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Full Length Research Paper

Antibacterial activity of *Sargassum polycystum* C. Agardh and *Padina australis* Hauck (Phaeophyceae)

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Seaweeds are used in pharmaceutical and biochemical applications as they possess interesting biological activities that contribute to the discovery of natural therapeutic agents. In this study, the antibacterial activity of n-hexane, dichloromethane and methanolic extracts of brown seaweeds (Phaeophyceae), *Sargassum polycystum* C. Agardh and *Padina australis* Hauck, was examined using the disc diffusion and broth microdilution methods. The bioactivity of the seaweed extracts was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The antibacterial activity against Gram-negative bacteria (beta-lactamase positive and negative *Escherichia coli*, *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus cereus*) was discussed. Gram-positive bacteria especially *B. cereus* was more susceptible to the seaweed extracts (MIC = 0.130 to 0.065 mg/ml). Generally, *S. polycystum* extracts exhibited higher bacteriostatic activity (lower MICs) against all the tested bacterial strains when compared with *P. australis*. However, *P. australis* extracts showed a narrow spectrum of bactericidal activity against *B. cereus*. n-Hexane extracts of *S. polycystum* exhibited promising bacteriostatic agents against *B. cereus* (MIC = 0.065 mg/ml) with MIC value lower than the standard MIC of potential antimicrobial drug (0.100 mg/ml). Since only crude seaweed extracts were tested in this study, further purification and isolation of bioactive compounds from the extracts are essential in future studies in order to optimize their antibacterial activity.

Key words: Phaeophyceae, disc diffusion test, minimum bactericidal concentration (MBC), minimum inhibition concentration (MIC).

INTRODUCTION

Recently, the use of antibiotic for resistance of pathogenic bacteria has increased in an alarming rate. However, due to the widespread, over prescription and misuse of antibiotics, it has led to the evolution and adaptation of pathogenic bacteria strains to conventional drug treatments (Spicer, 2008). Thus, alternative prevention and treatment are implied and natural sources such as plants and seaweeds are increasingly used. Despite the extensive studies of antibacterial activity in

higher plants, seaweeds may be used as a new and promising source of bioactive compounds in the field of medical and biochemical applications (Leary et al., 2009).

The antimicrobial activity in seaweed extracts has been reported since 1917. Biological compounds extracted from some seaweed species, namely, Phaeophyceae, Rhodophyceae and Chlorophytceae, were proven to have potentials medicinal activities such as, antibacterial, antiviral, antitumour, antifungal, antiprotozoa, and mosquito and larva control (Bansemir et al., 2006). To date, only certain antibacterial activities of brown seaweed species have been studied in details [evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)]. Brown seaweeds like

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Dictyota (Jebakumar and Satheja, 2008; Stirk et al., 2007) and *Sargassum* (Kim and Lee, 2008) have been studied and they showed promising antibacterial activity. Phenolic compounds which play a major role in antibacterial and antifungal activities are found abundantly in brown seaweeds when compared with the green and red seaweeds (Chkhikvishvili and Ramazanov, 2000).

Malaysia with its rich seaweeds resources and a total of 356 taxa as reported (Phang, 2006), offers a great opportunity for researchers to look into their potential bioactive compounds. Thus, in this study, antibacterial activity of two species of Phaeophyceae (*Sargassum polycystum* C. Agardh and *Padina australis* Hauck) against five pathogenic bacteria (*Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 6538) was studied.

MATERIALS AND METHODS

Identification of seaweeds

S. polycystum samples were collected from Teluk Kemang (TK) and Cape Rachado (CR), while *P. australis* samples were collected from CR in February 2009. All samples were identified by using the standard taxonomy key (Trono and Fortes, 1988; Tsutsui et al., 2005). Morphology of the seaweeds was observed and cross sections of *P. australis* thallus were prepared and observed under the microscope to confirm the identification. Herbarium specimens of both species were deposited at the Faculty of Engineering and Science, UTAR.

Preparation of extracts

Seaweed samples were washed thoroughly and rinsed with distilled water, then air-dried and ground. The samples (15 g) were then soaked in three different solvents (150 ml) with decreasing polarity, namely, methanol, dichloromethane and n-hexane. Maceration was conducted for three days with constant agitation in orbital shaker. The extracts were filtered through the Whatman filter paper No. 3 and concentrated using a rotary evaporator (Buchi Rotavapor R-200, Switzerland) at 40°C. The concentrated extracts were dried and stored at -20°C in tightly sealed vials prior to antibacterial assays.

Preparation of media

Nutrient agar (NA, Difco™, France), Mueller Hinton agar (MHA, Difco™, France) and Mueller Hinton broth (MHB, BBL™, France) were prepared according to their respective standard procedure. Prepared media were stored in a sterile environment at room temperature for further use.

Bacterial isolation

Two Gram-positive bacteria, namely, *S. aureus* ATCC 6538, *B. cereus* ATCC 11778 and three Gram-negative bacteria, namely, *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853, were used in this study. Colonies were selected from the bacterial stock and cultured on NA and incubated at 37°C for 24 to 48 h. Following incubation, a pure colony of bacteria was selected for each tested organism prior to the antibacterial assays.

0.5 McFarland standard

Three to five isolated bacteria colonies were selected from the pure isolate plate using a sterile inoculating loop and inoculated into 3 ml of Mueller-Hinton broth (MHB) in a sterile tube and mixed well. The turbidity of the bacteria suspensions were adjusted according to the turbidity of the commercially prepared 0.5 McFarland standard (bioMerieux® SA, France). The accurate turbidity of the bacterial suspension was confirmed by using a spectrophotometer (Genesys 20, Thermo Fisher Scientific). The spectrophotometer was blanked using the sterile MHB, measured at wavelength of 625 nm. The absorbance value was in the range of 0.08 to 0.10 which corresponded to 10⁷ to 10⁸ number of bacteria per ml of MHB.

Disc diffusion susceptibility testing

Each of the seaweed extracts was dissolved in 60% methanol to a concentration of 10 mg/ml and the solutions were vortexed at high speed. Bacterial inoculums were adjusted according to 0.5 McFarland standard and 0.1 ml of suspension was pipetted out from each bacterial inoculums and lawn onto their respective MHA plate. Solubilised extract (20 µl) with the concentration of 10 mg/ml was pipetted onto a commercial blank disc (Oxoid, UK) measuring 6 mm in diameter and the disc was placed onto the bacterial field using a sterile forceps. Blank disc impregnated with 20 µl of 60% methanol was set as the negative control and a commercial antibiotic disc (Oxoid, UK) impregnated with 10 µg of gentamycin was set as the positive control. The plates were incubated at 37°C and the zones of inhibition were measured after 24 h. Each set of testing was carried out in triplicates. A diameter larger than 6 mm indicates the presence of inhibition. Percentage inhibition (%) of all the seaweed extracts towards each type of bacterial strain was calculated using the following formula:

$$\text{Percentage Inhibition (\%)} = \frac{N}{T} \times 100 \%$$

Where, N = number of seaweed extract developed inhibition zone; T = total number of seaweed extract tested = 9.

MIC assay

A final concentration of 10 mg/ml of seaweed extracts were prepared by dissolving the concentrated seaweed extracts into 60% methanol and the solutions were vortexed at high speed. Bacterial inoculums were prepared from a 24 h culture according to 0.5 McFarland standard. Each inoculum was then diluted by transferring 50 µl of bacterial suspension into 4950 µl of MHB. After dilution, the bacterial suspension achieved a concentration of 1 × 10⁶ colony-forming units (cfu)/ml. Preparation of bacteria suspension was carried out only after two-fold dilution of the extracts was completed.

Under sterile condition, 96-well plate was prepared by first adding a volume of 50 µl of MHB into all the test wells and positive control wells, 75 µl into the negative wells and 100 µl into the sterility control wells. 25 µl of extract solution was added into the negative control well. Seaweed extract (50 µl) was added into the first row of the test well and 50 µl of 60% methanol was added to the first positive control well. Seaweed extracts and 60% methanol were serially diluted two-folds down their respective column. The prepared bacterial suspension (50 µl) with a concentration of 1 × 10⁶ cfu/ml was added to each of the test well and positive well to achieve a final concentration of 5 × 10⁵ cfu/ml. The plate was covered with sterile plastic cover and placed on orbital shaker for 15 min. The plate was then incubated for 24 h at 37°C.

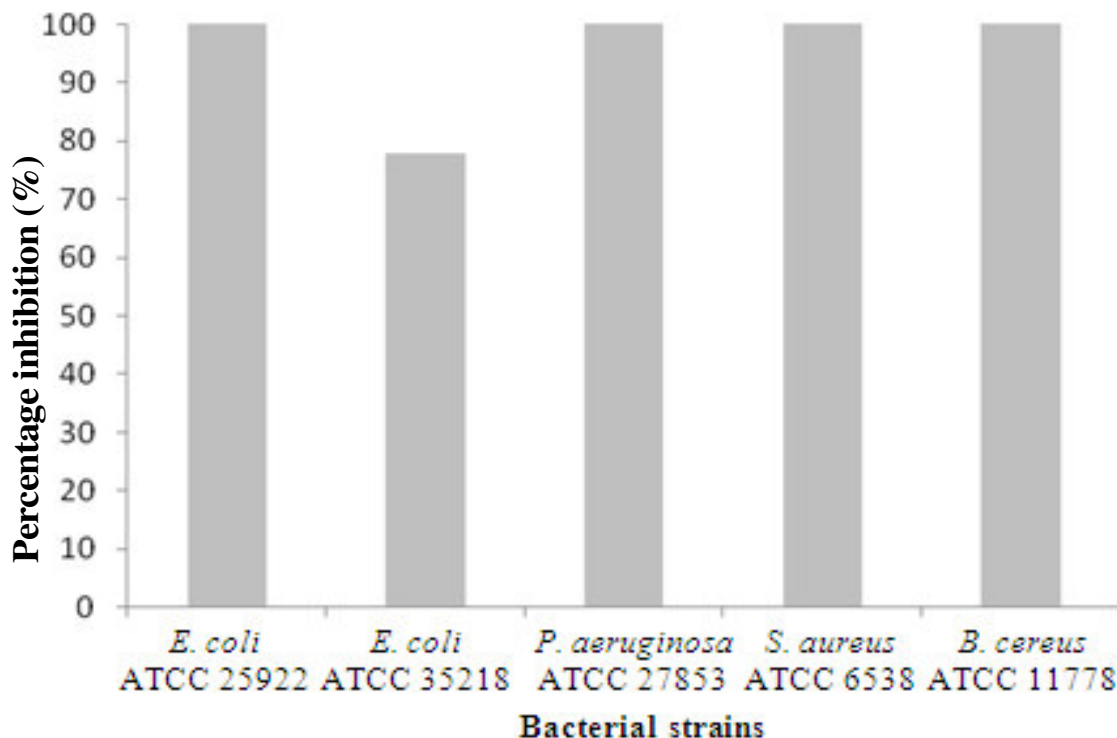


Figure 1. Percentage inhibition (%) of the nine seaweed extracts towards the five types of pathogenic bacterial strains.

Following an overnight incubation, 20 μ l of iodinitrotetrazolium (INT) (0.2 mg/ml) was added to each well and incubated for 30 min at 37°C. Results were observed after 30 min incubation. No change in color indicated no bacterial growth, while color change to pink or red indicated presence of bacterial growth. The highest dilution (lowest concentration) showing no visible growth of bacteria was taken as the MIC value expressed in mg/ml. Each set of assay was carried out in triplicates.

MBC assay

Solutions showing no visible growth of bacteria in MIC assay were pipetted out (20 μ l) from their respective test wells and spread evenly onto a new MHA. The plates were incubated for 18 to 24 h at 37°C. Following incubation, the colony-forming units (cfu) of the bacteria were counted. MBC is defined as the concentration which results in 99.9% reduction in mg/ml of the original bacterial inoculums, which also means that MBC only allow 0.1% of the bacteria to survive when re-cultured onto the new MHA (Taylor et al., 1983). Hence, the number of bacteria colony corresponding to 0.1% of the original bacterial inoculums in broth microdilution test was calculated, where 0.1% is approximately 8 cfu. The highest dilution (lowest concentration) of seaweed extract showing less than or equal to eight visible growth of bacteria colony on the MHA was taken as the MBC value expressed in mg/ml.

RESULTS

Disc diffusion susceptibility testing

Figure 1 shows the percentage of inhibition (%) of nine seaweed extracts against the five types of pathogenic

bacterial strains. All of the nine tested seaweed extracts showed inhibition (corresponding to 100% inhibition) towards beta-lactamase negative *E. coli* ATCC 25922, *P. aeruginosa*, *S. aureus* and *B. cereus*. Inhibition zones were only absent in two out of the nine seaweed extracts (methanolic and dichloromethane extracts of *P. australis*) towards the beta-lactamase positive *E. coli* ATCC 35218. Hence, the percentage of inhibition of the nine seaweed extracts against *E. coli* ATCC 35218 corresponded to 77.78% of inhibition.

MIC

Table 1 shows the MIC values of the nine types of seaweed extracts against the five pathogenic bacterial strains. The lowest MIC obtained from *P. australis* extracts against *E. coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa*, *S. aureus* and *B. cereus*, were 0.833, 1.667, 0.261, 0.417, and 0.130 mg/ml, respectively. The lowest MIC obtained from *S. polycystum* extracts against *E. coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa*, *S. aureus* and *B. cereus*, were 0.729, 0.417, 0.104, 0.117 and 0.065 mg/ml, respectively. MICs of *S. polycystum* showed a similar trend compared to *P. australis* where both strains of *E. coli* were least susceptible with *B. cereus* the most susceptible to the seaweed extracts. Generally, most of the *S. polycystum* (CR) extracts exhibited highest bacteriostatic activity

Table 1. MIC values of the nine types of seaweed extracts towards five pathogenic bacterial strains.

Bacteria strain	Gram	<i>P. australis</i> , CR			<i>S. polycystum</i> , TK			<i>S. polycystum</i> , CR		
		Methanol	Dichloromethane	n-Hexane	Methanol	Dichloromethane	n-Hexane	Methanol	Dichloromethane	n-Hexane
<i>E. coli</i> ATCC 25922	-	0.833±0.29	1.042±0.29	1.250±0.00	0.833±0.29	1.042±0.29	1.042±0.29	<u>0.729±0.29</u>	0.833±0.29	<u>0.729±0.29</u>
<i>E. coli</i> ATCC 35218	-	2.083±0.59	2.083±0.59	1.667±0.59	0.833±0.30	1.042±0.29	0.625±0.00	0.521±0.15	0.833±0.29	<u>0.417±0.15</u>
<i>P. aeruginosa</i> ATCC 27853	-	0.261±0.07	0.261±0.07	0.729±0.39	0.208±0.07	0.208±0.07	<u>0.104±0.04</u>	0.208±0.07	0.208±0.07	<u>0.104±0.04</u>
<i>S. aureus</i> ATCC 6538	+	0.417±0.15	0.417±0.15	1.042±0.29	0.313±0.00	0.313±0.00	0.156±0.00	0.261±0.07	0.261±0.07	<u>0.117±0.06</u>
<i>B. cereus</i> ATCC 11778	+	0.130±0.04	0.208±0.07	0.365±0.19	0.208±0.07	0.208±0.07	<u>0.065±0.02</u>	0.208±0.07	0.208±0.07	<u>0.065±0.02</u>

MIC values were expressed in mg/ml; + = positive; - = negative. Underline figures represent the lowest MIC value (highest bacteriostatic activity) among the nine seaweed extracts towards the respective pathogenic bacteria strain. No change of color was observed in all the positive control wells; color changes from clear to pinkish red was observed in all the negative control wells.

(lowest MICs) towards all the tested bacterial strains as compared to *S. polycystum* (TK) and *P. australis* (CR) (Table 3).

MBC

Table 2 shows the MBC values of the nine types of seaweed extracts towards five pathogenic bacterial strains. MBC was only detected in Gram-positive bacteria, where MBCs of *S. polycystum* (CR and TK) were detected in both *S. aureus* and *B. cereus*, however MBC of *P. australis* extracts was only detected in *B. cereus*. MBCs towards the other tested bacterial strains exceeded the lowest dilution which was 2.5 mg/mL. n-Hexane extract of *S. polycystum* (CR) exhibited the lowest MBC against *S. aureus* (0.521 mg/mL) while methanolic extract of *P. australis* exhibited the lowest MBC against *B. cereus* (0.182 mg/mL) (Table 3).

DISCUSSION

Antibacterial activity of the nine brown seaweed

extracts was analyzed with three types of assays, namely, disc diffusion susceptibility testing, MIC and MBC assays. Disc diffusion susceptibility testing was a preliminary screening of antibacterial activity of the extracted compounds as it can only give qualitative but not quantitative assessment. The size of the inhibition zone may not correspond to their antibacterial activity. Black (2005) suggested that factors like various diffusion rate of the extracts in the bacteria lawn due to different polarity, molecular size and molecular mass among the extracted components might affect the results. The introduction of MIC and MBC assays are essential in this study in order to obtain a more conclusive and quantitative result on the antibacterial potential of the tested extracts. As reported by Andrews (2006), MIC assay is used to judge the performance of all other methods of susceptibility testing and are used in diagnostic laboratories to give a definitive answer when a borderline result is obtained by other methods of testing or when disc diffusion methods are not appropriate.

MIC and MBC assays were conducted to assess the bacteriostatic and bactericidal

concentration of the seaweed extracts against the respective pathogenic bacterial strains. The lower the MIC and MBC values, the higher the antibacterial potential of the plant extracts. The MBC values obtained in this study were greater than their corresponding MIC values. This indicated that a higher concentration of the seaweed extracts was needed in order to exhibit bactericidal effect on the bacteria.

In this study, methanol, dichloromethane and n-hexane were used in which their polarity indexes (PI) varied from low (n-hexane, PI = 0.1), intermediate (dichloromethane, PI = 3.1) to high (methanol, PI = 5.1). Different extraction solvents were used in order to determine the right solvent that yielded an extract with a higher antibacterial activity. According to Kaufman et al. (1999), the primary consideration in antibacterial activity study is the choice of the extraction solvent being used. The purpose of the general screening of the antibacterial activity is to extract as many potentially active constituents as possible by using various types of solvents ranging from low to high polarity and selection of the best extraction solvents for each algae species which gave

Table 2. MBC values of the nine types of seaweed extracts towards five pathogenic bacterial strains.

Bacterial strain	Gram	<i>P. australis</i> , CR			<i>S. polycystum</i> , TK			<i>S. polycystum</i> , CR		
		Methanol	Dichloromethane	n-Hexane	Methanol	Dichloromethane	n-Hexane	Methanol	Dichloromethane	n-Hexane
<i>E. coli</i> ATCC 25922	-	Na	Na	Na	Na	Na	Na	Na	Na	Na
<i>E. coli</i> ATCC 35218	-	Na	Na	Na	Na	Na	Na	Na	Na	Na
<i>P. aeruginosa</i> ATCC 27853	-	Na	Na	Na	Na	Na	Na	Na	Na	Na
<i>S. aureus</i> ATCC 6538	+	Na	Na	Na	1.042±0.29	1.667±0.59	0.833±0.29	0.833±0.29	1.250±0.00	0.521±0.15
<i>B. cereus</i> ATCC 11778	+	0.182±0.10	0.208±0.07	0.417±0.15	0.417±0.15	0.521±0.15	0.417±0.15	0.833±0.29	1.667±0.59	0.625±0.00

MBC values were expressed in mg/ml. Underline figures represent the lowest MBC value (highest bactericidal activity) among the nine seaweed extracts towards the respective pathogenic bacteria strain. Na indicate no bactericidal activity or the bactericidal concentration was beyond the tested concentration; + = positive; - = negative.

potential antibacterial activity (Karmegam et al., 1997). Majority of the n-hexane extracts of *S. polycystum*, exhibited higher antibacterial activity than dichloro-methane and methanol extracts. However, methanolic extract of *P. australis* showed the highest activity when compared with the dichloromethane and n-hexane extracts. Hence, there is no consensus on the best solvents for the extraction of antibacterial compounds due to the differences between the characteristics of active chemicals among plants (Shalan et al., 2005). This suggests that seaweeds should be extracted in different solvent systems in order to optimize their antibacterial activity by selecting the best solvent system.

In the assessment of bacteriostatic activity of the seaweed extracts (MIC assay), the sequence of susceptibility was: both strains of *E. coli* < *S. aureus* < *P. aeruginosa* < *B. cereus*. The development of resistance towards antimicrobial agents may be due to the high adaptive mutation rate of *E. coli* which is 1000 times faster than the previous estimates (Perfeito et al., 2007). In MBC assay, MBCs less than 2.5 mg/ml could only be observed in the Gram-positive bacteria. This showed that Gram-positive bacteria were more

susceptible to the extracts than Gram-negative bacteria (Mahboubi and Haghi, 2008; Al-Haj et al., 2009; Ibtissam et al., 2009). It may be due to the hydrophobic lipopolysaccharide in the outer membrane of Gram-negative bacteria which provides protection against different agents. Michael et al. (2002) suggested that the absence of target structure in bacteria and the ability of bacteria to alter the structure of the extracts into active forms and genetic changes in bacteria lead to the alteration of metabolic pathway that is originally blocked by the antibacterial agents.

By comparing the MICs and MBCs values obtained from *P. australis* and *S. polycystum*, *S. polycystum* showed better bacteriostatic potential than *P. australis*. The MICs of *S. polycystum* were lower than the MICs of *P. australis* towards all the five bacterial strains tested. Besides, *S. polycystum* had a broader bactericidal spectrum when compared with the *P. australis*, *S. polycystum* exhibited bactericidal potential on both *S. aureus* and *B. cereus*, while *P. australis* only exhibited its bactericidal potential on *B. cereus*. This might be due to the presence of higher potential antimicrobial compounds in *S. polycystum* than *P. australis*. As supported by the

study of Matanjun et al. (2008), these authors showed that the phenolic contents of *S. polycystum* collected from Malaysia were higher compared with other seven species of seaweeds including *Padina* sp. However, the bacteriocidal potential of *P. australis* towards *B. cereus* was higher than *S. polycystum* as it exhibited the lowest MBC. This suggests that *P. australis* showed a narrow spectrum of activity with its effectiveness against one specific type of bacteria (*B. cereus*) in the study.

S. polycystum collected from two different locations were tested in this study. Generally, *S. polycystum* collected from CR exhibited higher bacteriostatic activity than *S. polycystum* collected from TK. Lighter color was observed on the blades of *S. polycystum* collected from CR as it grows in shallow areas (high intertidal zone) and exposed to sunlight for a longer period during low tides (tide level of 0.0 to 0.5 m above sea level; exposed to the sun range from 2 to 5 h) when compared with *S. polycystum* collected from TK which grew in deeper water of low or middle intertidal zone with shorter time of exposure to the sun during low tides (tide level of 0.0 to 0.3 m above sea level; exposed to the sun range from 2

Table 3. Comparison on the lowest MIC and MBC values obtained from seaweed extracts towards five types of pathogenic bacteria.

Bacterial strain	<i>P. australis</i> (CR)		<i>S. polycystum</i> (TK)		<i>S. polycystum</i> (CR)	
	MIC	MBC	MIC	MBC	MIC	MBC
<i>E. coli</i> (ATCC 25922)	0.833±0.29(M)	--	0.833±0.29 (M)	--	0.729±0.29 (M, H)	--
<i>E. coli</i> (ATCC 35218)	1.667±0.59 (H)	--	0.625±0.00 (H)	--	0.417±0.15 (H)	--
<i>P. aeruginosa</i>	0.261±0.07(M, D)	--	0.104±0.04(H)	--	0.104±0.04(H)	--
<i>S. aureus</i>	0.417±0.15 (M, D)	--	0.156±0.00 (H)	0.833±0.29(H)	0.117±0.06(H)	0.521±0.15(H)
<i>B. cereus</i>	0.130±0.04 (M)	0.182±0.10 (M)	0.065±0.02 (H)	0.417±0.15(M, H)	0.065±0.02(H)	0.625±0.00(H)

MIC and MBC values were expressed in mg/ml. -- indicate no bactericidal activity or the bactericidal concentration was beyond the tested concentration. M = Methanolic extract; H = n-hexane extract; D = dichloromethane extract.

to 3 h). Plouguerne et al. (2006) reported that the production of phenolic content of *Sargassum* species increased with the increased exposure to solar radiation in order to protect them from the UV radiation. As suggested by Zubia et al. (2008), the great variation observed in the potential antimicrobial components in seaweeds could be due to the external environmental factors such as herbivory, light, depth, salinity and nutrients of their growing environment. All of these factors could act on the spatiotemporal regulation on metabolic expression of the active compounds leading to marked qualitative and quantitative variations among similar species at a smaller scale than different species. Thus, this might be some of the reasons that led to the higher bacteriostatic activity in *S. polycystum* collected from CR.

S. polycystum (CR and TK) are considered as promising bacteriostatic agents against *B. cereus* with MIC values of 0.065 mg/ml which was lower than the standard MIC of potential antimicrobial drug which is 0.100 mg/ml (Kuate et al., 2007). *S. polycystum* extracts exhibited higher bacteriostatic activity against *B. cereus* when compared with other higher medicinal plants, namely, *Citrus aurantifolia* (MIC = 0.560 mg/ml), *Citrus hystrix* (MIC = 0.560 mg/ml) (Chanthaphon et al., 2008) and exhibited compatible activity to *Syzygium*

jambolanum (MIC = 0.064 mg/ml) (Chandrasekaran and Venkatesalu, 2004).

In conclusion, antibacterial activity of the seaweeds varies according to their species and extraction solvent. In this study, n-hexane extracts of *S. polycystum* exhibited promising bacteriostatic activity against *B. cereus*

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