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Antibacterial Activity of the Active Compound of *Ulva lactuca* Against MRSA Bacteria

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ABSTRACT

Bacteria with high resistance to various antibiotics, including *Staphylococcus aureus*, have posed significant challenges in healthcare. Some strains of *Staphylococcus aureus* have evolved into methicillin-resistant *Staphylococcus aureus* (MRSA). *Ulva lactuca*, a marine natural resource, is relatively unknown to the public but has shown potential for producing compounds with antibacterial activity. In this study, the method used was bioautography thin-layer chromatography (TLC), which identifies compounds responsible for forming inhibition zones against MRSA. Active compounds were separated using preparative TLC, and the isolated compounds were tested for purity and identified using UV-Vis spectrophotometry and FTIR. The antibacterial activity of the isolated compounds was evaluated by measuring the inhibition zones formed around the discs with a caliper. Based on the interpretation of UV and FTIR spectra, the compound isolated from *Ulva lactuca* was identified as belonging to the pigment group, specifically pheophytin. Antibacterial activity testing revealed that the average inhibition zone diameters at 25% and 50% concentrations were 10.38±0.25 and 16.49±0.07mm, respectively. ANOVA results showed a significant difference in antibacterial activity ($P< 0.05$) between the isolates at 25 and 50% concentrations.

INTRODUCTION

Antibacterial resistance is currently a major issue due to the high levels of resistance observed in bacteria causing common infections and the complexity of the consequences of bacterial resistance **(Prestinaci *et al.*, 2015)**. One of the bacteria with high resistance to various antibiotics is *Staphylococcus aureus* **(Mancuso *et al.*, 2021)**. The 2022 Global Antimicrobial Resistance and Use Surveillance System (GLASS) report highlights alarming levels of resistance among pathogenic bacteria, including *Staphylococcus aureus*, with several strains having evolved into *Methicillin-Resistant Staphylococcus aureus* (MRSA) **(WHO, 2023)**.

Ulva lactuca, also known as green algae, is classified as a macroalga within the phylum Chlorophyta. Green algae contain secondary metabolites such as alkaloids, triterpenoids, steroids, saponins, and flavonoids **(Ardita *et al.*, 2021)**. *Ulva lactuca* contains pigments, which are natural colors in photosynthetic plants formed based on light absorption at specific wavelengths. Plant pigments have various benefits for humans. They exhibit biological activities such as antioxidant, anti-cancer, anti-diabetic, anti-inflammatory, and antibacterial properties **(Pesang *et al.*, 2020)**. Pigment identification using UV-Vis spectrophotometry has shown the presence of β-carotene, fucoxanthin, chlorophyll a, and pheophytin a **(Hasanela *et al.*, 2020)**. *Ulva lactuca* is illustrated in Fig. (1).

Fig. 1. *Ulva lactuca* from Sundak Beach Gunung Kidul Yogyakarta, Indonesia

According to research, the diethyl ether extract of *Ulva lactuca* has been reported to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), with minimum inhibitory concentrations (MIC) against MRSA CCARM3115 (12.5µg/ mL), MRSA CCARM3104 (12.5µg/ mL), and MRSA CCARM3089 (50µg/ mL) **(Kim *et al.*, 2007)**. Furthermore, research by **Ardita *et al.* (2021)** reported that *Ulva lactuca* extract possesses anti-inflammatory and antibacterial properties that can combat resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) and accelerate tissue growth during the wound healing process.

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MATERIALS AND METHODS

Extraction process

Five hundred grams of powdered *Ulva lactuca* were carefully measured. The *Ulva lactuca* powder was macerated using 96% ethanol as a solvent in a ratio of 1:4 in a closed vessel **(Widyaningsih *et al.*, 2016)**. The powder was soaked for three days with occasional stirring, and the solvent was replaced every 24 hours. The macerate was separated, and the filtrate (ethanol extract) was evaporated using a rotary evaporator.

Fractionation

A total of 10 grams of the concentrated extract was dissolved in 100ml of distilled water and transferred into a separatory funnel. The aqueous fraction was then added to 100ml of n-hexane and shaken until homogeneous. The mixture was separated into two layers: an aqueous fraction and an n-hexane fraction. This process of adding 100ml of n-hexane to the aqueous fraction was repeated until the n-hexane phase became clear.

The separated aqueous fraction was mixed with 100ml of ethyl acetate, transferred to a separatory funnel, and shaken until homogeneous. The mixture was separated into two layers: an aqueous fraction and an ethyl acetate fraction. The addition of 100ml of ethyl acetate to the aqueous fraction was repeated until the ethyl acetate phase became clear. The three resulting fractions—n-hexane, ethyl acetate, and aqueous—were evaporated using a waterbath.

Compound identification

Compound identification was performed for flavonoids and terpenoids/steroids in the n-hexane and ethyl acetate fractions. The identification was confirmed using thin-layer chromatography (TLC).

TLC-bioautography antibacterial test

This test was conducted to identify the chemical components responsible for antibacterial activity. The method used in the TLC bioautography test was the contact method, where the TLC plate was placed on the agar medium that had been inoculated with the test bacteria **(Paputungan *et al.*, 2019)**. The procedure began by preparing the sample, which was spotted onto a TLC plate using an appropriate mobile phase. The plate was then analyzed under UV light at 245nm. Prior to testing, the TLC plate was ensured to be completely dry. For the antibacterial test, 25ml of MSA medium was prepared and mixed with 1µl of bacterial suspension before allowing it to solidify. The chromatogram, resulting from the separation of compounds on the TLC plate, was placed on top of the solidified medium and left for 60 minutes. Subsequently, the TLC plate was removed, and the medium was incubated for 24 hours at 37°C.

Separation of active compounds using preparative TLC

The separation of active compounds using preparative TLC was performed on fractions that exhibited bacterial inhibition zones in the TLC bioautography test. Fractions identified to have antibacterial activity in the bioautography test were applied as bands onto a silica gel GF254 TLC plate. The plate was then eluted in a chamber pre-

saturated with a suitable mobile phase. After elution, the plate was dried and observed under UV light at 254nm. The result of the preparative TLC separation was visualized as spots or bands. The elution process was repeated multiple times to obtain sufficient isolates. All bands suspected to contain active compounds were scraped off and extracted using methanol as the solvent, with the extraction repeated three times. The solvent was then evaporated at room temperature until crystal-like precipitates of the isolates were formed.

Purity test

The purity of the obtained isolates was tested using the multi-eluent TLC method and boiling point determination. The isolates were dissolved and spotted onto three silica gel GF254 TLC plates. Each plate was eluted using mobile phases of different polarities, which are Mobile phase 1: n-hexane : ethyl acetate (4:1 v/v) **()**, Mobile phase 2: n-hexane : ethyl acetate (7:3 v/v), and mobile phase 3: n-hexane : diethyl ether : acetone (8:5:3 v/v) **(Naselia *et al.*, 2020; Pesang *et al.*, 2020; Wutsqa *et al.*, 2021)**. The isolate was considered pure if only a single spot was observed on each chromatogram.

Identification of active compound spectrum patterns using UV-Vis spectroscopy

The spectrum measurement was conducted within a wavelength range of 350–700nm. Methanol p.a. was used as the blank solvent. The result obtained was a spectrum representing the relationship between wavelength (λ) and absorbance.

Identification of functional groups in active compound isolates using FTIR

The characterization of the isolates was performed using FTIR by directly placing the sample onto the prism of the FTIR instrument. The transmitted energy was measured as the IR beam passed through the sample. The results were obtained in the form of absorption bands corresponding to the functional groups, which were analyzed by observing the wavenumbers in the IR spectrum **(Putra *et al.*, 2020)**.

Antibacterial activity test of *Ulva lactuca* active compounds using the disk diffusion method

This test was conducted by adding 1µl of MRSA bacterial suspension to 25mL of MSA medium, which was then poured into a Petri dish and allowed to solidify. The discs were immersed in the prepared sample concentrations of 25, 50%, and DMSO solution as the negative control. The discs were soaked in the samples for 15 minutes. Ciprofloxacin antibiotic discs were used as the positive control. All the discs were placed on the solidified MSA medium and incubated at 37°C for 24 hours. After incubation, the inhibition zone diameter of methicillin-resistant *Staphylococcus aureus* (MRSA) was measured using a caliper.

Data analysis

The data obtained included the characteristics of the flavonoid isolate and the antibacterial activity test results, which were represented by clear inhibition zones measured with a caliper, as well as the results from SPSS. The research data on the inhibition zone diameter were analyzed using ANOVA. If significant differences were found, post-hoc tests were conducted using SPSS version 23.

RESULTS

The extraction process was performed using maceration with 96% ethanol as the solvent. The purpose of maceration was to enhance the extraction efficiency, ensuring the maximum yield of the extract. The yield obtained in this study was 22.33%.

The results of the phytochemical screening of the n-hexane and ethyl acetate fractions are presented in Table (1). The confirmation of compounds was performed using TLC. The results of TLC analysis were used for TLC bioautography method, as shown in Fig. (2).

Table 1. Phytochemical screening of the n-hexane fraction and ethyl acetate fraction of *Ulva lactuca*

Coumponds	Reagent	Literature	Results	
			n-hexane fraction	Ethyl acetate fraction
Flavonoids	Sample + Mg + $HCl_{(p)}$+ Amyl alcohol	The solution changes color to red, yellow, dark red (Robinson, 1995), green to blue (Markham, 1988)	+ Dark green color on the amyl alcohol layer	+ Dark green color on the amyl alcohol layer
Terpenoids	Sample +acetic acid anhidrat+$H_2SO_{(p)}$	Tritepenoids produce a red-purple color while steroids produce a green-blue color (Habibi *et al*., 2018)	+ Dark green color	+ Dark green color

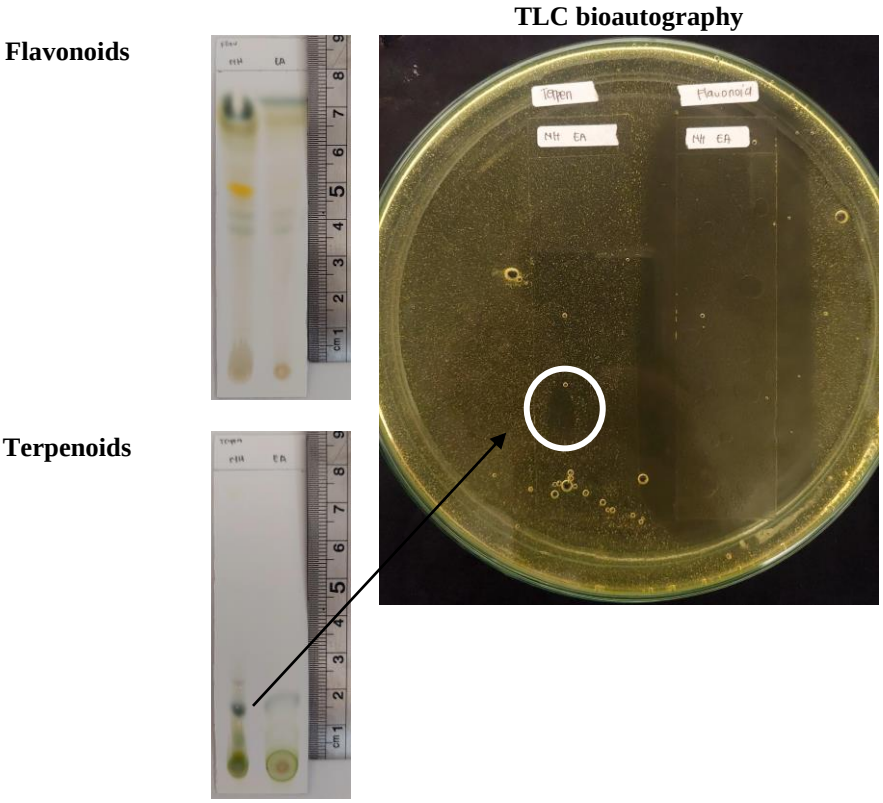


Fig. 2. Bioautography methods from flavonoids and terpenoids compounds against MRSA bacteria

Preparative TLC is aimed at facilitating the isolation of compounds. The n-hexane fraction was spotted onto a preparative TLC plate in a straight line using stationary phase silica gel GF254 (Fig. 3).

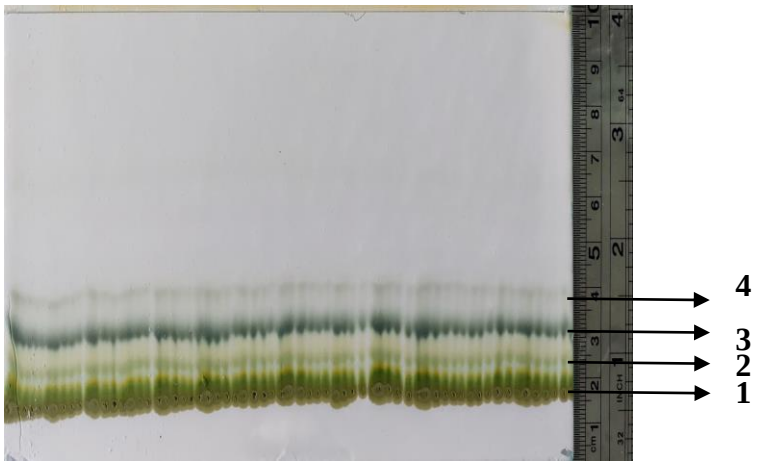
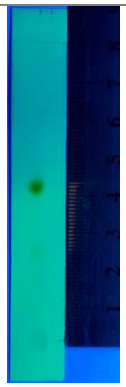
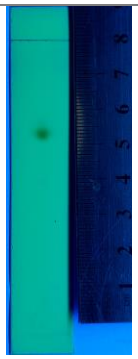


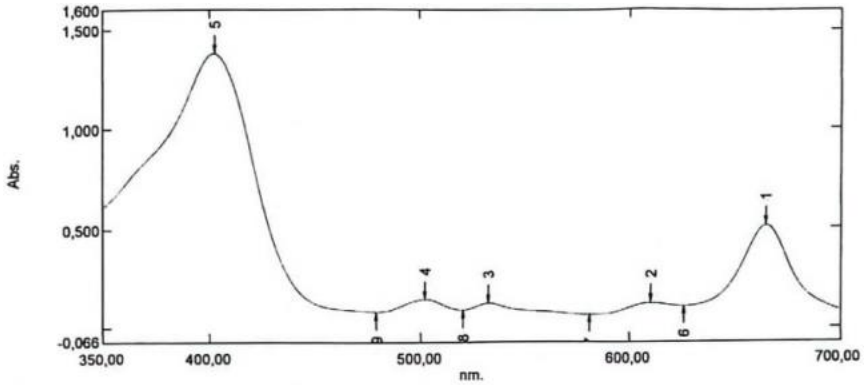
Fig. 3. Isolation of compounds by preparative TLC

The purity of the isolate was assessed using thin-layer chromatography (TLC) with three different mobile phase variations. The isolate obtained from preparative TLC was spotted onto a TLC plate and subjected to the three mobile phases (Table 2).

Table 2. Isolate purity test results using TLC with 3 different systems

Mobile phase	Visual	UV 254	Spotted Appearance (Anisaldehid-H_2SO_4)
n–hexane:ethyl acetat (4:1)			
n–hexane:ethyl acetat (7:3)			
n–hexane:diethyl eter: acton (8:5:3)			

The identification of isolate band three was first carried out by analysis using a UV-Vis spectrophotometer. The spectrum was measured in the wavelength range of 350-700nm (Fig. 4).



No.	P/V	Wavelength	Abs.	Description
1	⊕	665.20	0.510	
2	⊕	610.00	0.126	
3	⊕	532.90	0.123	
4	⊕	501.80	0.144	
5	⊕	403.10	1.386	

Fig. 4. Results of analysis of the band 3 isolate spectrum using UV-Vis spectrophotometry

The identification of isolate band three was carried out by analysis using FTIR (Fig. 5).

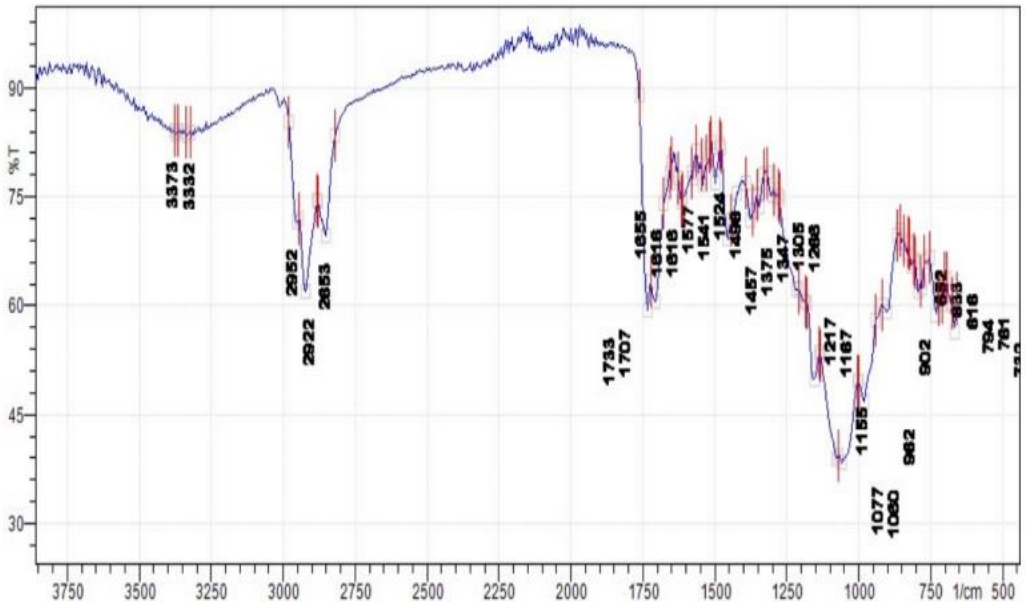


Fig. 5. Results of analysis of band 3 isolate spectrum using FTIR spectrophotometry

Table 3. Bonds wave number of isolate *Ulva lactuca*

No.	Vmax (cm^{-1})	Vmax (cm^{-1}) (Fathurrohmah dkk., 2023)	Functional group
1.	3373 cm^{-1}	3393 cm^{-1}	N-H
2.	2922 cm^{-1}	2921 cm^{-1}	CH_3-CH_2
3.	2853 cm^{-1}	2852 cm^{-1}	CH_2
4.	1618 cm^{-1}	1621 cm^{-1}	Carbonyl
5.	1060 cm^{-1}	1062 cm^{-1}	C-O, C-C
6.	1375 cm^{-1}	1377 cm^{-1}	C-N

The antibacterial activity of the phaeophytin isolate was tested against methicillin-resistant *Staphylococcus aureus* (MRSA). The diameter of the inhibition zone results were presented in Fig. (6) and Table (4).

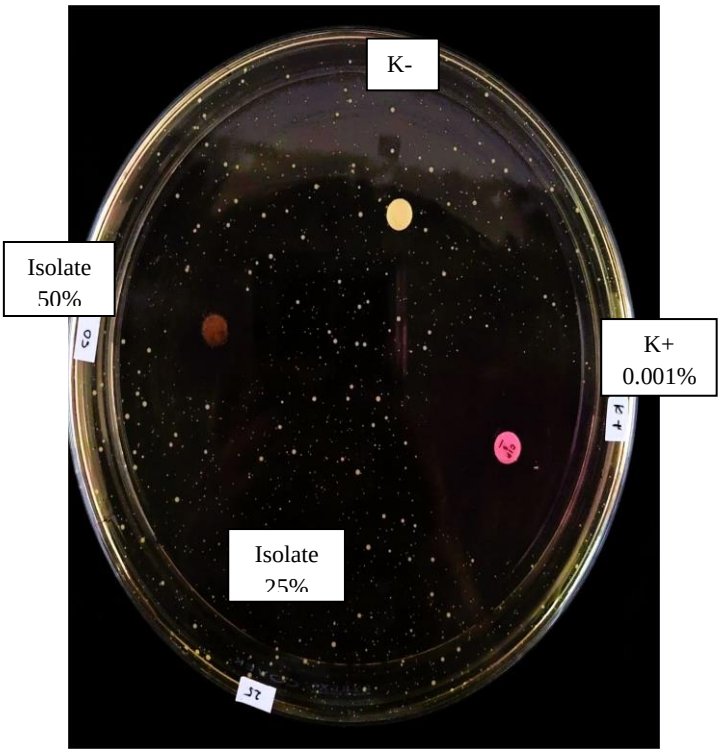


Fig. 6. Antibacterial activity of the pheophytin from *Ulva lactuca*

Table 4. Inhibition zone of pheophytin *Ulva lactuca*

Replication	Inhibitor zone (mm)			
	DMSO	K+ (Ciprofloxacin)	25%	50%
1.	0.000	34.68	7.72	17.21
2.	0.000	35.74	12.64	15.85
3.	0.000	34.06	10.78	16.41
Average	0.000	34.83±0.09	10.38±0.25	16.49±0.07

DISCUSSION

The aim of TLC bioautography is to identify which chemical components are responsible for the antibacterial activity. The method used in TLC bioautography is the contact method, where the TLC plate is placed on top of an agar medium inoculated with the test bacteria **(Paputungan *et al.*, 2019)**. The results of the TLC bioautography showed an inhibition zone for the terpenoid/steroid compounds in the n-hexane fraction.

Preparative TLC is aimed at facilitating the isolation of compounds. The resulting spots were scraped and placed into a vial, with solvent added for further identification. From the preparative TLC, four bands were obtained. However, only band three showed antibacterial activity in the TLC bioautography test. Band three exhibited a grayish-green color. This band was then subjected to a purity test to confirm that the compound was pure.

The purity of the isolate was assessed using thin-layer chromatography (TLC) with three different mobile phase variations. The isolate obtained from preparative TLC was spotted onto a TLC plate and was subjected to the three mobile phases. Based on the TLC analysis, which showed a single spot, the isolate was considered pure.

The results of the UV-Vis spectrophotometric analysis of the isolated bioactive compound showed two prominent absorbance peaks: one in the UV range (λmax- 403nm) and another in the visible range (λmax- 665nm). These peaks are characteristic of pheophytin, as reported in previous literature **(Milenković *et al.*, 2012; Zhang & Ruan, 2015; Sathyanathan *et al.*, 2016)**. Each plate was eluted using mobile phases of different polarities, which are Mobile phase 1: n-hexane : ethyl acetate (4:1 v/v) **(Wutsqa *et al.*, 2021)**. Subsequently, Mobile phase 2: n-hexane : ethyl acetate (7:3 v/v) **(Naselia *et al.*, 2020)**, and mobile phase 3: n-hexane : diethyl ether : acetone (8:5:3 v/v) **(Pesang *et al.*, 2020)**. The isolate was considered pure if only a single spot was observed on each chromatogram.

The spectrum from the UV-Vis analysis is shown in Fig. (4). The result showed that pattern and the maximum wavelength at 403, 501, 532, 610 and 665nm is the same as the literature of pheophytin **(Jeffrey *et al.*, 1997; Kusmita *et al.*, 2015)**.

Identification of functional groups in active compound isolates using FTIR

The pheophytin compound, where several functional groups are observed at the wavenumbers 1618 and 1060 cm^{-1}, indicates the presence of carbonyl and C-O groups. The amide group is also observed in the pheophytin spectrum at a wavelength of 3373cm^{-1}, with the N-H bond. This bond is formed after the release of Mg^{+} ions from the chlorophyll, which is substituted by free H^{+} ions, resulting in the formation of pheophytin, which affects the acidity of chlorophyll **(Wiyono *et al.*, 2023)**. One of the factors contributing to the degradation of chlorophyll is the effect of light (photodegradation). The process of chlorophyll degradation begins with the loss of the magnesium (Mg) atom at the center of the chlorophyll molecule. Chlorophyll that loses its magnesium component is called pheophytin **(Dimara *et al.*, 2018)**.

The results of the antibacterial activity test are presented in Fig. (6) and Table (4). Phaeophytin contains photosensitive porphyrins. Porphyrins can generate reactive oxygen species (ROS), which interact with lipids in bacterial biomembranes **(Wang *et al.*, 2019)**. These ROS cause bacterial cell death through several mechanisms, including oxidation of membrane lipids and amino acids in proteins, protein crosslinking, and oxidative damage to nucleic acids, resulting in the disruption of normal microbial function. The antibacterial activity of porphyrins is attributed to the high susceptibility of Gram-positive bacteria, which possess a relatively porous peptidoglycan layer and lipoteichoic acids in their cell walls, allowing porphyrin molecules to diffuse into the target sites within the cell. In contrast, the cell walls of Gram-negative bacteria contain negatively charged lipopolysaccharides (LPS), which hinder the permeability of neutral or anionic porphyrins from the external environment into the bacterial cell **(Tautua *et al.*, 2019)**.

The inhibition zone diameter of the phaeophytin isolate was statistically analyzed using SPSS (Statistical Product and Service Solution) version 23. The data in this study are normally distributed and homogeneous, allowing for further ANOVA testing. The results of the ANOVA test showed a significance value of 0.000 ($P < 0.05$), indicating that there were significant differences between the concentrations.

CONCLUSION

Based on the results of the study, it was found that the active compound from *Ulva lactuca* is phaeophytin. The active compound was identified as phaeophytin isolate based on the interpretation of UV and FTIR spectra. The results of the antibacterial activity test showed an average inhibition zone diameter of 10.38±0.25 and 16.49±0.07mm at concentrations of 25 and 50%, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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