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Evaluation of the Microalga *Arthrospira platensis* as an Antibacterial Against Multidrug Resistance (MDR) Bacteria Causing Wound Infections

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ABSTRACT

Wound infections caused by multidrug-resistant (MDR) bacteria are a significant concern due to the high morbidity and mortality rates. This study aimed to evaluate the antibacterial activity of *Arthrospira platensis* extract against MDR bacteria that cause wound infections. Antibacterial activity was evaluated by determining the values of minimum inhibitory concentration (MIC) and minimum *b*actericidal concentration (MBC) with the microdilution method. The results from the study showed that *A. platensis* extract has antibacterial activity against *Methicillin-resistant Staphylococcus aureus* (MRSA), MDR *Enterococcus faecalis*, MDR *Escherichia coli*, MDR *Klebsiella pneumonia*, and MDR *Pseudomonas aeruginosa*. In this study, the MIC and MBC values of each *A. platensis* extract were ≥6.25mg/ mL. *A. platensis* has the potential to be developed as an antibacterial agent. To further explain the antibacterial activities of *A. platensis* extracts, more *in vivo* research and the identification of mechanisms of action are necessary.

INTRODUCTION

Wound infections can be caused by a variety of factors, including bacterial, viral, and fungal pathogens. *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* are the commonly identified bacterial species in infected wounds **(Prastiyanto *et al*., 2024a)**. One of the treatments suggested for bacterial infections is the use of antibiotics **(Bologa *et al*., 2013)**. However, inappropriate use of antibiotics according to applicable regulations can increase the prevalence of bacterial strains resistant to antibiotics or multi-drug resistant bacteria (MDR) **(Prastiyanto *et al*., 2024b)**. In 2019, the five leading pathogens causing resistance-related deaths

(*Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*) were responsible for 929000 (660000–1270000) deaths attributable to antimicrobial resistance and 3.57 million (2.62–4.78) deaths associated with antimicrobial resistance **(Murray *et al*., 2022)**. As a result of the numerous deaths brought on by MDR bacteria, alternate supplies of antibiotics made of natural substances are required.

Alternative sources of antibiotics derived from natural ingredients can be obtained from marine bacteria **(Kusmita *et al*., 2021; Prastiyanto *et al*., 2022a; Prastiyanto *et al*., 2024c; Prastiyanto *et al*., 2024d)** and plants **(Prastiyanto, 2021; Prastiyanto *et al*., 2021)**. Furthermore, of all previously listed natural components, microalgae stand for another natural ingredient that has the potential to function as a substitute antibiotic. *Arthrospira platensis*, commonly known as spirulina, is one species of microalgae that is beneficial for health to prevent or treat several diseases in humans, from infections to chronic wounds **(Wollina *et al*., 2018)**

Regarding its possible antibacterial properties, certain studies are interested in *A. platensis* extracts. The research conducted by **Abdel-Moneim *et al*. (2022)** stated that *A. platensis* methanol extract has better antibacterial activity compared to other extracts (N-hexane extract and acetone extract) against *Bacillus cereus*, *S. aureus*, *Listeria monocytogenes*, *E. coli*, *Salmonella typhi*, and *K. pneumonia*. Additionally, the esearch by **Singh *et al*. (2021)** showed that each extract of acetone, methanol, and ethyl acetate *A. platensis* concentration of 100% has antibacterial activity against *S. typhi*, *S. aureus* ATCC 25923, and *E. coli* ATCC 25992. **Usharani *et al*. (2015)** reported that the methanol extract of *A. platensis* has better antibacterial activity against *S. aureus*, *Streptococcus pyogenes*, *Streptococcus epidermidis*, *Proteus mirabilis*, *Bacillus cereus*, *K. pneumoniae*, and *Shigella flexneri* compared to extracts with other solvents (acetone solvent, ethanol solvent, N-hexane solvent, and petroleum ether solvent) and has antifungal activity against some pathogenic fungi.

Based on research conducted by several researchers, they only used standard bacteria (ATCC), and research has never been done against MDR bacterial strains. In addition, in previous studies, the solvents used to extract compounds contained in *A. platensis* were solvents methanol, acetone, ethyl acetate, and petroleum ether. Therefore, the use of other solvents such as ethanol, chloroform, and N-hexane representing each polarity of polar, semipolar, and nonpolar solvents, and research related to the antibacterial activity of *A. platensis* extract against MDR bacteria needs to be conducted. This study aimed to determine the antibacterial activity of *A. platensis* extract against MDR bacteria that cause wound infection and determine the minimum inhibitory concentrations (MIC) value and minimum bactericidal concentrations (MBC).

MATERIALS AND METHODS

Collection and extraction of *Arthrospira platensis*

A. platensis powder was obtained from Algaepark Indonesia Mandiri Ltd. located in Jalan Pedan, Karangdowo District, Klaten Regency, 57464, Central Java, Indonesia on December 24, 2022. Extraction of *A. platensis* was carried out by maceration method **(Prastiyanto *et al*., 2021)** using 96% ethanol solvent, 100% chloroform, and 100% N-hexane. The extraction began by dissolving 100 grams of *A. platensis* powder into 300mL of 96% ethanol solvent, 100% chloroform, and 100% N-hexane each in a 500mL beaker, then homogenizing on a magnetic stirrer at 260rpm overnight (24 hours) at room temperature. The coupling of each solvent was daily carried out until the solution became clear, assuming no more bioactive compounds in the *A. platensis* powder. The supernatant was then filtrated through Whatman filter paper number 1. The *A. platensis* extract filtrate was then evaporated using a rotary evaporator at 45°C until the filtrate volume decreases a lot, then the filtrate was accommodated and inserted in a water bath device until the *A. platensis* extract filtrate has a thick consistency like a paste (Fig. 1). The extract was then made in a concentration of 100mg/ mL by weighing the extract by 100mg, then put into a 1mL microtube, and then added with DMSO up to the limit mark of 1mL.

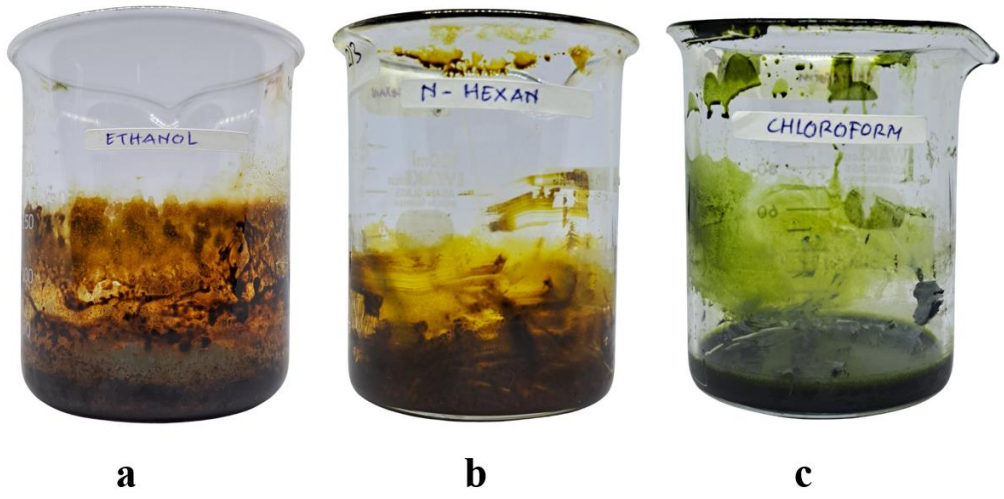

Fig. 1. Extraction of *A. platensis* carried out by maceration method using different solvents: a) Ethanol; b) Chloroform; c). N-hexane

Bacteria preparation and antibiotic sensitivity test

MDR bacteria used in this study include MRSA, MDR *Enterococcus faecalis*, MDR *Escherichia coli*, MDR *Klebsiella pneumonia*, and MDR *Pseudomonas aeruginosa*. All bacteria were obtained from the Microbiology Laboratory of RSUD K.R.M.T. Wongsonegoro Hospital, Semarang, Indonesia, and were the cause of wound infections.

Subsequently, bacteria were identified and antibiotic sensitivity tests were performed. MDR bacteria were subcultured on Blood Agar Plate (BAP) media overnight (24 hours) at 35±2°C. MDR bacterial colonies were homogenized and adjusted to the 0.5 McFarland standard ($1.5x10^8$ CFU/mL) using a densitometer **(Prastiyanto *et al*., 2024a)**.

Antibacterial activity test against MDR bacteria

Minimum inhibitory concentration (MIC)

MIC test against test bacteria was carried out using the *Mueller-Hinton Broth* (MHB) microdilution method **(CLSI, 2020)**. Determination of MIC value was carried out using serial dilution technique utilizing microwell plates. A total of 100µL of MHB was inserted in each well, and 100µL of *A. platensis* extract was inserted in the first well, followed by a series of dilutions until it reached the 8th well. Then, a total of 10µL of McFarland standard 0.5 MDR bacterial suspension was added to each well except in the negative control well line. Next, the microwell plate was incubated for 18 hours at a temperature of 35±2°C. After incubation, resazurin was added as much as 20µL to each well, and then incubated for 10-15 minutes **(Dian *et al*., 2019)**. Furthermore, color changes were observed in each well. The MIC value was determined as the lowest concentration of extract that was able to inhibit the growth of MDR bacteria determined by seeing no discoloration to purplish pink after being added with resazurin compared to controls. Moreover, the best antibacterial activity of the extract was indicated by the lowest value of MIC.

Minimum bactericidal concentration (MBC)

Wells from the MIC test was subcultured on blood agar plate (BAP) media and incubated in an incubator with a temperature of 35±2°C for 16-20 hours. The MBC value was determined by looking at the presence or absence of bacterial colony growth on BAP media. The lowest concentration of extracts that can kill bacteria was evidenced by the absence of bacterial colony growth, representing a way to determine the MBC value **(Prastiyanto *et al*., 2022b)**

Phytochemical screening of *Arthrospira platensis* extract

A. platensis extracts were phytochemically tested for the presence of secondary metabolite compounds, including flavonoids, tannins, saponins, alkaloids, steroids, and phenol using the methods described earlier **(Eve *et al*., 2020)**.

RESULTS AND DISCUSSION

Extract *Arthrospira platensis* yield

The yield of *A. platensis* extract with ethanol solvent is 96% higher compared to other extracts (Table 1). This can be caused by differences in the polarity of the solvent used, where according to the principle of "like dissolve like" polar solvents will dissolve

polar compounds, semi-polar solvents will dissolve semi-polar compounds, and non-polar solvents will dissolve non-polar-compounds **(Deshmukh *et al*., 2019)**. In addition, according to **Tavakoli *et al*. (2021)**, differences in yield values between one extract and another can be caused by differences chemical composition of the sample used.

Table 1. Extract *A. platensis* yield

Solvent	Yield (%)
Ethanol	3.25
Chloroform	0.95
N-hexane	0.57

Antibiotic sensitivity to MDR bacteria

Antibiotic sensitivity testing showed that the bacteria from wound infection identified were MDR bacteria (Fig. 2) since they were resistant to at least three or more classes of antibiotics. MRSA shows resistance to the antibiotic classes lincosamides (clindamycin), macrolides (erythromycin), fluoroquinolones (ciprofloxacin), aminoglycoside (gentamicin), and penicillin (oxacillin). MDR *Enterococcus faecalis* is resistant to the antibiotic classes macrolide (erythromycin), fluoroquinolones (ciprofloxacin, levofloxacin), tetracycline (tetracycline), glycopeptide and lipoglycopeptide (vancomycin). Moreover, MDR *Escherichia coli* is resistant to the antibiotic classes aminoglycoside (gentamicin), monobactam (aztreonam), cephalosporin (ceftazidine), penicillin (ampicillin), and sulfonamides (Trimethoprim-Sulfamethoxazole). Furthermore, MDR *Klebsiella pneumonia* is resistant to the antibiotic classes penicillin (ampicillin), monobactam (aztreonam), sulfonamides (Trimethoprim-Sulfamethoxazole), aminoglycoside (gentamicin), and cephalosporin (ceftazidine). MDR *Pseudomonas aeruginosa* is resistant to the antibiotic classes cephalosporin (ceftazidine), monobactam (aztreonam), aminoglycosides (gentamicin), and fluoroquinolone (ciprofloxacin).

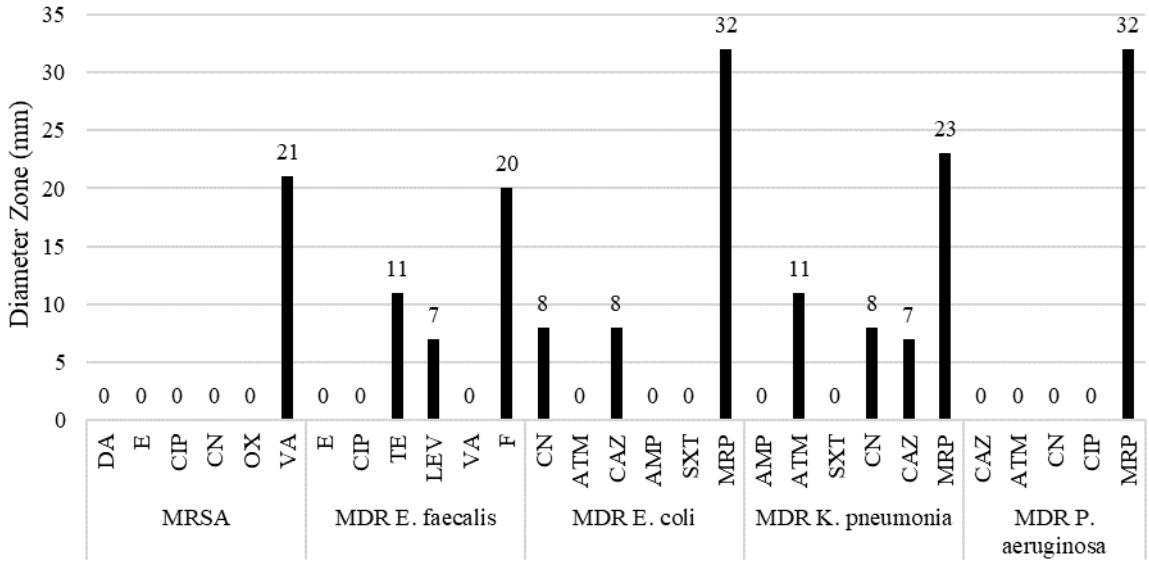

Fig. 2. Sensitivity test of some antibiotics to MDR bacteria. DA: Clindamycin, E: Erythromycin, CIP: ciprofloxacin, CN: gentamicin, OX: oxacillin, VA: vancomycin, TE: tetracycline, LEV: levofloxacine, F: nitrofurantoin, ATM: aztreonam, CAZ: ceftazidine, AMP: ampicillin, SXT: trimethoprim-sulfamethoxazole, MRP: meropenem

Antibacterial activity

The best MIC value is defined as the lowest concentration of *A. platensis* extract containing antibacterial compounds that can inhibit the growth of test bacteria. Determination of the MIC value is carried out by looking at the color change in the microwell plate. The best MBC value is defined as the lowest concentration of *A. platensis* extract capable of killing test bacteria. In this study, the MIC and MBC values of each *A. platensis* extract ranged from ≥6.25 mg/mL (Table 2 & Figs. 3, 4).

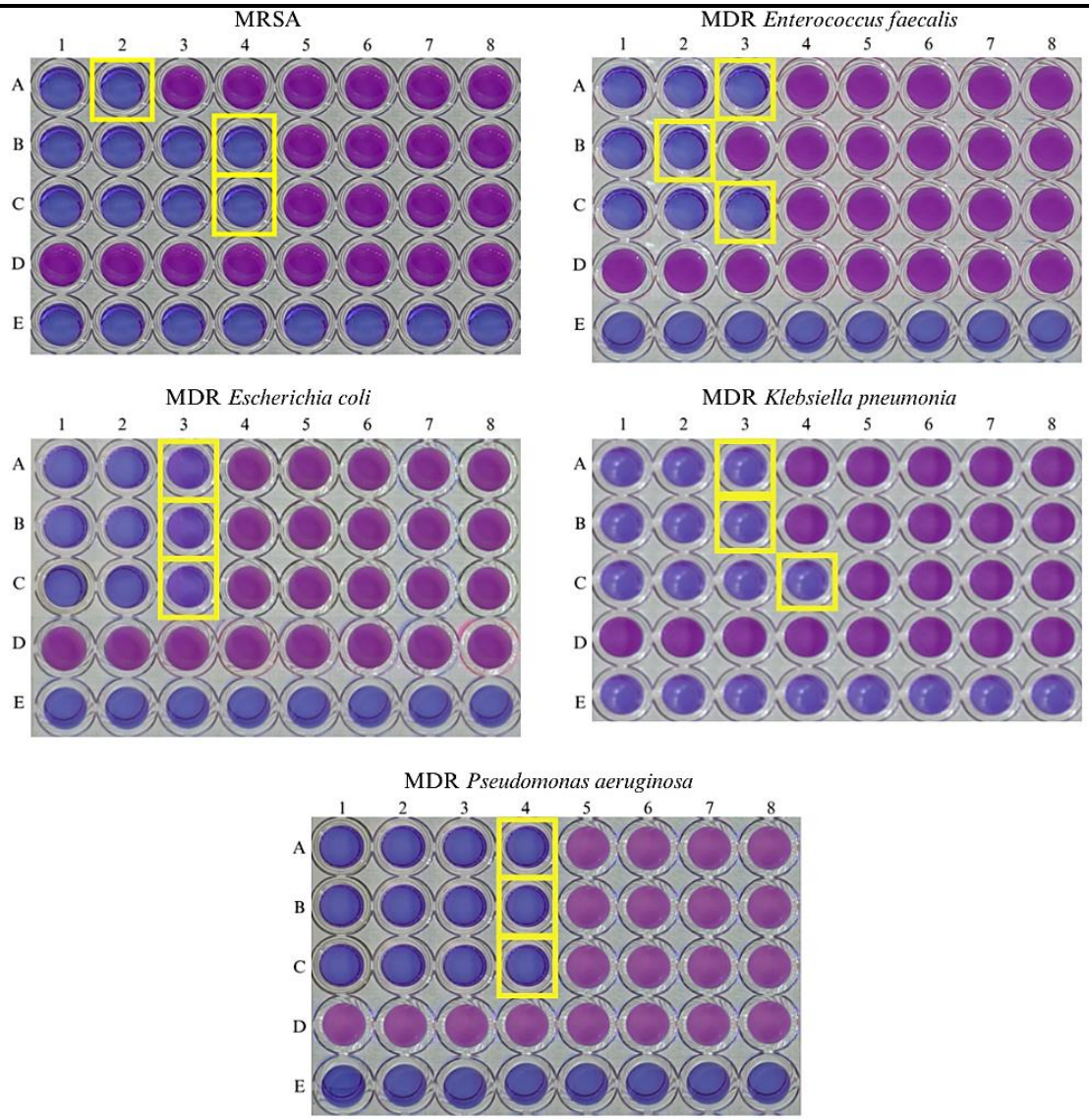

Fig. 3. The MIC test extract of *A. platensis* ethanol solvents, chloroform, and N-hexane against bacteria MDR. A. ethanol solvent, B. chloroform, C. N-hexane solvents, in sequence 1-8 concentrations of extract *A. platensis* 50; 25; 12.5; 6.25; 3.13; 1.56; 0.78; and 0.39mg/ mL. D. positive control, E. negative control. Yellow box: MIC value

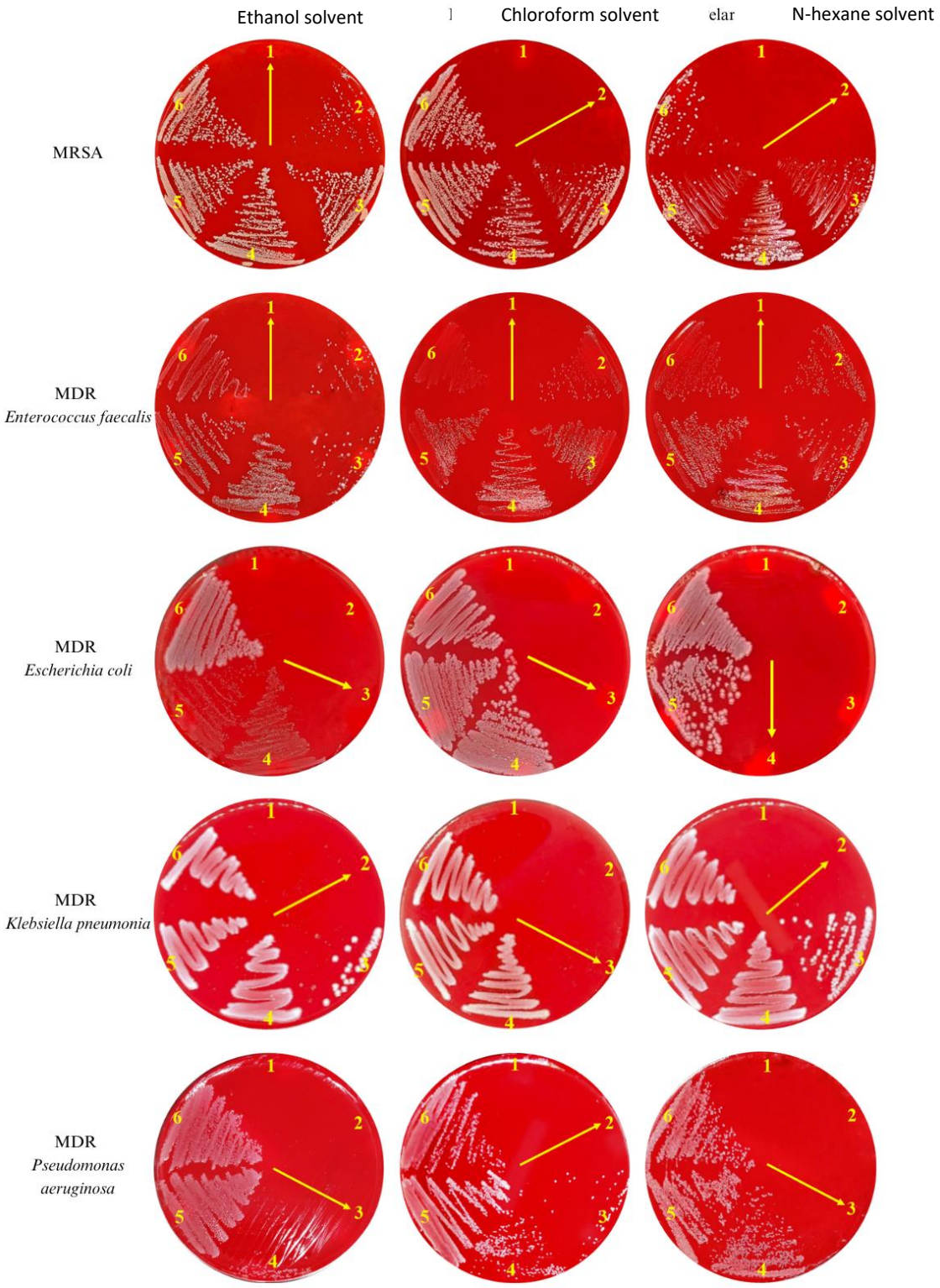

Fig. 4. MBC tests of *A. platensis* extract with ethanol solvents, Chloroform, and N-hexane against MRSA. A. ethanol solvent, B. chloroform, C. N-hexane solvent, sequentially 1-6 concentrations *A. platensis* extracts 50; 25; 12.5; 6.25; 3.13; 1.56mg/mL. Yellow arrow: MBC value

Table 2. MIC and MBC values of *Arthrospira platensis* extract against MDR bacteria

Bacterial species	Ethanol		Chloroform		N-hexane	
	MIC	MBC	MIC	MBC	MIC	MBC
MRSA	≥25,0	≥50,0	≥6,25	≥25,0	≥6,25	≥25,0
MDR *Enterococcus faecalis*	≥12,5	≥50,0	≥25,0	≥50,0	≥12,5	≥50,0
MDR *Escherichia coli*	≥12,5	≥12,5	≥12,5	≥12,5	≥6,25	≥6,25
MDR *Klebsiella pneumonia*	≥12,5	≥25,0	≥12,5	≥25,0	≥12,5	≥12,5
MDR *Pseudomonas aeruginosa*	≥6,25	≥12,5	≥6,25	≥25,0	≥6,25	≥12,5

In MRSA, the best MIC value and MBC value are shown by *A. platensis* extract chloroform solvent and N-hexane solvent, which was 6.25mg/ mL for MIC value and 25.0mg/ mL for MBC value. In MDR bacteria *Enterococcus faecalis*, the best MIC value is shown by *A. platensis* extract of ethanol solvent and N-hexane solvent, which was 12.5mg/ mL for MIC value. The best MBC value *of A. platensis* extract against MDR bacteria *Escherichia coli* is found in each A. *platensis extract* with ethanol solvent, chloroform solvent, and N-hexane solvent, which was 50.0mg/ mL. The best MIC and MBC values of *A. platensis* extract against *Escherichia coli MDR* bacteria were found in *A. platensis* extract with N-hexane solvent, which was 6.25mg/ mL. In MDR *K. pneumonia* bacteria, the best MIC values were shown by each ethanol solvent *A. platensis* extract, chloroform solvent, and N-hexa solvent, which was 12.5mg/ mL. N-hexane solvent *A. platensis* extract showed the best MBC value against *K. pneumoniae* MDR bacteria, which was 12.5mg/ mL. The best MIC value of *A. platensis extract* against MDR bacteria *P. aeruginosa* was found in each *A. platensis* extract with ethanol solvent, chloroform solvent, and N-hexane solvent, which was 6.25mg/ mL. While, the best MBC value *of A. platensis* extracts against MDR bacteria *P. aeruginosa* was found in each A. *platensis* extract, ethanol solvent, and N-hexane solvent.

The results of antibacterial activity tests of MIC and MBC diffusion methods in this study provide better information than several previous studies. Previous research conducted by **Singh *et al*. (2021)** only tested the antibacterial activity of each *A. platensis* extract of acetone solvent, methanol solvent, and ethyl acetate solvent by disc diffusion method against several ATCC strain bacteria (*Salmonella typhi*, *S. aureus* ATCC 25923, and *E.coli* ATCC 25992) and from this study it was known that the best extract that can inhibit bacterial growth is acetone extract *A. platensis* concentration of 100% against *Salmonella typhi* with an inhibitory zone diameter of 12mm. Research by **Usharani *et al*. (2015)** reported that methanol extract of *Spirulina platensis* has better antibacterial

activity against *S. aureus*, *Streptococcus pyogeners*, *Streptococcus epidermidis*, *Proteus mirabilis*, *Bacillus cereus*, *K. pneumoniae*, and *Shigella flexneri* with a MIC value of 1.25mg/ mL compared to extracts with other solvents (acetone solvent, ethanol solvent, n-hexane solvent, and petroleum ether solvent).

Another study conducted by **Abdel-Moneim *et al*. (2022)** evaluated the antibacterial potential of each methanol, acetone, and n-hexane extract of *A. platensis* against several strains of ATCC bacteria, where the results of the study found that the best antibacterial activity was found in methanol solvent *A. platensis* extract which was able to inhibit the growth of *Bacillus cereus*, *S.aureus, Listeria monocytogenes*, *E. coli*, *S. typhi*, and *K. pneumonia* with MIC values ranging from 1-2mg/ mL. **Abdel-Moneim *et al*. (2022)** explained that good antibacterial activity in methanol solvent *S. platensis* extract can be associated with high total phenolic content, which can damage the integrity of bacterial cells and increase cell permeability causing leakage of contents from the cytoplasm.

Phytochemical screening of *Arthrospira platensis* extract

Phytochemical screening of each *A. platensis* extract ethanol solvent, chloroform solvent, and N-hexane solvent included flavonoids, tannins, saponins, alkaloids, steroids, and phenol (Table 3). Phytochemical screening results show that all extracts contain flavonoids and steroids. In addition, alkaloid compounds are contained only in each extract with chloroform solvent and N-hexane solvent.

Table 3. The phytochemical content of extract *A. platensis*

Solvent	phytochemical content					
	Flavonoids	Tannins	Saponins	Alkaloids	Steroids	Phenols
Ethanol	+	-	-	-	+	-
Chloroform	+	-	-	+	+	-
N-hexane	+	-	-	+	+	-

Description: +=contained, - = not contained

The presence of steroid content in each extract of *A. platensis* ethanol solvent and chloroform solvent can be caused by the dipole moment polar and semipolar compounds which will induct into nonpolar molecules that do not have dipole-dipole activity, so that nonpolar compounds can dissolve in semipolar and polar solvents **(Agustini *et al*., 2015)**. In addition, several factors can affect the presence of compounds in the extract including, varietal factors and species of samples extracted, seasonal variation factors, growing or cultivation condition factors, and processing and storage factors **(Pyo *et al*., 2014)**

Flavonoids act as compounds that have antibacterial activity through several mechanisms, namely, inhibiting the function of bacterial cell membranes **(Dzoyem *et al*., 2013)**, inhibiting the synthesis of bacterial nucleic acids **(Hidhayati *et al*., 2022)** as well as the process of energy metabolism **(Shamsudin *et al*., 2022)**. The mechanism of action of alkaloids as antibacterial compounds is by disrupting the constituent components of

peptidoglycan in bacterial cells, so that the bacterial cell wall layer becomes damaged and causes death in bacterial cells **(Pratita *et al*., 2019)**. The mechanism of action of steroids as antibacterials, namely steroids associated with lipid membranes and sensitivity to steroid components, causes leakage in bacterial liposomes **(Epand *et al*., 2007)**. Steroid content in the extract can interact with cell phospholipid membranes that are permeable to lipophilic compounds, causing cell membrane integrity to decrease and cell membrane morphology to change resulting in fragile cells and lysis **(Epand *et al*., 2007)**.

CONCLUSION

Arthrospira platensis extracts in ethanol solvent, chloroform solvent, and n-hexane solvent were shown to have the potential to be developed as alternative antibiotics from natural materials against MDR bacteria that cause wound infections. Furthermore, *in vivo* studies and mode of action determination need to be conducted to elucidate the antibacterial effect of *A. platensis* extracts

Authors contribution

Fandhi Adi Wardoyo: Writing (original draft), Methodology, Formal analysis, Data curation, Funding acquisition.
Eko Yulianto: Writing (original draft), Methodology, Formal analysis.
Dhiyananda Widnyani: Writing (original draft), Methodology, Formal analysis.
Afifah Khairunnisa: Writing (original draft), Methodology.
Sri Darmawati: Writing (review & editing), Validation, Supervision, Conceptualization.
Rizal Maarif Rukama, Haily Liduin Koyou, Ahmad Naqib, and Mohd Nazil Salleh: Review & editing, Validation.
Muhammad Evy Prastiyanto: Writing (review & editing), Validation, Formal analysis.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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