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Original Research Article

Optimization of Antimicrobial Peptide (AMP) Extraction from *Justicia gendarussa* Leaves Using Box-Behnken Design

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ABSTRACT

This study aimed to determine the optimal conditions for extracting antimicrobial peptides (AMPs) from *Justicia gendarussa* Burm. F. leaves and to identify AMPs that have potential antibacterial activity. Extraction optimization was performed using a Box-Behnken design, and total protein levels were measured using the Bradford method. SDS-PAGE was used to identify AMPs, and the well diffusion method was used to test their antibacterial activity. The results showed that the optimum extraction conditions were an acetic acid concentration of 14.9%, an extraction time of 2.9 h, and a sample-to-solvent ratio of 1:3. The AMP from *J. gendarussa* leaves was predicted to have a molecular weight of ~11 kDa. The crude extracts exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, with inhibition zones of 8.61 mm and 22.85 mm, respectively. These findings suggest that AMP derived from *J. gendarussa* leaves possesses promising antibacterial properties, thus demonstrating its potential as a viable source for developing antibacterial agents in the context of the antibiotic industry.

Keywords: Antimicrobial peptide, optimize, Box-Behnken design, *Justicia gendarussa* Burm. F. leaves

Introduction

The imprudent utilization of antibiotics has resulted in the emergence of antibiotic-resistant, which have been declared a global public health threat by the World Health Organization. Antibiotic resistance has been identified as a significant global concern due to its detrimental impact on economic and human welfare, particularly in poorer nations. According to estimations, the prevalence of antibiotic-resistant diseases is projected to result in around 10 million fatalities annually by the year 2050.[1] Additionally, if proper measures are not implemented, there is a forecasted total economic decline of $100.2 trillion in gross domestic product by the same year.[2] Notwithstanding the various associated risks with antibiotic resistance, it is evident that the current clinical pipeline for developing antimicrobial drugs is inadequate in addressing this pressing concern. Hence, there exists a pressing necessity for the advancement of novel pharmaceuticals that possess mechanisms of action that mitigate the development of resistance.

Antimicrobial peptides (AMPs) have emerged as a significant focus of research in antibiotics. AMPs serve as effectors in the innate immune system and modulators in the adaptive immune system.[3] The invention and utilization of AMPs as a novel class of antibiotics has garnered significant attention due to their wide-ranging efficacy against various types of bacteria, fungi, parasites, viruses, and drug-resistant microorganisms.[4]

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Moreover, AMPs have demonstrated a slower propensity to induce bacterial resistance, thus enhancing their appeal in antibiotic research.[5,6] AMPs exhibit a broad spectrum of ways by which they exert their activity, posing challenges for bacteria to build resistance against them.[7,8]

The utilization of AMPs sourced from plants presents considerable potential due to their vast biodiversity and the relative ease of procurement and extraction compared to animal-derived AMPs.[9] It has been reported by the Data Repository of Antimicrobial Peptides (DRAMP) that there are 824 AMPs derived from plants.[10] These AMPs are highly stable and resistant to chemical, thermal, and enzymatic degradation due to 4-12 cysteine residues forming disulfide bonds, which stabilize their tertiary and quaternary structures.[11] AMPs can be found in various parts of plants, including leaves, roots, seeds, flowers, and stems.[11] For instance, AMPs derived from plant leaves have shown antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.[12]

Justicia gendarussa Burm. F., a medicinal plant, is known to have AMP as a potential source. It is thought to originate from China and is extensively found in Sri Lanka, India, Indonesia, and Malaysia. The Indonesian people utilize the leaves of this plant for its medicinal properties. This plant possesses various therapeutic properties, including antioxidant,[13,14] hepatoprotective,[13] antimicrobial,[14] and anti-HIV.[15] This substance is commonly employed in conventional medicine for the management of an assortment of medical conditions such as fever, cough, aphthae, arthritis, headache, liver disorders, chronic rheumatism, inflammation, vaginal discharge, dyspepsia, facial paralysis, earache, bronchitis, and ophthalmic disorders.[16,17]

The efficacy of plant AMP extraction depends on various factors, including the choice of solvent, such as acetic acid, the pH level of the surrounding environment, and the proportion of solvent to solid materials.[18] Several researchers have optimized the extraction of plant AMP by using fractionation in *Canastra artisanal* and optimizing ethanolic and acid extraction in *Capsicum annuum*.[19,20] However, until now, no one has conducted AMP studies on *J. gendarussa* leaves. In addition, extraction optimization of this plant is only limited to

secondary metabolite compounds, not AMP.[21–24] Therefore, this study will optimize the extraction of AMP compounds from *J. gendarussa* leaves with acetic acid concentration, pH, and solvent-to-solids ratio factors. The characterization of AMP was further evaluated using bioassays as antibacterial against *Escherichia coli* and *Staphylococcus aureus*, as well as using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify the molecular weight of the AMP obtained.

Materials and Methods

Plant collection and identification

J. gendarussa leaves with voucher number BMK0173092016 were obtained from the Tropical Biopharmaca Research Center (6º3'49"S and 106º42'57"E), IPB University in Bogor, West Java, Indonesia. The plant was identified by Taufik Ridwan and deposited in the Tropical Biopharmaca Research Center in September 2016.

Plant material preparation

The leaves were harvested three months after being planted in June 2023. They were cleaned with running water and dried through aeration. Afterward, the leaves were blended and sieved with a mesh size of 100, resulting in 149 micro-sized particles. The sample's water content was then determined to be less than 10%, and it was stored in a closed container at room temperature until required for peptide extraction.

Box-Behnken design and crude peptide extraction

The study utilized the Box-Behnken design, specifically the Design Expert 13.0 software developed by Stat-Ease Inc. in Minneapolis, USA, to optimize the extraction process. Multiple variables were considered in the optimization, including the concentration of the acetic acid solvent (5%, 10%, and 15%), the duration of extraction (1 h, 2 h, and 3 h), and the ratio of sample to solvent (1:3, 1:5, and 1:7 mg/mL). Consequently, 17 formula points were obtained (Table 1).

The process of extracting peptides from the leaves of *J. gendarussa* was described in a study conducted by Mijiti *et al*.,[25] with several modifications.[25] A 2-gram sample was subjected to extraction using acetic acid. The extraction process involved stirring the samples at 4°C using a magnetic stirrer. The experimental design employed for this extraction included varying the solvent concentration, the extraction duration, and the ratio between the solvent and sample, as per the Box-Behnken design (Table 1). Subsequently, the mixes were centrifuged (Kitman-T24, Tomy Kogyo CO. Ltd., Tokyo) at 10,000 g for 10 min at 4°C. The resulting liquid fraction, known as supernatant, was collected and preserved at 4°C. The supernatant was analyzed to identify the appropriate protein extraction by assessing the total protein content.

The total protein content of each sample was measured using the protocol described by Bradford.[26] This method presented significant advantages, being cost-effective and user-friendly, with a heightened sensitivity to protein dye. The Bradford test showcased an impressive sensitivity level ranging from 0 to 0.01 mg. This made it 10 times more sensitive than the Lowry method and 100 times more sensitive than the Biuret method.[27] Briefly, the procedure involved diluting a 10 µL aliquot of the crude extract with 90 µL of distilled water in a microplate well, followed by shaking for 30 s. Next, 100 µL Bradford reagent was added, and the mixture was shaken for 30 s. The reaction mixture was then incubated for 10 min in the dark at room temperature. Using a spectrophotometer, the total protein content was determined by measuring the absorbance at a wavelength of 595 nm. Standard bovine serum albumin was employed in this study. The protein concentration of the samples was expressed in milligrams per milliliter (mg/mL).

The collected data on the response of total protein were then utilized to ascertain the optimal approach for maximizing the concentration of acetic acid, pH level, and volume-to-solids ratio in extracting peptides from *J. gendarussa* leaves. Data analysis was conducted using Design Expert 13.0 software. The optimal solution was chosen based on its desirability value, which approached 1. In addition, the accuracy of the projected total protein value obtained from the resulting solution was assessed by comparing it with the actual value using the residual standard error (RSE) expressed as a percentage.[22] Subsequently, the protein extract that exhibited the highest level of effectiveness was subjected to molecular weight analysis using SDS-PAGE, as well as evaluation of its antibacterial activity.

Determination of molecular weight

The crude extract of the protein selected was concentrated using trichloroacetic acid (TCA). The TCA precipitation method was employed using TCA solution at a final concentration of 25%. The mixture was then incubated at −20°C for 1 h. SDS-PAGE was conducted following the Laemmli method as described in the reference.[28] The samples designated for analysis were dissolved in a 2X loading buffer solution (containing 5% β-mercaptoethanol). Subsequently, the samples were subjected to boiling for 10 min at a temperature of 100 °C. The gel was then filled with samples of 10 µL each. The gel was composed of a stacking gel with a concentration of 5% and resolving gels with concentrations of 10% and 15%, respectively. Electrophoresis was conducted using a Bio-Rad system (Mini-PROTEAN Tetra Cell, BIO-RAD, USA) by applying a voltage of 90 V until the sample migrated to the lowermost region of the gel. The gels were subjected to Coomassie blue staining to detect proteins. The molecular weight of the protein was determined using a protein marker provided by CSL-BBL.

Antibacterial activity

The antibacterial activity of crude protein extracts of *J. gendarussa* leaves was evaluated utilizing the standard well diffusion method.[29] The test bacterial strains were *E. coli* ATCC 8739 and *S. aureus* ATCC 6535, cultivated in Nutrient Broth medium at 37°C for 24 hours. Following incubation, the bacteria were suspended in distilled water and adjusted to a McFarland standard of 0.5. A volume of 20 µL of a crude extract containing *J. gendarussa* leaves protein extract at a concentration of 0.511 mg/mL was introduced into the wells of Mueller Hinton Agar medium, which had a diameter of 6 mm. The same positive and negative control volume was also loaded to the well. The positive control was chloramphenicol at a concentration of 0.08 mg/mL. An acetic acid solution of the same pH of the protein extract was used for the negative control. The specimens were incubated for 24 h at 37°C. Subsequently, the extent of inhibition was assessed using a caliper to measure the diameter of the zone in millimeters. The repetition of the inhibition zone measurement for each sample and control was triplicate.

Results and Discussion

Effect of acetic acid concentration, extraction time, and sample-to-solvent ratio on total protein content

No universal techniques are available to extract AMPs from all types of plant structure families.[18] However, the optimized extraction scheme using 10% acetic acid as solvent allows for extracting AMPs from different families.[18] Aside from choosing solvent, extraction duration and sample-solvent ratio vary from research to research.[18] To determine the optimal condition for AMP extraction from *J. gendarussa* leaves, we used an experimental design involving three variables: concentrations of acetic acid, extraction durations, and sample-to-solvent ratios. The Box-Behnken Design requires three levels of each variable. We used the lower and higher number of the mainly used parameter to set the level for each variable, resulting in varying concentrations of acetic acid (5, 10, and 15%), variable extraction durations (1, 2 and 3 hours), and varying sample-to-solvent ratios (1:3, 1:5, and 1:7 w/v).

Table 1 displays the experimental design and response data about the total protein content extracted from the *J. gendarussa* leaves. Combining an acetic acid concentration of 10%, extraction period of 1 h, and sample-to-solvent ratio of 1:7 resulted in the lowest recorded total protein level of 0.3437 mg/mL. The maximum protein concentration observed was 0.5109 mg/mL, achieved using an acetic acid concentration of 15%, an extraction period of 2 h, and a sample-to-solvent ratio of 1:3. The findings about the total protein content indicate that the concentration of acetic acid, duration of extraction,

and ratio of the sample to the solvent have an impact on the protein content acquired. The model chosen by Design Expert v.13.0 demonstrates the effects of the acetic acid concentration, extraction time, and sample-to-solvent ratio. The preferred model is quadratic. The choice of this model is determined by various criteria, including a high F value, an adequate precision value greater than 4, a *p*-value less than 0.05, and a difference of less than 0.2 between the Adjusted R^2 and Predicted R^2 values.[30,31] The calculated R^2 (coefficient of determination) was 0.9750, as shown in Table 2. The coefficient of determination (R^2) quantifies the degree of association between the chosen study variables and observed outcomes. According to the source,[31] an R^2 value is considered outstanding if it exceeds 0.8. This finding demonstrates that the components chosen in this investigation are suitable for maximizing the protein yield.

The relationship between the concentration of acetic acid, duration of extraction, and ratio of the sample to the solvent, as illustrated in the quadratic model, can be elucidated using the coefficients derived from Equation 1. The equation presented in this context includes the coefficients for the acetic acid concentration (A), extraction time (B), and sample-to-solvent ratio of sample to solvent employed (C). These coefficients are denoted as 0.0029A, 0.0090B, -0.0604C, 0.0007AB, 0.0085AC, 0.0069BC, $0.0235A^2$, $0.0088B^2$, and $0.0261C^2$, respectively. The interplay between various factors leads to the emergence of distinct coefficients. Specifically, the coefficients associated with acetic acid concentration and extraction time were positive, indicating a favorable impact on the overall protein yield. Conversely, the coefficients linked to the sample-to-solvent ratio exhibited a negative value, suggesting that this factor did not positively influence the total protein output.[32]

$$\begin{aligned} Total\ protein\ content \\ &= 0.0029A + 0.0090B - 0.0604C + 0.0007AB \\ &+ 0.0085AC + 0.0069BC + 0.0235A^2 \\ &+ 0.0088B^2 \\ &+ 0.0261C^2 \end{aligned} \quad (1)$$

Where, A = acetic acid concentration, B = time extraction, and C = sample-to-solvent ratio.

The elevated total protein concentration acquired through the extraction process utilizing a 15% acetic acid solution can be attributed to the comparatively lower pH associated with this concentration than the other concentrations. An increase in the acidity of the pH can lead to an increase in protein solubility. In contrast, an acidic pH can result in the degradation of complex proteins into more superficial protein structures, such as peptide components and amino acids.[33] The concentration was identified as the primary determinant of protein content variation. The relationship between several factors influencing the levels of total proteins can be observed using a two-dimensional counter plot (Figure 1). The elliptical lines observed in the 2D counterplot suggest a significant interaction between components, whereas fewer elliptical lines imply a less significant interaction between variables.[34] The contour map in two dimensions illustrates the optimal interaction between solvent concentration and extraction time, as indicated by the formation of elliptical lines. The counter plot in two dimensions exhibits a range of colors. The blue indicates the lowest total protein level, whereas red indicates the highest total protein level.

Solution optimization and verification

The optimal solution was derived by analyzing the protein levels obtained at each stage of the extraction process. The optimization solution refers to the desired state achieved by evaluating and comparing the most favorable outcomes or optimal approaches based on previously obtained results. The chosen optimization solution exhibited the highest desirability value. In this context, desirability refers to approaching the response by optimizing the components to be utilized.[35] According to previous research,[36] a desirability value of one represents the highest level of attractiveness, indicating the optimal performance of the component under analysis.

Table 1: Design experimental of Box–Behnken in three independent variables (acetic acid concentration, time extraction, and ratio sample to solvent) and total protein content response of *J. gendarussa* leaves

Run	Acetic acid (%)	Time (h)	Ratio of sample to solvent (g/mL)	Total protein content (mg/mL)
1	10	1	1:7	0.3437
2	10	3	1:7	0.3725
3	15	2	1:3	0.5109
4	5	1	1:5	0.4038
5	15	3	1:5	0.4185
6	10	3	1:3	0.4685
7	10	2	1:5	0.3665
8	15	1	1:5	0.3958
9	15	2	1:7	0.3624
10	5	2	1:3	0.4761
11	5	2	1:7	0.3614
12	10	2	1:5	0.3700
13	5	3	1:5	0.4235
14	10	2	1:5	0.3902
15	10	1	1:3	0.4675
16	10	2	1:5	0.3836
17	10	2	1:5	0.3801

Table 2: ANOVA for protein extraction of *J. gendarussa* leaves

Response	F-Value	*p*-Value	R^2	Adjusted R^2	Predicted R^2	Adeq Precision
Total protein content	30.33	0.0001	0.9750	0.9428	0.7472	18.3640

Conversely, a desirability value of zero signifies an unfavorable response provided by the component. The resulting desirability value in this study characterizes the optimal point, which is determined by the combination of the solvent concentration, extraction time, and sample-to-solvent ratio. A desirability rating of 1 signifies the optimal combination for achieving the maximum total protein levels. The desirability function method transforms an estimated response into a scale-free value called desirability. This method allows for optimizing objectives, whether it involves maximizing, minimizing, or achieving a specific target value for the response. Different desirability functions can be employed based on the goal in question. Desirability functions are assigned values between 0 and 1; a value of 0 indicates an unfavorable response generated by the factors, while a value of 1 signifies optimal performance for the components being considered.[36]
The optimization solution for the total protein content of *J. gendarussa* leaves had a desirability value of 1, indicating a highly favorable outcome. The anticipated total protein content was 0.511 mg/mL (Table 3). The model's accuracy was assessed using the percentage value of the Residual Standard Error (%RSE). A lower percentage of relative standard error (%RSE) indicated a higher level of accuracy in the produced model. It is generally accepted that an appropriate model should have an %RSE value below 5%.[37,38] The percentage of relative standard error (RSE) obtained from validating the total protein content in *J. gendarussa* leaves is reported as 0% (Table 4). This value indicates that the derived model is precise. An %RSE below 5% is deemed appropriate for assessing model precision because it signifies a low level of relative standard error. %RSE is a measure of the accuracy of a predictive model, and a value below 5% suggests that the model's predictions closely align with the actual experimental data. In practical terms, this indicates high precision and reliability in the model's ability to capture and reproduce the observed outcomes. A lower %RSE implies a smaller margin of error between predicted and actual values, making the model suitable for operational scenarios where a tight and dependable fit is crucial for making informed decisions or conducting reliable analyses. The optimal conditions for the extraction of AMP were determined to be a CH_3COOH concentration of 14.9%, extraction period of 2.9 h, and sample-to-solvent ratio of 1:3. Under these conditions, the total protein content of the crude AMP extract was 0.511 mg/ml. The crude AMP extract was used in further investigations. The concentration of CH_3COOH employed in this investigation was significantly higher than that used in the work conducted by Brahim et al. (2022), where a concentration of 1 M CH_3COOH was utilized.[39]

Molecular weight analysis by SDS-PAGE
The electropherogram results of AMP crude extract from *J. gendarussa* leaves are presented in Figure 2. The identification results are displayed in lanes 2 and 3 of the electropherogram. The protein concentration of AMP crude extract was too low (0.511 mg/mL) to be detected as a distinct band in the electropherogram (lane 3). Therefore, further treatment by TCA precipitation was required to enhance the protein concentration. As a result, the protein band of the suggested AMP appeared on the electropherogram (lane 2). Precipitation with TCA is employed to concentrate the protein and eliminate any impurities such as salt and detergent prior to downstream application such as SDS-PAGE.[40] Adding TCA to an aqueous protein solution will disrupt the hydrogen-bonded water molecules surrounding a protein, causing the protein to lose its solubility and can be recovered by centrifugation; the protein will also be in a denatured state.[40] However, TCA precipitation is not intended for protein purification. Surprisingly, this research observed a single protein band (Figure 2, lane 2). These findings do not indicate the purity of the protein. Further investigation, such as 2D-gel, should be performed to verify. The band observed in lane two was determined to be a putative antimicrobial peptide (AMP) with an estimated molecular weight of 11 kilodaltons (kDa). The molecular weight of the anticipated antimicrobial peptide (AMP) is comparable to those of *Scots pine* seeds (11 kDa) and *Triticum aestivum* (13 kDa).[41,42]

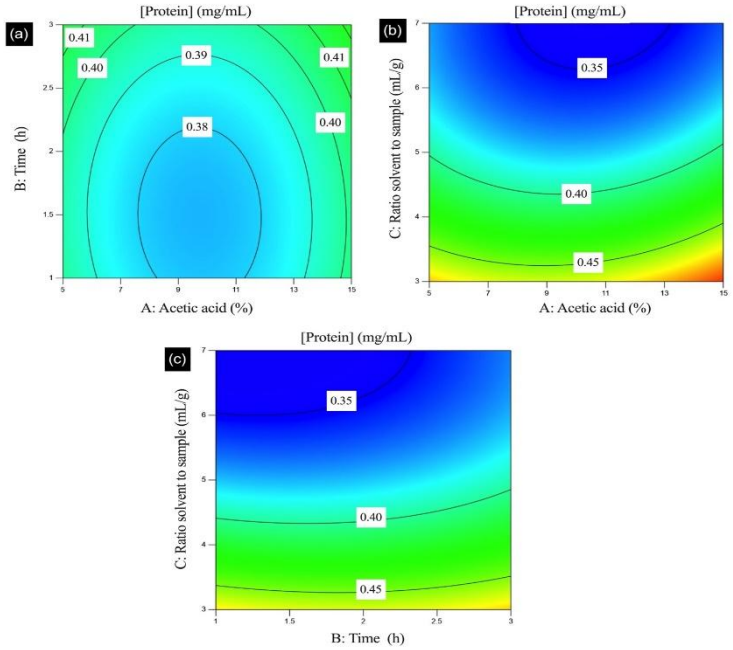

Figure 1: The 2D contour plot depicts the predicted results of the quadratic model response surface analysis for total protein extraction, illustrating the interplay between concentration and extraction time (a), the impact of the sample-to-solvent ratio on concentration (b), and the combined effects of extraction time and sample-to-solvent ratio (c).

Table 3: Extraction optimization solution of total protein with Box-Behnken design from *J. gendarussa* leaves

Run	Acetic acid (%)	Time (h)	Ratio of sample to solvent (g/mL)	Total protein content (mg/mL)	*Desirability*
1	14.9	2.9	1:3	0.511	1.000
2	15.0	2.9	1:3	0.511	0.999
3	14.9	3.0	1:3	0.511	0.998

Table 4: Extraction optimization verification result of total protein with Box-Behnken design from *J. gendarussa* leaves

	Factor			Protein (mg/mL)	*Desirability*
	Acetic acid (%)	Time (h)	Ratio of sample to solvent (g/mL)		
Prediction	14.9	2.9	1:3	0.511	1,000
Verification	14.9	2.9	1:3	0.511	

%RSE	0

Plant antimicrobial peptides (AMPs) can be categorized into many families include thionins (consisting 45–48 amino acids), defensins (consisting 45–54 amino acids), hevein-like peptides (29–45 amino acids), knottin-type peptides (approximately 30 amino acids in length), *α*-hairpinins, cyclotide (ranging 28–37 amino acids), lipid transfer protein (ranging70–90 amino acids), and snakin (comprising 63-66 amino acids).[11,43,44] Lipid transfer protein and snaking are families of cysteine-rich peptide-AMP with molecular weight higher than 7 KDa and are considered proteins.[38] Based on the anticipated molecular weight, inferring that the AMP derived from the outcome corresponds to either a lipid transfer protein or a snakin is reasonable. However, it is challenging to decide the type of AMP solely based on its molecular weight because the classification of plant AMP is usually based on their amino acid sequence similarity, cysteine motifs, disulfide bond patterns, and their tertiary structure.[11,38] Additional research is necessary to ascertain the precise molecular weight and characterize the amino acid sequence and structure to validate this data.

Antibacterial activity

The antibacterial activity of crude AMP extract against *E. coli* and *S. aureus* were evaluated using the standard well diffusion method. Chloramphenicol at a concentration of 0.08 mg/mL was used as positive control, resulting in inhibition zones measuring 20.86 mm and 16.40 mm against E. coli and S. aureus, respectively (Figure 3). Chloramphenicol is a widely employed positive control in antibacterial activity assays because it is a well-established and potent broad-spectrum antibiotic.[45] The negative control, consisting of CH_3COOH adjusted to the same pH as the extraction findings, exhibited no observable zone of inhibition when tested bacteria studied, as indicated in Figure 3. Conducting this test is essential because of the substantial impact of pH on bacterial growth. pH can potentially impede bacterial growth by disrupting the functionality of enzymes within the bacteria, thereby inhibiting their growth. Additionally, pH fluctuations can induce alterations in metabolic processes.[46] The experiment evaluated the antimicrobial activity of a crude protein extract at a concentration of 0.5111 mg/mL against *E. coli* and *S. aureus*. The results showed that the AMP crude extract produced inhibition zones of 8.61 mm and 22.85 mm for *E. coli* and *S. aureus*, respectively. Antimicrobial peptides (AMP) derived from *J. gendarussa* leaves exhibit enhanced efficacy against gram-positive bacteria, specifically *S. aureus*, and demonstrate higher comparable action to chloramphenicol (significant at $p<0.001$). In contrast, the compound's efficacy against gram-negative bacteria was lower than that against chloramphenicol (significant at $p<0.001$). These findings indicate that AMP derived from *J. gendarussa* leaves may possess a distinct range of antibacterial properties against gram-positive bacteria. However, further investigation using a broader antimicrobial activity assay is required to confirm these results.

The antimicrobial activity of AMP derived from the leaves of *J. gendarussa* was found to be similar to that of the AMP obtained from *Anthyllis sericea* leaves, which showed better activity on *S. aureus* than *E. coli* with inhibition zones measuring 10 mm and 22.5 mm. AMP derived from the leaves of *Anthyllis sericea* was extracted using a 1 M solution of CH3COOH. Subsequent purification of the extract yielded a protein content of 302.03 mg/mL.[39]

The antibacterial activity of AMP crude extract from *J. gendarussa* leaves is comparable to the antibacterial activity of its phytochemicals extract. The inhibition zone of AMP crude extract against *E. coli* and *S. aureus* was 8.61 mm and 22.85 mm, respectively. Meanwhile, according to Nirmalraj et al., the inhibition zone of the phytochemicals extracts of *J. gendarussa* leaves ranged between 5 mm to 12 mm against *E. coli* and 4-7 mm against *S. aureus*.[18] The AMP crude extract seems to have a better activity on Gram-positive bacteria; on the contrary, the phytochemicals extract shows a better activity on Gram-negative bacteria.

Further study on the mechanism of AMP action should be performed to explain this result. However, a general mechanism could be applied. The outer cell envelopes of Gram-positive and Gram-negative bacteria are significantly different. Gram-positive bacteria have a thick matrix comprising a crosslinked peptidoglycan layer decorated with negatively charged teichoic acid surrounding the cytoplasmic membrane. Nano-sized pores penetrate the peptidoglycan layers, allowing AMPs to diffuse through.[47] On the contrary, the peptidoglycan layer in Gram-negative bacteria is much thinner and less cross-linked, but it has an additional outer membrane consisting purely of phosphate lipids in the inner leaflet and a coat of lipopolysaccharide in the outer leaflet. LPS molecules comprise many negatively charged phosphate groups engaged in salt bridges with divalent cations, resulting in an electrostatic network.[48] This electrostatic region is a primary barrier to most hydrophobic antibiotics, resulting in low permeability. Therefore, the details of how AMPs penetrate Gram-positive and Gram-negative bacteria must vary in their atomistic interactions. In the case of Gram-positive bacteria, AMPs need to diffuse across the peptidoglycan matrix first and then act on the cytoplasmic membrane. In contrast, killing Gram-negative bacteria involves perturbation or disruption of both outer and cytoplasmic membranes—inability to permeabilize or disrupt the outer membrane results in the loss of antimicrobial activity.[49]

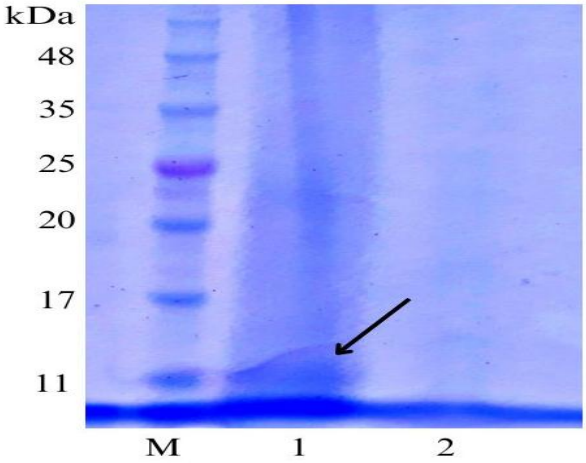

Figure 2: Electrophoregram of crude protein extract from *J. gendarussa* leaves. Lane M= protein ladder, 1 = crude protein extract precipitated with TCA, 2 = crude protein extract.

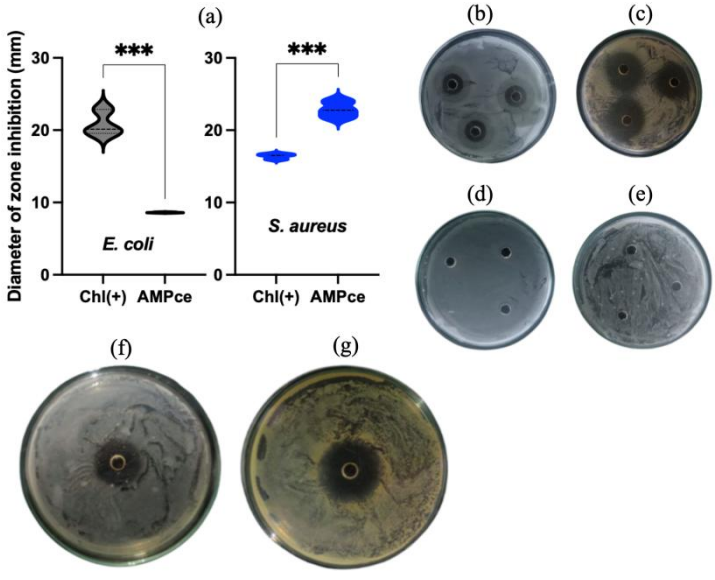

Figure 3: Diameter of zone inhibition from antimicrobial peptide crude extract (AMPce) of *J. gendarussa* leaves and

chloramphenicol (Chl(+)) (a), inhibition zone observation results of AMPce (b, c), negative control (d, e), and Chl(+) (f, g) samples against *E. coli* and *S. aureus* respectively. ***significat at p<0.001.

Conclusion

This study successfully identified optimal conditions for extracting antimicrobial peptides (AMPs) from *J. gendarussa* leaves and demonstrated their potent antibacterial activity. The AMPs extracted showed promising results against *E. coli* and *S. aureus*, suggesting their potential for development as antibacterial agents.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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