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Original Article

Carrageenan from Balinese Red Seaweed (*Gracilaria* sp). as Antibacterial against Eight Pathogenic Bacteria

Karagenan Rumput Laut Merah Bali (*Gracilaria* sp.) sebagai Antibakteri terhadap Delapan Bakteri Patogen

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Abstract

The increase in infection cases every year, coupled with the rise in resistance cases, increases the urgency of exploring antimicrobial agents. *Gracilaria* sp. is a red seaweed that is widely known as one of the potential producers of active metabolites sulfated polysaccharides. Carrageenan is one form of sulfated polysaccharide that has antimicrobial activity. This study was conducted to evaluate the antibacterial activity of carrageenan derived from *Gracilaria* sp. against several pathogenic bacteria, including *Escherichia coli, Staphylococcus aureus*, *Staphylococcus epidermidis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Streptococcus pyogenes*, and *Salmonella typhi*. Carrageenan was extracted from Gracilaria sp. samples obtained from the South Denpasar area (Bali) using KOH. An antibacterial activity assay of carrageenan was carried out using an agar well diffusion method with nutrient agar media. Tests were conducted at concentrations of 1 and 5 mg/mL (*E. coli* and *S. epidermidis*), 20, 30, 40 mg/mL (*S. aureus, S. pyrogenes, E. faecalis,* and *S. typhi*), 50, 75, and 100 mg/mL (*P. aeruginosa* and *K. pneumoniae*). Incubation was carried out for 24 hours at ± 37℃. Data were analyzed statistically by One-Way ANOVA and Kruskal Wallis with a 95% confidence level. The results showed that carrageenan extracted from *Gracilaria* sp. exhibited significant antibacterial activity against several pathogenic bacteria, especially gram-negative bacteria. The antibacterial activity produced in this study was proportional to the increase in concentration, where the inhibitory activity produced increased as the concentration of carrageenan increased. It can be concluded that carrageenan has the potential to be an antibacterial.

Abstrak

Peningkatan kasus infeksi dan resistensi setiap tahun, meningkatkan urgensi untuk mengeksplorasi agen antimikroba. *Gracilaria* sp. merupakan rumput laut merah yang dikenal secara luas sebagai salah satu penghasil metabolit aktif potensial yaitu polisakarida tersulfasi. Karagenan merupakan salah satu bentuk sulfated polisakarida yang memiliki aktivitas antimikroba. Penelitian ini dilakukan untuk menguji aktivitas antibakteri karagenan dari *Gracilaria* sp. terhadap beberapa bakteri patogen yaitu *E. coli, S. aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Pseudomona aeruginosa, Enterococcus faecalis, Streptococcus pyogenes,* dan *Salmonella typhi.* Karagenan diekstraksi dari sampel *Gracilaria* sp. yang diperoleh dari daerah Denpasar Selatan (Bali) dengan menggunakan KOH. Pengujian aktivitas antibakteri karagenan dilakukan dengan metode difusi agar sumuran dengan media nutrien agar. Pengujian dilakukan pada konsentrasi 1 and 5 mg/mL (*E. coli* dan *S. epidermidis*), 20, 30, 40 mg/ml (*S. aureus, S. pyrogenes, E. faecalis,* dan *S. typhi*) 50, 75, and 100 mg/mL (*P. aeruginosa* dan *K. pneumoniae*). Inkubasi dilakukan selama 24 jam pada suhu ± 37℃. Data dianalisis secara statistik dengan One-Way ANOVA dan Kruskal Wallis dengan taraf kepercayaan 95%. Hasil penelitian menunjukkan karagenan yang diekstrak dari *Gracilaria* sp. menunjukkan aktivitas antibakteri yang signifikan terhadap beberapa bakteri patogen, terutama bakteri gram negatif. Aktivitas antibakteri yang dihasilkan pada penelitian ini sebanding dengan peningkatan konsentrasi, dimana aktivitas penghambatan yang dihasilkan semakin meningkat seiring dengan meningkatnya konsentrasi karagenan. Dapat disimpulkan bahwa karagenan memiliki potensi sebagai antibakteria.

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INTRODUCTION

Infectious diseases are one of the significant public health problems in developed and developing countries. The World Heart Organization (WHO) states that in 2017, as many as 15% of deaths of children

under 5 years old were caused by infectious diseases.[1] Infection management is carried out through the administration of antibacterials. Antibacterials are substances that can inhibit or kill the growth of microorganisms. Antibacterials can also be classified based on the effect given, whether antibacterials can kill the growth of bacterial cells or antibacterials can only inhibit the growth of bacterial cells.[2]

The use of antibacterials or antibiotics often causes several problems, one of which is resistance. Resistance occurs when the effectiveness of drugs to prevent or treat infections decreases. Bacterial resistance to antibiotics increases the urgency of finding new antibiotics. Natural materials are sources that are widely explored for various biological activities, including antibacterials. One of the natural materials explored is the seaweed plant. Seaweed is one of the seafood commodities that has an essential role in Indonesia because it has extensive advantages, such as being used for food ingredients, the pharmaceutical industry, the cosmetic industry, the textile industry, medicines, and others, and has been marketed both domestically and abroad.[3]

Seaweeds have various pharmacological activities derived from primary or secondary metabolites. One of the primary metabolites of seaweed is carrageenan. Carrageenan is a natural polysaccharide derived from red algae with a structure characterized by long linear chains of D-galactose and D-anhydrogalactose with anionic sulfate groups, contributing to its bioactivity, including antibacterial effects.[4] The antimicrobial activity of carrageenan has been attributed to its ability to interact with bacterial cell membranes, leading to cell lysis and growth inhibition.[5,6] Several studies demonstrated the anttibacterial activity of carrageenan against several pathogens, such as *Staphylococcus aureus* and *Escherichia coli*.[7–9]

Regarding the background of Bulung Sangu (*Gracilaria* sp.), it is thought that carrageenan derived from *Gracilaria* sp.) also has antibacterial activity against several pathogenic bacteria. This study was conducted to evaluate the antibacterial activity of carrageenan derived from Bulung Sangu (Gracilaria sp.) against several pathogenic bacteria, including *E. coli, S. aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterococcus faecalis, Streptococcus pyogenes,* and *Salmonella typhi*.

MATERIAL AND METHOD

Research Materials and Instruments

Instruments Autoclave, Mc.Farland reader.

Materials Samples of red seaweed (*Gracilaria* sp.) obtained from the waters of Serangan (South Denpasar, Bali) nutrient agar (Merck), bacterial culture obtained from the Kerthi Bali Sadhajiwa Health Laboratory Center of Bali Province, sterile distilled water, chloramphenicol antibiotic (Merck), Mg powder, acidified ethanol, amyl alcohol, HCl 2N, Dragendorf reagent, Mayer reagent, $FeCl_3$1%, NaOH 1N.

Research Procedures

1. Carrageenan extraction

Fresh seaweed samples were cleaned of impurities and washed thoroughly under running water several times to reduce salt content. The seaweed samples were then processed into simplicia. Fifteen grams of simplicia was heated with KOH 6% at 60℃ for 60 minutes in a beaker glass covered with aluminum foil. The ratio of sample and solvent was kept at 1:30. After heating, the seaweed was washed with running water until it reached pH 7-8. pH was tested on the seaweed using a universal pH. Seaweed with pH 7-8 was again heated with the same heating process. After heating, the solvent was mixed in cold 96% ethanol at as much as 3x the heating solvent's volume, stirred continuously until carrageenan fibers were formed, then allowed to stand for 30 minutes. The carrageenan fibers formed were taken out, aerated, and then oven-dried at 60℃ until a constant weight was obtained. The carrageenan obtained was blended and sieved into a homogeneous fine powder. The carrageenan samples were used for secondary metabolite identification testing by tube reaction to confirm the absence of secondary metabolites (flavonoid, alkaloid, saponin, tannin, quinone) in the samples.

2. Antibacterial assay

An antibacterial assay of carrageenan was carried out using the agar well diffusion method with nutrient agar media. The negative control was sterile distilled water, and the positive control was chloramphenicol 15 µg. Bacterial suspension was used at Mc. Farland turbidity 0.5 equivalent to 1.5 x 10^8 (Colony Forming Unit) CFU/mL. The carrageenan powder was dissolved in distilled water to obtain concentrations of 1 and 5 mg/mL (for *E. coli* and *S. epidermidis* antibacterial assay), 20, 30, 40 mg/ml (for *S. aureus, S. pyrogenes, E. faecalis,* and *S. typhi*) 50, 75, and 100 mg/mL (for *P. aeruginosa* and *K. pneumoniae*). Incubation was carried out for 24 hours at ± 37℃. The assay was done in triplicate.

Data Analysis

Measurement of the inhibition zone was carried out with a caliper after the incubation process and calculated with the following calculation:

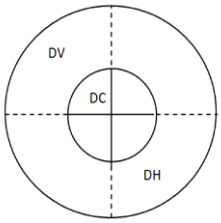

Figure 1. Measurement of the inhibition zone

Inhibition zone = $\frac{(Dv-Ds)+(Dh-Ds)}{2}$ ..... (1).

Dv: vertical diameter

Dh: horizontal diameter

Dc: agar well diameter (5 mm)

The diameter of the inhibition zone obtained was classified according to the following table.

Table 1. Inhibition zone classification

Zone diameter (mm)	Inhibition
>20	Very strong
10-20	Strong
5-10	Moderate
<5	Weak

The diameter of the inhibition zone (mm) obtained was compared statistically using SPSS (IBM Statistics version 25) with One-Way ANOVA followed by Bonferroni Post-Hoc Test and Kruskal Wallis followed by Mann Whitney at 95% confidence level by excluding the value of the negative control.

RESULT AND DISCUSSION

The development of antibacterial agents from marine products has attracted significant interest due to the unique biodiversity of the marine ecosystem, which serves as a rich source of bioactive compounds. Marine organisms, including bacteria, fungi, and macroalgae, have been shown to produce a variety of antimicrobial substances that exhibit potent antibacterial activity against various pathogens, including those resistant to conventional antibiotics.

In this study, the antibacterial activity of one marine product, carrageenan, obtained from one of the red algae in Bali, *Gracilaria* sp. This seaweed is known by the regional name Bulung Sangu and is currently widely used in the production of polysaccharide products and daily consumption.[10] The results of the antibacterial

activity assay of carrageenan against several pathogenic bacteria are shown in **Table 2**. In this study, identifying secondary metabolites in carrageenan showed negative results in all metabolites tested. Carrageenan is a form of polysaccharide, a primary metabolite, so secondary metabolite testing shows negative results. These test results indicate that no secondary metabolites are also extracted, which can affect the results of this study.

Table 2. Inhibition zone (mm) of carrageenan

Bacteria	Concentration			Negative Control	Positive Control
	50 mg/mL	75 mg/mL	100 mg/mL		
P. aeruginosa	18.33 ± 3.21^{a} (VS)	23.66 ± 1.15^{ab} (VS)	24.00 ± 2.65^{ab} (VS)	0.00 ± 0.00	28.66 ± 1.53^{b} (VS)
K. pneumoniae	28.33 ± 2.52^{a} (VS)	33.33 ± 4.16^{ab} (VS)	32.67 ± 1.53^{ab} (VS)	0.00 ± 0.00	36.33 ± 1.15^{b} (VS)
	1 mg/mL	**5 mg/mL**			
E. coli	18.00 ± 1.15^{a} (S)	33.00 ± 1.00^{b} (VS)		0.00 ± 0.00	23.00 ± 2.52^{c} (VS)
S. epidermidis	17.50 ± 1.80^{a} (S)	30.30 ± 1.20^{b} (VS)		0.00 ± 0.00	27.00 ±1.30^{b} (VS)
	20 mg/mL	**30 mg/mL**	**40 mg/mL**		
S. aureus	19.02 ± 2.62^{a} (S)	24.72 ± 1.31^{b} (VS)	22.53 ± 4.01^{b} (VS)	0.00 ± 0.00	13.50 ± 0.61^{a} (S)
S. pyogenes	11.08 ± 0.28^{a} (S)	17.10 ± 0.52^{b} (S)	18.35 ± 0.82^{b} (S)	0.00 ± 0.00	10.25 ± 0.05^{c} (S)
E. faecalis	12.08 ± 4.80^{a} (S)	11.70 ± 5.90^{a} (S)	17.80 ± 2.80^{a} (S)	0.00 ± 0.00	5.20 ± 0.60^{b} (M)
S. typhi	11.10 ± 0.81^{a} (S)	16.98 ± 1.28^{a} (S)	17.05 ± 3.50 ^{a} (S)	0.00 ± 0.00	11.91 ± 1.78^{a} (S)

Results are expressed as mean ± standard deviation (n=3). Letters in superscript following the inhibition zone diameter values indicate statistical results where different letters indicate significant differences ($p<0.05$) between concentrations in the same group of bacteria. Letters behind the values indicate the classification of antibacterial activity (VS (very strong), S (strong), M (moderate), W (weak)).

The data revealed that carrageenan from Balinese red seaweed (*Gracilaria* sp.) showed significant potential against several pathogenic bacteria. The diameter of the inhibition zone varied depending on the concentration of carrageenan and the type of bacteria tested (**Figure 2**). Antibacterial activity classified as very strong, based on the classification in **Table 1**, was observed at high concentrations against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, with inhibition zone diameters reaching 24.00 mm and 32.67 mm at 100 mg/mL concentration, respectively. Similar activity was also observed against *Escherichia coli* and *Staphylococcus epidermidis* bacteria at a 5 mg/mL concentration. However, the activity against bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Salmonella typhi* were classified as strong to moderate at the lower concentration range. In this study, gram-negative bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes,* and *Enterococcus faecalis)* generally showed higher sensitivity to carrageenan than gram-positive bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Salmonella typhi)*. The results of this study are excellent for further development, considering that in some explorations of antibacterial agents, gram-negatives show higher resistance compared to gram-positive bacteria. Gram-negative bacteria have an outer membrane, a formidable barrier against many antibiotics. This outer membrane consists of lipopolysaccharides and phospholipids, which significantly reduce the permeability of these bacteria to various antimicrobial agents, making them inherently more resistant to various classes of antibiotics compared to gram-positive bacteria that do not have such an outer membrane.[11,12] The peptidoglycan layer in gram-positive bacteria is thicker and more accessible to many antibiotics, such as beta-lactams, which target cell wall synthesis. In contrast, gram-negative bacteria have a thinner peptidoglycan layer but are protected by their outer membrane, which limits the effectiveness of these agents.[13] This structural barrier is a significant reason why many antibiotics, including vancomycin, are ineffective against gram-negative bacteria.[14]

This result also indicates that carrageenan has concentration-dependent antibacterial activity. Antibacterial activity increased as the concentration of carrageenan increased, with the largest inhibition zone diameter at the highest concentration (100 mg/mL). However, the limitation of this study is that the antibacterial activity was not tested at the same concentration, so the activity produced at the same concentration cannot be compared. This study used different concentrations with the concern that there would

be overlapping in the test, disrupting the process of reading the results. Testing through the dilution method is recommended to obtain each pathogenic bacteria's minimum inhibitory concentration (MIC) value.

This activity is thought to be related to the ability of carrageenan to disrupt the bacterial cell membrane through electrostatic interactions between the sulfate groups in carrageenan and the bacterial cell wall, causing cytoplasmic leakage. Carrageenan is a *sulfated polysaccharide*. Sulfated polysaccharides are the most common part of seaweed cell walls.[15] Carrageenan contains primary metabolite compounds such as vitamins, minerals, fiber, alginate, and agar. It is classified into three types based on its structure: kappa, lambda, and iota carrageenan. Carrageenan has the same structure as carbohydrates, where the acid groups contained in carbohydrates will interact electrostatically with the cell membrane which can disrupt the bacterial cell wall so that it leaks into the bacterial cytoplasm.[16]

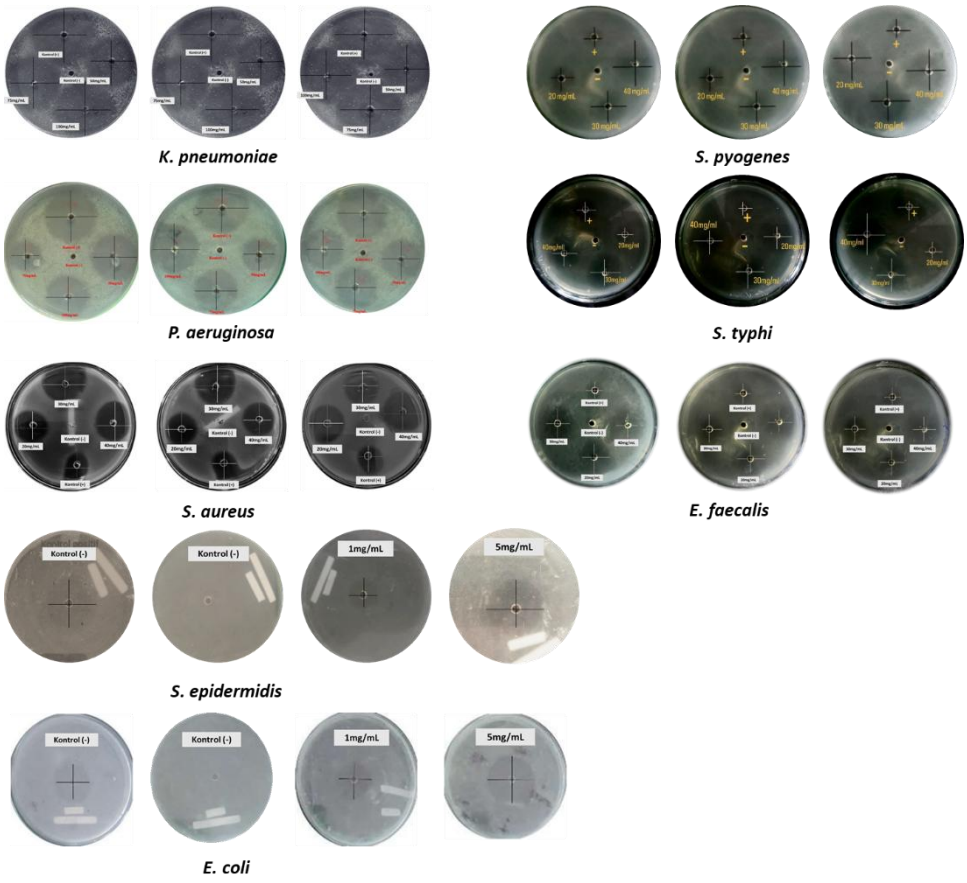

Figure 2. Inhibition of carrageenan against some pathogenic bacteria

The electrostatic interactions between the sulfate groups of carrageenan and the bacterial cell wall are fundamental to its antibacterial activity. Studies have shown that carrageenan can cause cytoplasmic leakage in bacteria, leading to cell lysis.[17] This effect is primarily due to the binding of negatively charged carrageenan to the positively charged components of the bacterial membrane, which disrupts the integrity of the membrane and facilitates the leakage of cytoplasmic contents.[18,19] The presence of sulfate groups in carrageenan enhances its ability to form complexes with cationic substances, which can further destabilize bacterial membranes.[20] Moreover, the structural characteristics of different types of carrageenan, such as kappa (κ), iota (ι), and lambda (λ), influence their antibacterial efficacy. Kappa-carrageenan, for example, has been shown to possess a specific arrangement of sulfate groups that enhances its interaction with bacterial membranes.[21] The varying degrees of sulfation among these types of carrageenan contribute to their distinct mechanisms of action against bacteria. Kappa-carrageenan is known for its strong gelling properties and its ability to form stable complexes with proteins, which may also play a role in its antibacterial effects.[22,23]

In addition to direct interactions with bacterial membranes, carrageenan has been observed to modulate immune responses, which can indirectly affect bacterial viability. For example, carrageenan has been shown to influence the production of reactive oxygen species (ROS) in immune cells, which can enhance their bactericidal activity.[24] This dual mechanism, which includes the direct disruption of bacterial membranes and modulation of immune responses, highlights the potential of carrageenan as a therapeutic agent against bacterial infections.

Studies have shown that carrageenan can effectively inhibit the growth of both gram-positive and gram-negative bacteria, demonstrating its broad-spectrum antibacterial potential. Research conducted on oligosaccharide carrageenan also found there are various activities, including antitumor, antioxidant, and anticoagulant.[25–27] Research conducted by Wang et al. (2012) showed that carrageenan has antifungal activity in *Saccharomyces cerevisiae* with a strong category, while antibacterial activity in *Escherichia coli* and *Staphylococcus aureus* bacteria with a weak category, where the concentrations used were 1 mg/mL, 5 mg/mL, and 20 mg/mL. The MIC values for carrageenan against various bacterial strains have been reported, indicating its effectiveness at relatively low concentrations.[20] Several studies have also shown that carrageenan can increase a material's antimicrobial activity. Formulating carrageenan as an edible film inhibited *Staphylococcus aureus* sub sp. *aureus* CCM 7110 and *Escherichia coli* CCM 3954.[28] Other similar studies showed inhibition of carrageenan edible film against *S. aureus, Bacillus cereus, E. coli, Salmonella typhimurium,* and *P. aeruginosa.*[29] The structural characteristics of carrageenan, particularly the arrangement and density of sulfate groups, are crucial for its antibacterial efficacy. Different types of carrageenan, such as kappa (κ), iota (ι), and lambda (λ), exhibit varying degrees of antibacterial activity due to their distinct molecular structures.[30] However, this study's limitations include the inability to identify the structure of the carrageenan produced, so the exact mechanism underlying the antibacterial activity cannot be predicted. Research related to this can be carried out as follows to support the results of this study.

CONCLUSION

The results of this study showed that carrageenan extracted from *Gracilaria* sp. exhibited significant antibacterial activity against several pathogenic bacteria, especially gram-negative bacteria. The antibacterial activity produced in this study was proportional to the increase in concentration, where the inhibitory activity produced increased as the concentration of carrageenan increased. The antibacterial activity of carrageenan originates from the electrostatic interaction between the sulfate groups on the carrageenan structure and the bacterial cell wall, which causes membrane damage and cytoplasmic leakage.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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