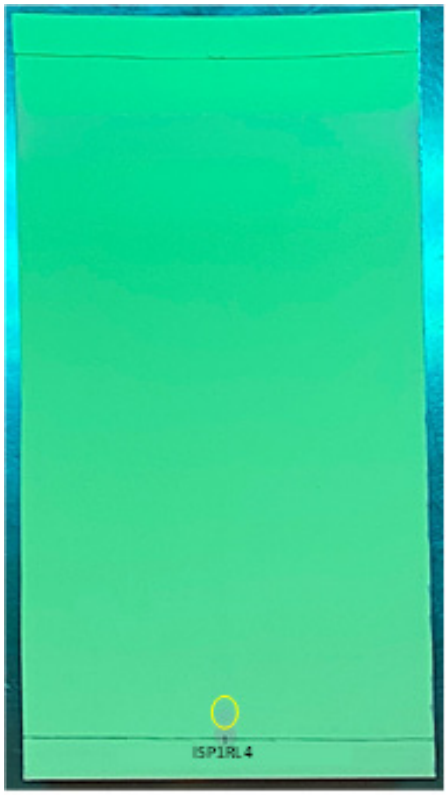

Fig. 4. Spot visualization of ISP1RL4 isolate extract fraction on GF_{254} silica gel plate
Notes: Yellow circle: spot on the fraction that had successfully appeared

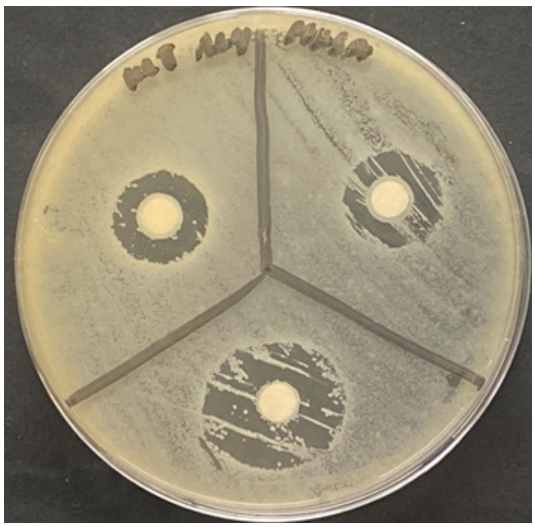

Fig. 5. Diameter zone of inhibition of the ethyl acetate fraction against MRSA

ISP1RL4 was more effective to inhibit the growth of Gram-negative multidrug resistant bacteria especially *E. coli* ESBL as shown in Table 1 compared to MRSA.

Fraction Analysis of Active Compound Extract Filtrate in ISP1RL4 Isolate

The results of TLC analysis (thin layer chromatography) revealed that there was only one spot (Figure 4) that appeared on the GF_{254} TLC plate (Table 2). Fraction F1 had an R*f* value of 0,06. The fraction was tested for antibacterial activity on the test bacteria. The highest inhibition of the fraction was observed against MRSA (Figure 5).

LC-HRMS Analysis of Active Compound in ISP1RL4 Isolate

The results of LC-HRMS analysis of the crude ethyl acetate extract isolate ISP1RL4 detected 381 compounds with different peak percentages and retention times which were successfully identified in the LC-HRMS chromatogram shown in Figure 6. Eleven antibacterial compounds detected in the ethyl acetate crude extract of ISP1RL4 isolate based on LC-HRMS could be seen in Table 3. The highest detected compound was 2-Amino-1,3,4-octadecanetriol (11,21 % relative abundance). The GC-MS analysis of the crude ethyl acetate isolate ISP1RL4 detected 11 antibacterial compounds as presented in Table 4.

DISCUSSION

The study investigated the antibacterial properties of a bacterial strain, ISP1RL4, isolated from *E. cottonii* seaweed. Microscopic analysis revealed that the isolate was rod-shaped and Gram-negative. Molecular identification confirmed that the isolate ISP1RL4 identified as *Pseudomonas aeruginosa*. The isolation of the bacterium from seaweed aligns with the known distribution of Pseudomonas species, which are frequently found in marine ecosystems[52,53]. Marine *P. aeruginosa* has been a subject of interest due to its diverse characteristics and potential applications. Studies have highlighted the adaptability of *P. aeruginosa* in various environments, including marine habitats, where it demonstrates unique patterns of cultivability and survival, indicating physiological adaptations to oceanic conditions[54].

Our study has provided further evidence to support the antibacterial potential of *P. aeruginosa* ISP1RL4, as initially suggested by block agar experiments[23]. The ethyl acetate extract derived from *P. aeruginosa* ISP1RL4 effectively suppressed the growth of both Gram-positive and Gram-negative MDR bacteria. Additionally, *P. aeruginosa* has been identified as a source of antimicrobial activity against multidrug-resistant pathogens,

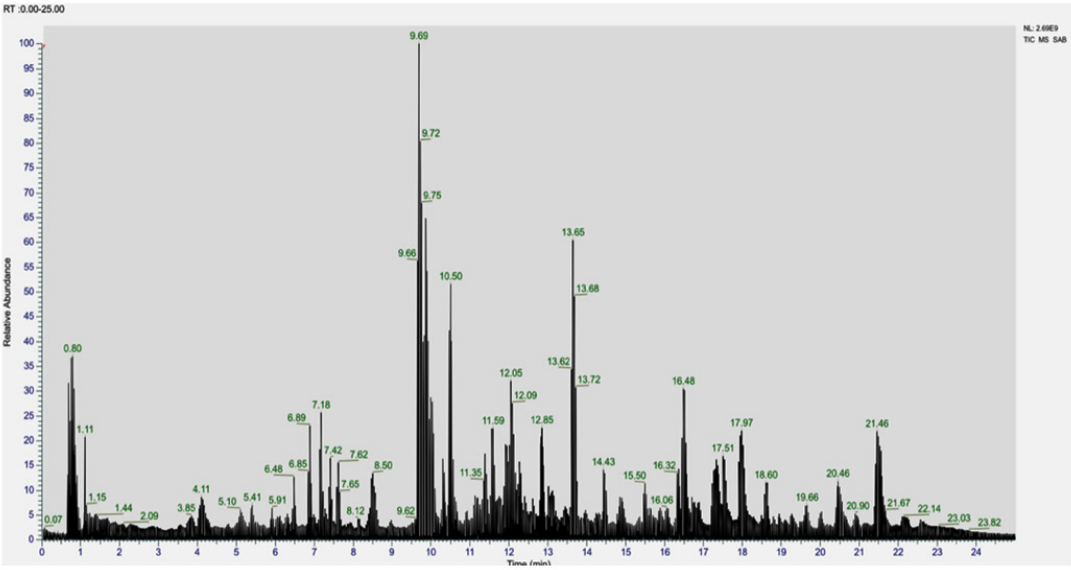

Fig. 6. LC-HRMS chromatogram of the ethyl acetate extract of ISP1LR4 isolate

Table 3. Antibacterial compounds detected in the ethyl acetate crude extract of ISP1RL4 isolate based on LC-HRMS results

Name	Formula	Cal. Molecular Weight	Compound characteristic	Activity	% relative abundance	Reference
2-Amino-1,3,4-octadecanetriol	$C_{18}H_{39}NO_3$	317.29191	Organic compound	Antibacterial	11.21	29
Bis(4-ethylbenzylidene) sorbitol	$C_{24}H_{30}O_6$	414.20368	Organic compound	Antibacterial	4.81	30
Dextromethorphan	$C_{18}H_{25}NO$	271.19315	Organic compound	Antibacterial	4.53	31
Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15142	Organic compound	Antibacterial	4.41	32
C14-Dihydroceramide	$C_{32}H_{65}NO_3$	511.49607	Organic compound	Antibacterial	4.17	33
C16-Dihydroceramide	$C_{34}H_{69}NO_3$	539.52751	Organic compound	Antibacterial	2.76	33
6-Methoxyquinoline	$C_{10}H_9NO$	159.06818	Organic compound	Antibacterial	2.10	34
D-(+)-Maltose	$C_{12}H_{22}O_{11}$	342.11637	Organic compound	Antibacterial	1.79	35
Propranolol	$C_{16}H_{21}NO_2$	259.15684	Organic compound	Antibacterial	1.63	36
1-Stearoylglycerol	$C_{21}H_{42}O_4$	358.30770	Organic compound	Antibacterial	1.52	37
Armillaramide	$C_{34}H_{69}NO_4$	555.52202	Organic compound	Antibacterial	1.12	33

Table 4. Antibacterial compounds detected in the ethyl acetate crude extract of ISP1RL4 isolate based on GC-MS results

Name	Formula	Molecular Weight (g/mol)	Compound characteristic	Activity	% relative abundance	Reference
2-Hexanol	$C_6H_{14}O$	102.17	Organic compound	Antibacterial	10.94	38
3-Hexanol	$C_6H_{14}O$	102.17	Organic compound	Antibacterial	9.86	39
3-Pentanol, 2-methyl-	$C_6H_{14}O$	102.17	Organic compound	Antibacterial	9.86	40
2-Hexanone	$C_6H_{12}O$	100.16	Organic compound	Antibacterial	8.60	41
Isobutyl acetate	$C_6H_{12}O_2$	116.16	Organic compound	Antibacterial	7.62	42
Cyclotrisiloxane, hexamethyl-	$C_{12}H_{22}O_{11}$	222.46	Organic compound	Antibacterial	7.12	43
3-Hexanone	$C_{18}H_{30}O_3S$	100.16	Organic compound	Antibacterial	4.56	44
Toluene	$C_6H_5CH_3$	92.14	Organic compound	Antibacterial	3.81	45
Ethylbenzene	C_8H_{10}	106.16	Organic compound	Antibacterial	2.40	46,47
o-xylene	C_8H_{10}	106.16	Organic compound	Antibacterial	2.40	48–50
Actinobolin	$C_{13}H_{20}N_2O_6$	135.21	Organic compound	Antibacterial	1.10	51

with certain marine isolates demonstrating efficacy against bacteria like *S. aureus*[55]. The production of antimicrobial metabolites by marine Pseudomonas strains further underscores their potential as sources of novel antibacterial agents. The antibacterial properties exhibited by the ethyl acetate extract suggest that its components have a wide range of antibacterial activity[56]. This result aligns with prior research highlighting the broad-spectrum antibacterial properties of *P. aeruginosa* against various bacterial species. In terms of the degree of inhibition, the extract exhibited moderate to strong antibacterial activity, with inhibition zones ranging from 9.8 to 11 millimeters[57]. Notably, the purified extract effectively suppressing MRSA bacterial target compared to that of other test bacteria. This finding highlights the selective antibacterial activity of the purified extract, demonstrating a particular efficacy against MRSA compared to other bacterial strain. In addition, discrepancy diameter zone of inhibition could be due to the absence of an outer membrane with lipopolysaccharide in Gram-positive bacteria, making them more susceptible to antibacterial compounds compared to Gram-negative bacteria[58,59]. The produced diameter zone of inhibition may vary which can be attributed to the different types of secondary metabolites produced, different chemical composition, concentration, and polarity. Additionally, the morphological and physiological characteristics of each bacterial strain influence these results[60].

Variations in the type of active compound content were found in the LC-HRMS and GC-MS results. Active compounds were found to be more diverse and numerous in LC-MS results than GC-MS. The ability of LC-HRMS to generate exact mass measurements and molecular formulas for unknown compounds in an extract could contribute to this observation. Additionally, this method excels at determining chemical structures with high sensitivity, even when working with small sample sizes and limited time[61,62]. Comparing to GC-MS, a commonly employed technique for characterizing chemotypes from a sample, the instrument was limited to analyzing non-polar and/or volatile compounds[63]. This had covered a broader spectrum of compounds found in LC-HRMS which had been shown by the identification of non-volatile compounds such as sorbitol and ceramides.

The active compound of *P. aeruginosa* ISP1RL4 ethyl acetate extract found in LC-HRMS was mainly dominated by 2-Amino-1,3,4-octadecanetriol (11.2%), which belong to the phytosphingosine compound class. Phytosphingosine is a long-chain sphingolipid base consisting an amino alcohol with 18 carbon atoms typical in plants, which has antibacterial properties. Phytosphingosine, at a concentration of 15.9 ìg/mL, effectively killed 95% of the three bacterial species: *P. syringae* pv. tomato, *A. tumefaciens*, and *R. radiobacter*[29]. C14-Dihydroceramide, C16-Dihydroceramide, and Armillaramide are ceramides reported for the first time that had been isolated from *Eucheuma cottonii*-associated bacterial. A study discovered that short-chain ceramides and a ù-azido-C6-ceramide exhibited antibacterial activity towards *Neisseria meningitidis* and *N. gonorrhoeae*[33]. Ceramides found in *Cissus incisa* leaves had an antibacterial activity against nine multidrug-resistant bacteria, with the most significant inhibition observed against Gram-negative bacteria, particularly carbapenem-resistant *Acinetobacter baumannii* at a concentration of 50 µg/mL[64]. Based on LC-HRMS results, a sorbitol compound was found in the ethyl acetate extract of *P. aeruginosa* ISP1RL4, namely Bis(4-ethylbenzylidene) sorbitol, which had also been found in black cumin extract with the best antibacterial activity against MRSA via in silico methods[30].

Despite the confirmed antibacterial potential of ethyl acetate extract of P. aeruginosa ISP1RL4, there are a number of limitations that need to be improved for future studies. Firstly, the observed activity resulted from crude extracts therefore an optimization is required such as performing Nuclear magnetic resonance (NMR) analysis to determine the exact active compounds[65]. Secondly, the fermentation volume at the current study was set at 100 mL which resulted in a low yield of an active extract, therefore a higher volume e.g. 1 L is essential to obtain more extracts. Thirdly, the current screening was mainly focused on measuring the diameter zone of inhibition, however no information is available on the median lethal dose (LD50) of the extract which is crucial for a more comprehensive antibacterial analysis. Fourthly, more MDR bacterial strains that are among human pathogens such as *Enterobacterium faecium*, *Enterobacterium*

faecalis, and *Streptococcus pneumoniae*[1] need to be included to evaluate antibacterial spectrum of the ethyl acetate extract of *P. aeruginosa* ISP1RL4. Lastly, the current study has not evaluated toxicity of the compounds, so the future study should be focused to screen for in vitro toxicity test such as MTT assays and lactate dehydrogenase. Such screening will ensure safety and efficacy of the extract.

CONCLUSION

In conclusion, this study has confirmed antibacterial potential of the isolate *Pseudomonas aeruginosa* ISP1RL4 against selected multidrug-resistant bacteria. Analysis of chemical profiles of the crude extract has identified a number of promising antibacterial molecules that potentially can be synthesized by *P. aeruginosa* ISP1RL4. Future studies would be focused to improve some limitations of the current results such as identifying the exact antibacterial compounds, increasing fermentation volume to obtain higher yield, analyzing LD50 of the extract, adding more MDR bacteria tests and performing in vitro toxicity tests. Nevertheless, this study adds a valuable insight on the potential of seaweeds-associated bacteria especially from the species of *P. aeruginosa* as the producer of (novel) antibacterial compounds.

ACKNOWLEDGMENT

Authors also thanks The Directorate of Laboratory Management, Research Facilities and Science and Technology Park, National Research and Innovation Agency (BRIN) Yogyakarta, Indonesia, for SEM and LC-HRMS instrument.

Funding Source

This research was funded by the Fundamental Research Grants awarded by Ministry of Education, Culture, Research, and Technology Republic of Indonesia, fiscal year 2024 to Anak Agung Gede Indraningrat under grant number: 110/E5/PG.02.00.PL/2024, 2927/LL8/AL.04/2024, 565/Unwar/DPPM/PD-13/2024.

Conflict of Interest

The author(s) do not have any conflict of interest.

Data availability Statement

Sequence data of ISP1RL4 bacterial isolate was deposited in GenBank under accession number PP783522. Data related to antibacterial screening, SEM and light microscopy observation, TLC, LC-HRMS and GC-MS can be accessed via the Figshare online repository http://surl.li/rpyonh

Ethics Statement

Experiment described in this manuscript has been ethically approved by the Ethics Commission of Faculty of Medicine and Health Sciences, Warmadewa University, Denpasar-Bali under ethics number: 345/Unwar/FKIK/EC-KEPK/I/2023 on 2 October 2023

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required

Clinical Trial Registration

This research does not involve any clinical trials

Author Contributions

AAGI designed experiments, provided consumables, compiled and performed final data analysis and wrote the draft manuscript. PPCPP performed lab works. EM performed LC-HRMS and SEM experiments. MDW performed data analysis on GC-MS and LC-HRMS. DAPSM cultured MDR bacteria. NLPEKS performed PCR and DNA sequencing. All authors read and reviewed the draft manuscript. All authors agreed with the manuscript before submission.

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