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ANTIBACTERIAL ACTIVITY OF *Chaetoceros calcitrans* AGAINST PATHOGEN *Staphylococcus aureus* AND *Staphylococcus epidermidis* CAUSING SKIN INFECTION

Aktivitas Antibakteri *Chaetoceros calcitrans* terhadap Patogen *Staphylococcus aureus* dan *Staphylococcus epidermidis* Penyebab Infeksi Kulit

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ABSTRACT

The microalgae Chaetoceros calcitrans *has potential as a natural antibacterial but is rarely applied to pathogens that cause skin infections such as* Staphylococcus aureus *and* S. epidermidis*. The purpose of this study was to determine the optimum concentration of* C. calcitrans *extract to inhibit the growth of* S. aureus *and* S. epidermidis*. The antibacterial activity of* C. calcitrans *was tested by the Kirby Bauer diffusion method. The results showed that* C. calcitrans *extract dissolved in DMSO with the concentrations of 5, 10, 15, and 25 mg mL^{-1} could produce inhibition zones on* S. aureus *and* S. epidermidis*. The average diameter of the largest inhibition zone resulted in the concentration of 25 mg mL^{-1}, namely 10.1 ± 0.5 mm in* S. aureus *and 9.3 ± 0.5 mm in* S. epidermidis*. It can be concluded that the extract of* C. calcitrans *has antibacterial activity against bacteria that cause skin infections* S. aureus *and* S. epidermidis *with the optimum concentration of 25 mg mL^{-1}.*

Keywords: Chaetoceros calcitrans*, Guillard F/2, Skin infection,* Staphylococcus aureus*,* Staphylococcus epidermidis

ABSTRAK

Mikroalga *Chaetoceros calcitrans* berpotensi sebagai antibakteri alami namun masih jarang diaplikasikan pada patogen penyebab infeksi kulit seperti *Staphylococcus aureus* dan *S. epidermidis*. Tujuan penelitian ini adalah menentukan konsentrasi ekstrak *C. calcitrans* yang paling optimum untuk menghambat pertumbuhan *S. aureus* serta *S. epidermidis*. Aktivitas antibakteri *C. calcitrans* diuji dengan metode difusi Kirby Bauer. Hasil penelitian menunjukkan bahwa ekstrak *C. calcitrans* yang dilarutkan pada DMSO dengan konsentrasi 5, 10, 15, dan 25 mg mL^{-1} dapat menghasilkan zona hambat pada *S. aureus* dan *S. epidermidis*. Rata-rata diameter zona hambat terbesar dihasilkan konsentrasi 25 mg mL^{-1} yaitu 10,1 ± 0,5 mm pada *S. aureus* dan 9,3 ± 0,5 mm pada *S. epidermidis*. Dapat disimpulkan bahwa ekstrak *C. calcitrans* memiliki aktivitas antibakteri terhadap bakteri penyebab infeksi kulit *S. aureus* dan *S. epidermidis* dengan konsentrasi optimum sebesar 25 mg mL^{-1}.

Kata Kunci: *Chaetoceros calcitrans,* Guillard F/2*,* Infeksi kulit*, Staphylococcus aureus, Staphylococcus epidermidis*

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INTRODUCTION

Skin diseases can harm a person's quality of life, from the physical to the mental, due to changes in physical appearance caused by swelling, redness, itching, and pain (Flohr and Hay 2021). Skin diseases are common and contribute significantly to the global disease burden (Flohr and Hay 2021). In 2011, skin disease was the third most common disease out of 10 diseases among healthcare outpatients in Indonesia, accounting for 48,576 new cases (Wandhita et al. 2022). Cases of skin disease continue to be reported in several Indonesian cities, including South Jakarta (Sahala et al. 2016), Lampung (Syahputri and Hasibuan 2015), and Teluk Nibung District, North Sumatra (Saragih et al. 2019).

One of the causes of skin disease is infection by bacteria, fungi, parasites, and viruses (Urban et al. 2021). Bacterial infections on the skin are more common than other pathogens, with a prevalence ranging from 0.2 to 35% (García et al. 2020). *S. aureus* and *S. epidermidis*, both opportunistic pathogens, are the most common bacteria that cause skin infections (Byrd et al. 2017). Antibiotics such as mupirocin, erythromycin, tetracycline, penicillin, and ampicillin can be used to treat infections caused by these two bacteria (Ghalehnoo 2018). Unfortunately, long-term antibiotic use can lead to bacterial resistance (Foster 2017, Aggarwal et al. 2019, Chabi and Momtaz 2019). *S. aureus* and *S. epidermidis* are capable of a variety of resistance mechanisms, including the production of lactase enzymes, the modification of antibiotics through glycosylation and phosphorylation processes, and the use of an efflux pump system (Foster 2017, Yılmaz and Aslantaş 2017). Therefore, we require a new compound component that can treat these bacteria-caused skin infections.

Several studies have shown that natural ingredients can be used to combat antibiotic resistance (Rossiter et al. 2017, Atanasov et al. 2021). Natural materials have several advantages over synthetic compounds, including higher molecular mass and stiffness (Atanasov et al. 2021). *Chaetoceros calcitrans* is one of the natural ingredients with antibacterial potential. Moreover, *C. calcitrans* is a marine microalgae with antibacterial activity against a variety of aquaculture pathogens, including *Vibrio* sp., *Aeromonas salmonicida*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Salmonella enterica*, and *Escherichia coli* (Fajardo et al. 2020). Furthermore, *C. calcitrans* contains several bioactive compounds that can inhibit bacterial growth, including phenolic groups, terpenoids, alkaloids, vitamins, and fatty acids (Maftuch et al. 2018). Seraspe et al. (2014) reported that the highest antibacterial activity was obtained by extraction with an n-hexane solution.

Unfortunately, only few studies have been conducted in Indonesia to evaluate the inhibitory strength of *C. calcitrans* extract against the growth of pathogenic bacteria in humans, especially those that cause skin infections. Salim (2018) only focused on the inhibition of *C. calcitrans* extract against *S. aureus* instead of *S. epidermidis*. The microalgae were grown on bold basal media (BBM), which limited their growth. Ramadhanty et al. (2020) reported that *C. calcitrans* will grow faster on Guillard F/2 media than on BBM. Guillard F/2 is an enriched seawater medium. This medium is commonly used to grow diatom marine algae because it contains major nutrients, trace metals and vitamins. Differences in nutrient content in growth media can also cause differences in the chemical composition of *C. calcitrans* such as carbohydrates, proteins and fats content (Prafanda et al. 2020). Therefore, the objective of this study was to cultivate *C. calcitrans* on Guillard F/2 media and determine the optimum concentration of *C. calcitrans* extract to inhibit the growth of *S. aureus* and *S. epidermidis*.

MATERIALS AND METHODS

Location and time

This study was conducted in May – August 2022 at The Bacteriology Laboratory of Medical Laboratory Technology, Faculty of Health Sciences and Technology, Jenderal Achmad Yani University.

Material

C. calcitrans, the microalgae used in this study, was obtained from the Aquaculture Center, Jepara, Indonesia. The bacterial strains tested for antibacterial activity were *S. aureus* ATCC 2913 and *S. epidermidis* ATCC 12228. A Guillard F/2 medium containing nitrate, phosphates, silicates, trace metals, and vitamins was used to create growth curves and cultivate *C. calcitrans*. Moreover, *S. aureus* and *S. epidermidis* were grown on agar medium (OxoidTM) with sheep's blood. Furthermore, Mueller Hinton Agar (OxoidTM) media was used for antibacterial testing.

Methods

Cultivation of *C. calcitrans*

C. calcitrans was grown in glass jars containing Guillard F/2 medium with pH 7, room temperature, the salinity of 34 g L^{-1}, and constant light (photoperiod 24/0). *C. calcitrans* was activated by growing it in Guillard F/2 media twice before cultivation for obtaining biomass. At 4 days of culture, cells were inoculated onto the cultivation medium at a rate of 1×10^6 cells mL^{-1}. Cells were counted using a hemocytometer once per day for 14 days, and a growth curve was created by plotting the length of cultivation time (days) against the number of *C. calcitrans* cells.

Biomass was harvested before the culture reached the stationary phase (at the end of the exponential phase). *C. calcitrans* was collected by centrifugation at 4,500 rpm for 5 minutes at 4ºC. Furthermore, *C. calcitrans* was cultivated and dried using the freeze-drying method to produce microalgae powder.

Extraction of *C. calcitrans*

The microalgae powder was weighed, liquid nitrogen was added to damage the cells by crushing. *C. calcitrans* was extracted by diluting the sample with n-hexane at a 1:6 (w/v) ratio. N-hexane was used to extract fatty acid as active compound. The extraction was carried out using the maceration method for 3×24 hours with stirring (Maftuch et al., 2018). Furthermore, the filtrate was separated from the pellet by centrifugation at 4,500 rpm for 15 minutes. After collecting the filtrate, the pellet was extracted with n-hexane again before being centrifuged again. This procedure was repeated three times. The collected filtrate was then evaporated using a rotary evaporator. Moreover, the extract was collected and dissolved in 1% Dimethyl sulfoxide (DMSO). Several concentrations were made from stock extract solution by adding 1% DMSO as solvent into concentration of 5, 10, 15, and 25 mg mL^{-1}.

Antibacterial test

The bacteria *S. aureus* and *S. epidermidis* were first activated by growing them on the blood agar medium. Bacteria were collected after 24 hours of incubation at 37ºC and dissolved in physiological NaCl until the turbidity reached 0.5 McFarland (1.5×10^8 CFU mL^{-1}). Moreover, the bacteria were inoculated on Mueller Hinton Agar (MHA) media by scratching the entire surface with a sterile swab. After that, the *C. calcitrans* extract with a concentration of 5, 10, 15, and 25 mg mL^{-1} embedded in a paper disc was affixed to the surface of the bacteria-filled agar. A 0.5 cm diameter paper disc containing the antibiotic ampicillin 10 µg mL^{-1} on MHA media served as the positive control for the antibacterial test, while the negative control was a 1% DMSO solvent addition. The treatments and controls were incubated for 24 hours at 37ºC. Furthermore, the inhibition zone that formed was measured using a caliper. The antibacterial activity test was carried out four times.

Data Analysis

The data was statistically analyzed using the IBM SPSS Statistics 23 program. The first step was to test the normality of the data. Then, a one-way ANOVA test was run after the data was found to be normally distributed. The Post Hoc Test was then used to determine whether there was a significant difference between treatments ($p \leqslant 0.05$).

RESULTS AND DISCUSSION

This study began with the plot of a growth curve for *C. calcitrans* (Figure 1). According to the growth curve, *C. calcitrans* reached the exponential phase (log) from the day 1 to day 4 (Figure 2). On the day 4, the cell density was 7.75×10^6 cells mL^{-1}. *C. calcitrans* reached the stationary phase from the fifth to the eighth day, and after the eighth

day, *C. calcitrans* reached the death phase, which was characterized by a decrease in cell density.

Several factors can influence the microalgae cultivation process, including light intensity (Akbarnejad et al. 2020), temperature (Kong et al. 2021), growth media (Velasco et al. 2016), and inoculum age (Cheng et al. 2018). *C. calcitrans* was successfully cultivated in this study using Guillard F/2 media. Guillard F/2

A growth curve must be plotted to figure out the exact age of the inoculum. Based on the growth curve in this study, *C. calcitrans* reached the end of the exponential phase with the highest cell density of 7.75 × 10^6 cells mL^{-1} in 4 days. Besides, an inoculum aged 4 days was used in this study because the optimal inoculum age was obtained prior to the stationary phase. Fatty acid was primary metabolites required for *C. calcitrans* growth as well as active compound in this study. Therefore, fatty acid content reached high amount in the end of exponential phase. In contrast, Velasco et al. (2016) reported that after 7 days of cultivation in Guillard F/2 media, *C. calcitrans* had a cell density of 8.5 × 10^6 cells mL^{-1}. Another study found that the cell density was 1.58 × 10^6 cells mL^{-1} on the

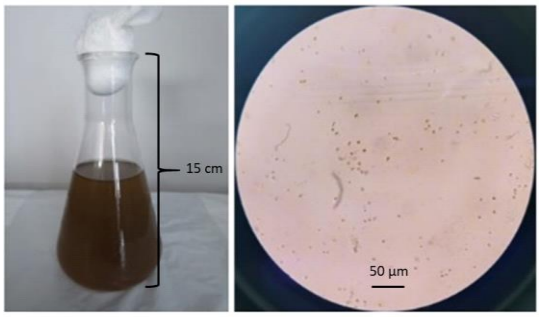


Figure 1. (A) *C. calcitrans* culture, (B) *C. calcitrans* cell

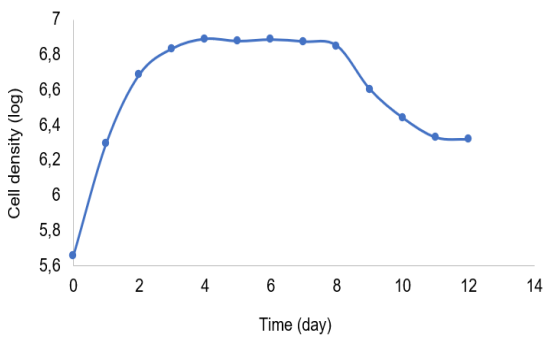


Figure 2. The growth curve of Microalgae *C. calcitrans*.

contains an optimal N:P ratio value of around 12 and 23 when compared to other media such as BBM (Salim 2018), triple 15 and humus extract (Velasco et al. 2016), allowing *C. calcitrans* to grow fast and produce a high cell density. The density of *C. calcitrans* cells in this study was quite higher than that in Salim's (2018) study, which grew *C. calcitrans* on BBM media to test its inhibitory strength against *S. aureus*.

eighth day of cultivation in Guillard F/2 media (Sureshkumar et al. 2014). Moreover, Akbarnejad et al. (2020) reported that *C. calcitrans* cell density on Guillard F/2 media can gradually increase over 5–10 days.

Because *C. calcitrans* was activated twice in this study, it was able to achieve the highest cell density in the shortest period. The results of the preliminary study showed that unactivated *C. calcitrans* had a slow growth rate (results not published). *C. calcitrans* was adapted to the growth medium during the activation process. It is possible that various enzymes involved in the metabolic process were already active when cultivation began, resulting in no adaptation phase (lag) and *C. calcitrans* growing faster. This also caused *C. calcitrans* to reach the stationary phase and die earlier in this study than in Sureshkumar et al. (2014) and Velasco et al. (2016). The

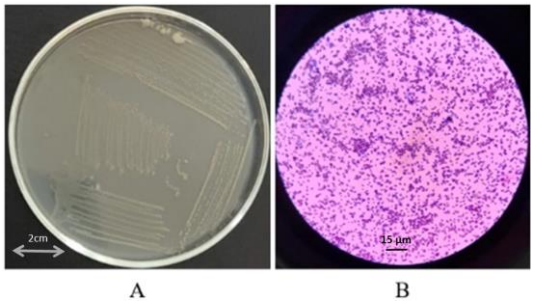


Figure 3. *(A) S. aureus culture, (B) S. aureus Gram stain*

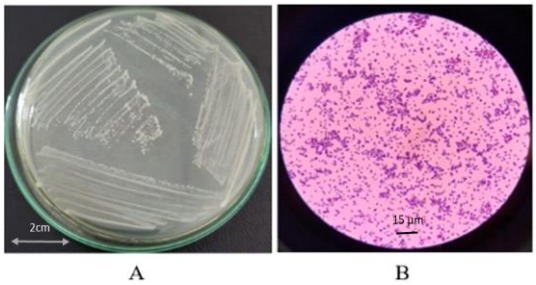


Figure 4. *(A) S. epidermidis culture, (B) S. epidermidis Gram stain*

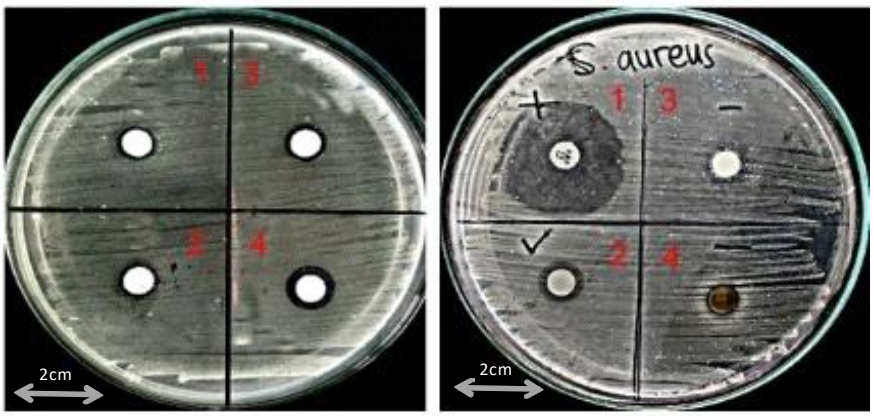
faster cell density increases, the quicker nutrients and growth sites are depleted.

The cultivation, biomass harvesting, and extraction processes were all completed successfully. Four concentration variants of C*. calcitrans* were tested on Gram-positive *S. aureus* (Figure 3) and Gram-positive *S. epidermidis* (Figure 4). The findings showed that an inhibitory zone formed in *S. aureus* (Figure 5A). The inhibition zone was produced by the four extract concentration variants, but the largest diameter of the inhibition zone resulted in a concentration of 25 mg mL^{-1}, which was 10.1 ± 0.5 mm (Table 1). The diameter of the inhibition zone produced by the 25 mg mL^{-1} *C. calcitrans* extract, on the other hand, was smaller than that of the positive control. The same results were obtained for *S. epidermidis* (Figure 6), with a *C. calcitrans* extract concentration of 25 mg mL^{-1} resulting in the largest inhibition zone diameter of 9.3 ± 0.5 mm (Table 1). Although the inhibition zone was smaller than the positive control, the difference was not significant. A 1% DMSO solution used as a solvent for *C. calcitrans* extract did not produce an inhibitory zone on *S. aureus* (Figure 5B) or *S. epidermidis* (Figure 6B). This showed that the inhibitory zone on the two bacteria was formed by the *C. calcitrans* extract without the influence of the solvent. Furthermore, the findings showed that non-extracted *C. calcitrans* could not produce inhibition zones for the two bacteria.

Based on the statistical analysis of the inhibition zones produced by four variants of the *C. calcitrans* extract concentration, the p-value = 0.000 was obtained. This value indicates a significant relationship between the independent and dependent variables. Thus, a significant difference in the diameter of the inhibition zone was discovered between the concentration variants of *C. calcitrans* extract against *S. aureus* (Table 2) and *S. epidermidis* (Table 3). Further analysis using the Pos Hoc test showed that the

Table 1. The inhibition zone diameter of *C. calcitrans* extract against *S. aureus* and *S. epidermidis*

Sample	Concentration (mg mL^{-1})	Average Inhibition Zone Diameter (mm)	
		S. aureus	*S. epidermidis*
C. calcitrans extract	5	5,6 ± 0,7	5,1 ± 0,1
	10	6,8 ± 0,5	6,2 ± 0,7
	15	8,3 ± 0,9	7,4 ± 0,5
	25	10,1 ± 0,5	9,3 ± 0,5
Positive Control	0,01	25	11
Negative Control	25	0	0



A B

Figure 5. The inhibition zone of *C. calcitrans* extract against *S. aureus*. A. At various extract concentrations (1: 5 mg mL^{-1}; 2: 10 mg mL^{-1}; 3: 15 mg mL^{-1}; 4: 25 mg mL^{-1}). B. The comparison of positive and negative controls (1: positive control; 2: *C. calcitrans* extract of 25 mg mL^{-1}; 3: negative control; 4: non-extracted *C. calcitrans*).

concentrations of 15 mg mL^{-1} and 25 mg mL^{-1} were significantly different.

C. calcitrans, which was grown and extracted successfully in this study, was found to have antibacterial activity against *S. aureus* at an optimum concentration of 25 mg mL^{-1}. This finding is in line with Salim (2018), reporting that a 25 mg mL^{-1} concentration of *C. calcitrans* extract can produce an inhibition zone of 10.5 mm on *S. aureus*. Although the use of Guillard F/2 media did not increase *C. calcitrans* antibacterial activity, the highest cell density was obtained more quickly. If *C. calcitrans* is produced as an alternative antibiotic, the growth rate will benefit production.

C. calcitrans extract can also inhibit the growth of *S. epidermidis* with an optimum concentration of 25 mg mL^{-1}. Until now, no other studies have investigated the antibacterial activity of *C. calcitrans* against *S. epidermidis*. Besides, *Thalasiossira* sp. has been tested against *S. epidermidis*. This microalga has a high content of fatty acids, similar to *C. calcitrans*. The diameter of the largest inhibition zone produced by an 8% extract of *Thalasiossira* sp. against *S. aureus* was 8.35 mm and 8.85 mm for *S. epidermidis* (Anggraeni et al. 2019). Furthermore, there was also *Navicula salinicola*, the microalga that was tested against *S. epidermidis* but did not show antibacterial activity when extracted with n-h exane (Kurnia et al. 2020). This indicated that *C. calcitrans* was more effective against *S. aureus* and *S. epidermidis* than the other two microalgae.

Although *C. calcitrans* can inhibit *S. aureus* and *S. epidermidis* growth, the inhibition zone produced was still smaller than that of the antibiotic ampicillin. This is due to the high purity of ampicillin, whereas

Table 2. The statistical analysis of variation in *C. calcitrans* extract concentration against the inhibitory zone of *S. aureus*

Concentration (mg mL^{-1})	Mean	SD	95% CI	P-*value*
5	5.75	0.95	4.23 – 7.27	
10	6.75	0.50	5.95 – 7.55	
15	8.25	0.95	6.73 – 9.77	0.000
25	10.0	0.81	8.70 – 11.3	

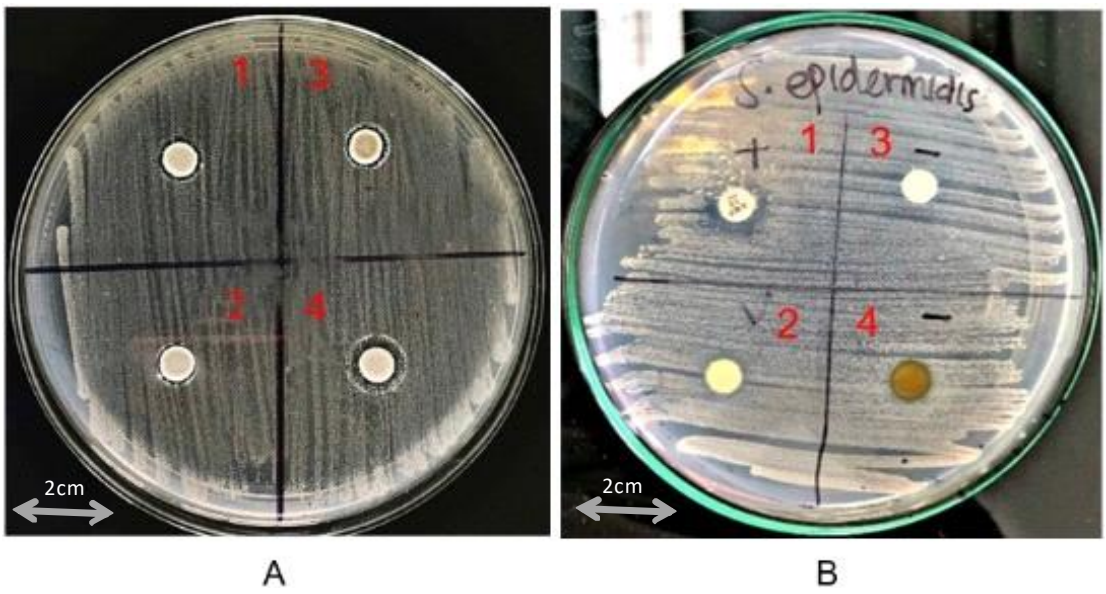


Figure 6. The inhibition zone of *C. calcitrans* extracts against *S. epidermidis*. A. At various extract concentrations (1: 5 mg mL^{-1}; 2: 10 mg mL^{-1}; 3: 15 mg mL^{-1}; 4: 25 mg mL^{-1}). B. The comparison of positive and negative controls (1: positive control; 2: *C. calcitrans* extract of 25 mg mL^{-1}; 3: negative control; 4: non-extracted *C. calcitrans*).

Table 3. The effect of variation in *C. calcitrans* extract concentration on the inhibitory zone of *S. epidermidis*

Concentration (mg mL^{-1})	Mean	SD	95% CI	P-*value*
5	5.00	0.00	5.00 – 5.00	
10	6.00	0.81	4.70 – 7.30	
15	7.25	0.50	6.45 – 8.05	0.000
25	9.25	0.50	8.45 – 10.0	

the C*. calcitrans* extract used in this study was still a crude extract, resulting in lower efficacy. Ampicillin is a broad-spectrum antibiotic that can inhibit both gram-positive and gram-negative bacteria (Peechakara et al. 2022). The action mechanism of ampicillin is also different from that of the *C. calcitrans* extract. Ampicillin inhibits cell wall synthesis by binding to penicillin-binding proteins involved in peptidoglycan synthesis (Maqbool et al. 2020). Cell lysis can occur when cell wall synthesis is inhibited. Besides, fatty acids are *C. calcitrans* compound components with antibacterial activity (Seraspe et al. 2014, Ramadhanty et al. 2020). Fatty acids in *C. calcitrans* can inhibit bacterial growth by reducing the permeability of cell membranes, leading to cell leakage and membrane damage (Dewi et al. 2018).

This study also indicated that the inhibition zone formed was completely due to the compounds contained in the *C. calcitrans* extract, with no influence from the DMSO solvent. The formation of an inhibition zone on disc paper soaked in non-extracted *C. calcitrans* and DMSO was ineffective. This finding is in line with Rahmi and Putri (2020), reporting that no inhibition zone was formed in *S. aureus*, *E. coli*, or *Candida albicans* after DMSO treatment. Several studies have also proven that the bioactive component of *C. calcitrans* which has a role as an antibacterial is a non-polar compound (Seraspe et al. 2012, Maftuch et al. 2018, Ramadhanty et al. 2020). One of the non-polar compounds which is believed to have the highest antibacterial activity is fatty acid. These fatty acids can be obtained by extracting *C. calcitrans* and dissolving it in n-hexane solution (Seraspe et al. 2012).

It is suggested that further study be conducted to test the activity of *C. calcitrans* extract against other skin infection-causing pathogens such as *Streptococcus pyogenes* bacteria or *C. albicans* fungi. It is recommended that bacteria resistant to antibiotics such as ampicillin or penicillin be used when testing for antibacterial activity. Furthermore, further research can purify the *C. calcitrans* extract to improve the inhibition. Numerous studies have shown that *C. calcitrans* extract can inhibit the growth of pathogens that cause skin infections, so *C. calcitrans* has the potential to be produced in commercial products such as face masks or ointments.

CONCLUSION

It can be concluded that *C. calcitrans* grew successfully on Guillard F/2 medium. *C. calcitrans* can reach the highest cell density of 7.75×10^6cells mL^{-1} in 6 days. With an optimum concentration of 25 mg mL^{-1}, *C. calcitrans* extract has antibacterial activity against bacteria that cause skin infections, *S. aureus* of 10.1 ± 0.5 mm and *S. epidermidis* 9.3 ± 0.5 mm.

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