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Antimicrobial effects of *Caulerpa sertularioides* extract on foodborne diarrhea-caused bacteria

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Abstract. – AIM: ***Caulerpa (C.) sertularioides*** **has many therapeutic uses in the practice of** ***traditional medicine*** **in Malaysia. Crude methanolic, diethyl ether extract, ethyl acetate extract and butanolic extract from** ***C. sertularioides*** **were subjected to antimicrobial screening including the three Gram-positive and three Gram-negative diarrhea-caused bacteria.**

MATERIALS AND METHODS: The antimicrobial activities were studied by using disc diffusion method and broth dilution method. The effect of the extract on the growth profile of the bacteria was examined via time-kill assay. In addition to the bactericidal effects study, microscopic observations using Scanning electron microscopy (SEM) was done to determine the major alterations in the microstructure of ***Bacillus (B.) subtilis*****.**

RESULTS: Ethyl acetate extract demonstrated antibacterial activity towards all the tested bacteria and produced inhibition zone ranging from ≤ 9 mm - ≥ 15 mm. However, all the tested bacteria were resistant to the butanolic extract treatment. ***B. subtilis*** **growth curve in the presence of the crude methanol extract at MIC showed bacteriostatic. The main abnormalities found from these microscopic observations were morphology alteration of the bacteria cells after exposure to the methanol extract.**

CONCLUSIONS: Data from this study revealed that ***C. sertularioides*** **may be potential antimicrobial agents against foodborne Gram-positive and Gram-negative bacteria particularly cause diarrhea, and also food spoilage microorganisms.**

Key Words:
Antimicrobial activity, *Caulerpa sertularioides*, Foodborne pathogens, Food Spoilage organisms, Diarrhea.

Introduction

Food poisoning illness occurs as a result of eating foods contaminated with bacteria or their toxins, and the symptoms include stomach upset to more serious symptoms such as diarrhoea, fever, vomiting, abdominal cramps and dehydration[1]. The symptoms generally start between 4-36 h after consuming the contaminated food. Diarrhoea is a condition which involves having three or more loose and watery stool, occurs for various reasons ranging from food allergy, infections, consumption of spoiled food or toxic chemicals in food, food poisoning to over dosage of antibiotics. Although diarrhoea causes discomfort and at times stomachaches, it is one of condition that should not be taken for granted as it can cause death, especially when dehydration occurs. Bacteria such as *Bacillus (B.) subtilis, cereus, Escherichia coli, Staphylococcus aureus MRSA* and *Acinetobacter anitratus* are considered as the most common cause of diarrhoea and food poisoning worldwide[2].

Even though there are many drugs that can be used to treat food poisoning and diarrhea, the interest in developing new types of effective and non-toxic antimicrobial compounds is increasing due to concern about health and safety. Consequently, interest in using natural antimicrobial compounds has become increasingly popular worldwide. Besides plant extracts, some of the marine macroalgae are also potential to be used to treat diarrhoea and food poisoning, especially the edible and those include genera of *Eucheuma, Gellidium, Gracilaria, Palmaria and Porphyra*[3,4]. These edible macroalgae usually contain significant amounts of protein, carbohydrate, vitamins, minerals and amino acids essential for human nutrition[5,6]. Furthermore, marine macroalgae also have been reported to be a potential source of bioactive compounds with broad range of biological activities such as antibacterial[7,8], antiviral[9], antifungal[10], antitumorals[11,12], anticancer[13] and antioxidants activities[6,14].

However, marine macroalgae or better known as seaweeds as a food in Malaysia is not as common in other countries like China, Korea and Japan. In Malaysia only two species are popularly consumed by people especially those who are living in the coastal areas especially along the

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east coast of Peninsular Malaysia and in East Malaysia, where they are occasionally eaten raw as a salad dish[4].

Caulerpa (C.) sertularioides is green feather like macroalgae in the group of Chlorophyta (green algae) and can be found in coastal and estuarine environments, and sometimes growing in sandy areas, seagrass beds or on mangrove prop roots[15]. It is reported to be used for the treatment of hypertension in traditional medicine and also as insect repellent agents[16]. In addition, the methanolic extract of *C. sertularioides* was reported to show a very strong telomerase inhibiting activity by acting on telomerase expression and inactivation after penetration of active compounds. The effect of this telomerase inhibiting activity can be led to an anti-cancer agent with very little side effects[17]. Rawat and Bhakuni[18] stated that *C. sertularioides* showed a central nervous system depression activity that enables potentiated pentobarbital hypnosis. They also reported that the depression of both spontaneous and locomotors activities was due to caulerpin that causes sedation. Both of facts revealed that *C. sertularioides* plays a very crucial role in the field of medicine.

Based on these advantages, hence, the present study was undertaken to evaluate and to relate the antibacterial activity of the *C. sertularioides* extracts against bacterial pathogens especially against food poisoning and diarrheal caused bacteria. This is a good approach in order to encourage the consuming of this macroalgae among people as this sea-vegetable are of nutritional interests, besides having medicinal value.

Materials and Methods

Collection and Processing of Algal Sample

The fresh sample of *C. sertularioides* was collected from Pulau Gedung, Penang, Malaysia. The sample was identified through its morphological characteristics according to the book of Rumpai Laut Malaysia[15] and authenticated by Associate Professor Dr. Shaida Fariza Sulaiman from the School of Biological Sciences, University Sains Malaysia.

Extraction Procedures

At the sampling site, the fresh algal samples were rinsed with seawater several times to remove associated debris and epiphytes. Once arrived at the laboratory, the samples were rinsed thoroughly under running tap water to remove all the unwanted epiphytes, necrotic parts and the salt from seawater. The clean samples were then dried in an oven at 45°C for 4-7 days until they were completely dried before grinding them into powder form. Approximately 40 g of dried powder form algal sample was soaked in 400 ml of 100% methanol at room temperature (30±2°C) for three consecutive days. The mixture was filtered using a muslin cloth and followed by Whatman No. 1 filter paper. A part of the methanol extract was concentrated using a rotary evaporator and the remainder extract was further partitioned with diethyl ether in a separating funnel. Subsequently the aqueous residue formed from the partitioning was further partitioned in ethyl acetate. Finally, the aqueous residue from the ethyl acetate partitioning was mixed with butanol. From this soaking and partitioning method, four extract preparations were obtained (methanol, diethyl ether, ethyl acetate and butanol extracts) and they were dried until dark paste was formed.

Microorganisms and Cultural Maintenance

Six bacterial species which were obtained from the Industrial Biotechnology Research Laboratory Culture Collection, School of Biological Sciences, University Sains Malaysia were used throughout the study. The bacterial cultures were maintained on nutrient agar slants at 37°C for 24 h. All the cultures were kept at 4°C until further used. Subculturing was done at every four weeks to maintain their survival.

Antibacterial Activity

The screening of the antibacterial activity was done using the disk diffusion method[19]. Bacterial suspensions were prepared by inoculating one loopful of a pure colony into 10.0 mL of sterile distilled water. The inoculum size was standardized by matching its turbidity to the McFarland 0.5 standard which equivalent to $1.5x10^5$ cells per mL.

One milliliter of the suspension was pipette into 15.0 mL of sterilized molten nutrient agar aseptically. The mixtures were mixed well by swirling the plates left and right and then they were left on the bench to solidify. The commercial antibiotic disk GF A (Whatman) with 6.0 mm diameter was used to screen the antibacterial activity. Each of the sterile disk was then impregnated with 20 μL of the extracts, which corresponding to 100.0 mg per mL of the extract stock. Chloramphenicol at the concentration of 30 μg per mL was used as a

positive control. 100% methanol was used as a negative control. All the impregnated disks were air dried before placing them on the agar surface. The plates were incubated at 37°C for 24 h and the antibacterial activity was determined by measuring the diameter of the inhibition zones formed around the disks.

Minimum inhibitory (MIC) and Minimum Bactericidal Concentrations (MBC) Determinations

The MIC values were studied for the microorganisms that were susceptible in the screening test. A serial of 2-fold dilutions of the extract were set up using sterile nutrient broth medium as diluents in 10.0 mL sterile test tubes containing 500 μL of the inoculums to give a final extract concentrations within the range of 0.31-40.00 mg per mL. All the tubes were incubated at 37°C for 24 h. MIC value was determined by comparing the turbidity of the whole tubes with negative control (which was nutrient broth inoculated with bacteria and without extract) and two positive control test tubes (nutrient broth only and nutrient broth with the extract only). The lowest dilution of the tube that showed no visual turbidity was taken as the MIC value. To determine the MBC value, one loopful of inoculums from all the non-turbid tubes was subcultured onto the agar and incubated for 24 h at 37°C. After the incubation period, the plates for each dilution subcultured were examined for colony growth. The lowest concentration at which there was no visible growth was taken as the MBC.

Time killing curves of B. subtilis in the Presence of Methanol Extract of C. sertularioides

Bacterial suspension of *B. subtilis* was prepared as described previously. One milliliter of the methanol extract stock was added into conical flasks containing 23.0 mL of sterilized nutrient broth and 1.0 mL of inoculum. The final concentrations of the extracts in the flasks were at 2.5 mg/mL (half MIC), 5.0 mg/mL (MIC) and 10.0 mg/mL (2 MIC). The control for this experiment was 23.0 mL of nutrient broth added with 1.0 mL of bacterial suspension and 1.0 mL of methanol. Each experiment was carried out in triplicate. All the flasks were incubated in an orbital shaker (Infors HT Ecotron) at 37°C with 150 rpm of agitation speed. One milliliter of the mixture within each flask was withdrawn at every 4 h intervals starting from 0 h until 48 h of incubation. Ten-fold or 100-fold dilutions of the mixture were performed in sterilized distilled water. The spread plate method was carried out and the inoculated plates were incubated at 37°C for 24 h. The number of colonies that grew on the agar plates was counted and only the plates that showed 30-300 colonies were counted as this is a standard protocol in order to get the most accurate reading as well valid statistically. The population growth (CFU/mL) of the bacteria was analyzed by plotting a logarithm of the number of the viable cells versus the incubation times. The generation time of the growth profiles was calculated as described previously[20].

Scanning Electron Microscope (SEM)

The bacterial suspension was prepared as described previously. For each sample, 1.0 mL of the 24 h old bacterial suspension was inoculated in a 50.0 mL conical flask containing 30.0 mL of sterilized nutrient broth and incubated in a shaker at 37°C, 150 rpm for 18 h. The bacterial suspension was then added to the extract stock solution (the final concentration in each flask was at the MIC value) and incubated at the required incubation time (12, 24 and 36 h). As for a negative control, a conical flask containing bacterial suspension was added with 1.0 mL of 100% methanol.

The SEM sample was prepared using the hexamethyldisilazane (HMDS) method[21] (Nation, 1983). The pellet of the bacterial suspension was fixed with McDowell-Trump fixative solution in 0.1 M phosphate buffer pH 7.2, for 2 h to fix the cell original condition. The sample was then resuspended in 1% osmium tetraoxide in phosphate buffer for 1 h, recentrifuged and finally the supernatant was discarded. The pellet was dehydrated using 50%, 75%, 95%, 100% ethanol and HMDS for 10 minutes, consecutively. Centrifugation was performed each time the samples were resuspended and the supernatant was also discarded after each centrifugation. Finally, after the HMDS solution was decanted, the Eppendorf tube containing the cells was left to air-dry in a desiccators at room temperature. The dried cells were then mounted on a specimen holder with a doubled-sided tape. The sample was coated with 5-10 nm of gold palladium alloy and viewed under a scanning electron microscope (Leica Cambridge, S-360, United Kingdom).

Statistical Analysis

The data were analyzed by Student *t*-test for comparing the extract on the tested bacterial vs

Chloramphenicol using Statistical Package for the Social Sciences (SPSS version 12.0) software (SPSS, Chicago, IL, USA). Statistical significance was assumed at the 0.05 levels ($p < 0.05$).

Results

Antibacterial Activity

Table I shows the extraction yield obtained from the partitioning method. From the 20 g of powdered algal sample used, 2.041 g of methanolic extract (10.2%) with dark green colored paste was obtained. Then, 0.901 g of diethyl ether extract (4.5%) with bright green colored paste was obtained. The ethyl acetate and butanol extracts obtained were 0.842 g (4.2%) and 0.787 g (3.9%) with dark yellow and brownish colored pastes, respectively.

The crude extracts of *C. sertularioides* exhibited a wide range of antimicrobial activity against the Gram positive and Gram negative bacteria. As shown in Table II, the methanolic, diethyl ether and ethyl acetate extracts of *C. sertularioides* showed good inhibition zones (9-14 mm) against *B. subtilis* and *Erwinia sp* ($p < 0.05$), whereas the diethyl ether and ethyl acetate extracts showed good inhibition zones against *MRSA*, *A. anitratus and B. cereus* ($p < 0.05$). However, *Yersinia sp.* was only inhibited by the ethyl acetate extract alone.

Effects of C. sertularioides Methanol Extract on the Growth Profile of B. subtilis

From the Figure 1, it is clearly shown that the number of viable cells was significantly reduced in the 2 MIC (10.0 mg per mL) if compared to control, ½ MIC (2.5 mg per mL) and at MIC (5.0 mg per mL) samples. Inhibition of bacterial cells for all samples was not apparent for the first 12 h as they experienced an exponential phase, similar to the control sample. However, after the 12 h of incubation time, the cells in the control samples increased tremendously until 36 h of incubation and then remained constant (which was the stationary phase). The highest colony numbers achieved for control (at the end of exponential phase) was 8.2×10^9 CFU/mL, whereas the highest colony numbers achieved by 1/2 MIC (also at the end of the exponential phase) was 2.2×10^9 CFU/mL. Therefore, the cell growth for 1/2 MIC was reduced than the control. Furthermore, the exponential phase of the control is longer than the 1/2 MIC. As for the concentration of 5 mg per mL (MIC value), the bacteria grew at a very slow rate and did not show distinct growth phases. At the concentration of 10.0 mg per mL (2 MIC value), there was no significant growth observed. The generation time recorded for the control was 41.7 min, whilst the generation time for 1/2 MIC and MIC were 1.03 h and 3.57 h, respectively. Hence, the addition of *C. sertularioides* extract was effective in prolonged the generation time of *B. subtilis*.

Structural Degeneration of the Methanol Extract Treated B. subtilis cells

Figure 2 shows the SEM micrograph of the *B. subtilis* cells after treatment with 5.0 mg per mL or at the MIC value. Figure 2A shows the control cells without any treatment. It shows typical normal cells of *B. subtilis*, with rod-shaped morphology. However, the cell surface became a bit rough and this could be due to the presence of a thin layer of slimy material that covered the whole cell surface. After 12 h of treatment (Figure 2B) there was a distinct formation of thick slimy material that covered the cells but the rod-shaped cells were still intact. Their cell structures were almost the same as in control. After 24 h of treatment (Figure 2C), most of the cells exhibited a very distinct structure compared to control. The formation of shrunken, distorted cells with cavities was observed on majority of the cells. The cells seem to lose their cell wall integrity and this could be due to lost or leakage of the cellular ma-

Table I. Extraction yield of different organic solvents for *C. sertularioides*.

Type of organic solvents	Weight of extract obtained (g) (20.0 g of powdered sample was used in each extraction)	Percentage (%)	Color
Methanol	2.041	10.2	Dark green
Diethyl ether	0.901	4.5	Bright green
Ethyl acetate	0.842	4.2	Dark yellow
Butanol	0.787	3.9	Brownish

Table II. Antimicrobial activities of the crude extract preparations of *C. sertularioides* on food poisoning and diarrhea-caused bacteria.

Microorganisms	ME	DE	EA	BU	C	N (mg/mL)	MIC (mg/mL)	MBC
Gram positive								
Bacillus subtilis	++	++	+	–	++	–	5.0	10.0
Bacillus cereus	–	++	+	–	++	–	5.0	10.0
MRSA	–	+	+	–	+	–	5.0	10.0
Gram negative								
Acinetobacter anitratus	–	++	+	–	++	–	10.0	20.0
Erwinia sp.	+	+	++	–	+++	–	10.0	20.0
Yersinia sp.	–	–	++	–	+++	–	10.0	20.0

Notes: The antimicrobial activity was determined based on the diameter of inhibition zone (mm). The results were recorded according to following scale: +++ = ≥ 15 mm, ++ = 10-14 mm, + = ≤ 9 mm, - = no inhibition zone. ME = Methanolic extract, DE = Diethyl ether extract, EA = Ethyl acetate extract, BU = Butanolic extract, C = Chloramphenicol, N = Methanol.

terials from the cells and therefore, the cells became crumpled and lysis. In fact, some of the cells lost their rod-shaped structure and had irregular shaped. After 36 h of treatment, there was a severe damage occurred to the cells which were in totally collapsed cells. The surface of the cells became crumpled and as shown in Figure 2D, the cavity formed on most of the cells which finally led to cell lysis and death. At this stage, the bacterial cells lost their metabolic activities and the growth of the bacteria was completely inhibited by the extract.

Discussion

The choice of solvent for extractions depends on the intention of studying the extract. Several types of organic solvents with different polarity range were used for extraction in order to obtain a diverse type of possible bioactive compounds. Due to the diversity of marine algae and their habitats, natural products from marine algae encompass a wide variety of chemical classes and the series of natural products can often contains a variety of chemical functionalities[22]. Each

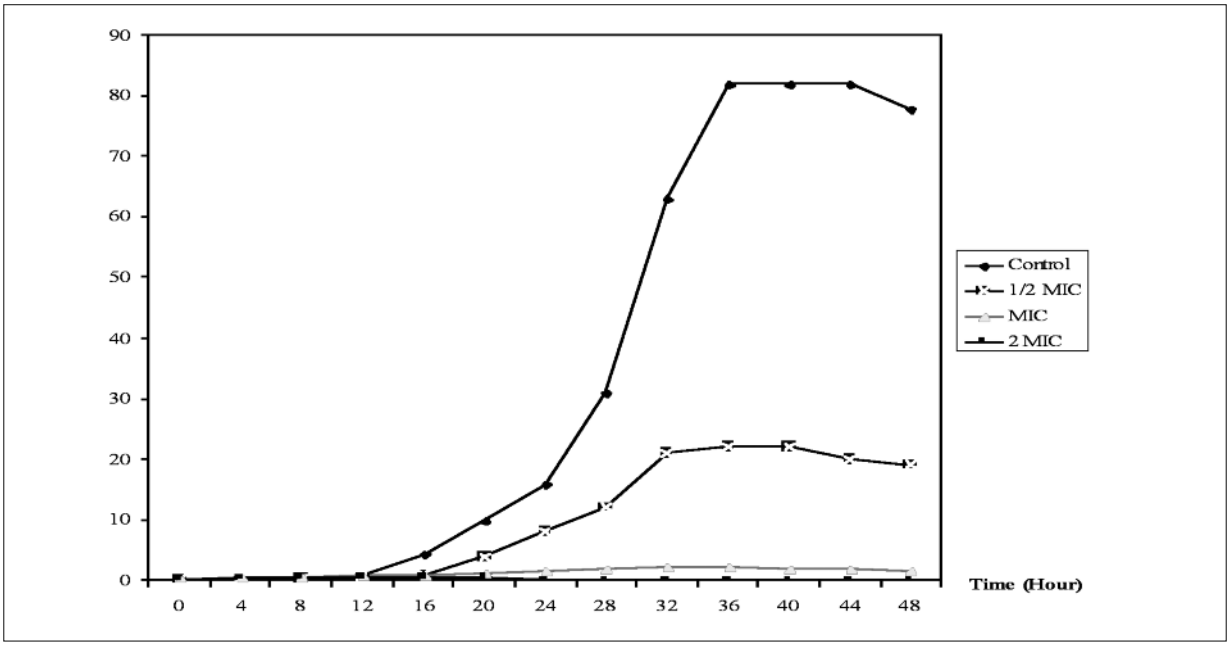

Figure 1. Growth profile for *B. subtilis* in nutrient broth without treatment of extract and 2.5 mg/ml (1/2 MIC), 5.0 mg/ml (MIC) and 10.0 mg/ml (2 MIC) concentration of *C. sertularioides* extract.

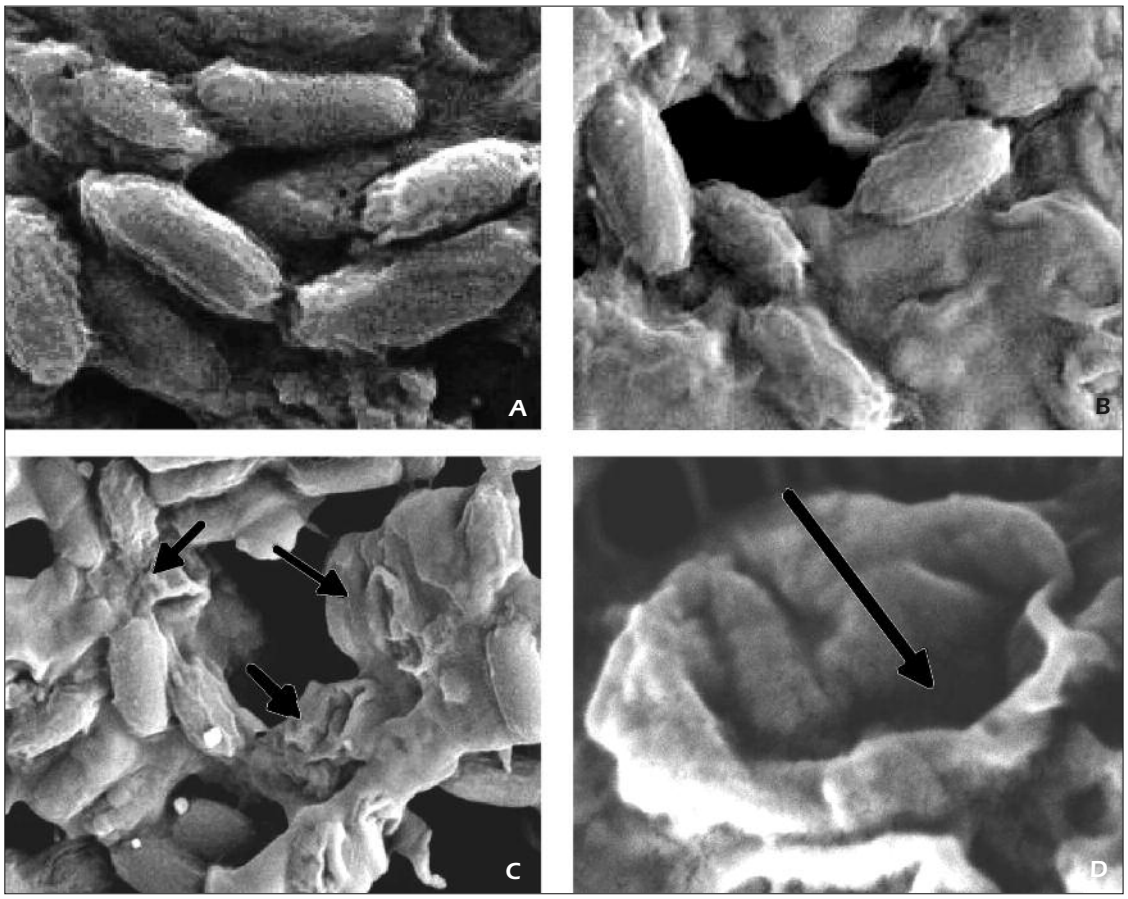

Figure 2. SEM micrograph showing the structural degeneration of the *C. sertularioides* methanol extract on treated *B. subtilis* cells. *Notes:* ***A,*** Control cells without treatment (5,000x). ***B,*** *B. subtilis* treated with extract for 12 hours (5,000×). ***C,*** *B. subtilis* treated with extract for 24 hours (3,000×). ***D,*** *B. subtilis* treated with *C. sertularioides* methanolic extract for 36 hours (35,000×). *The arrows indicated the formation of cavities on the baterial cells.

change in functionality has the potential to change the polarity of the compounds. Kandasamy and Arunachalam[23] stated that the type of organic solvent used for extraction is affecting the presence of bioactive compounds in the crude extracts and affecting its activity.

The antimicrobial test results in our study showed that the antibacterial activity depends on both, the algal and also the efficiency of the solvent as well as the extraction method. Supardy et al[24] for instant found that there was no antibacterial activity showed by the methanolic, diethyl ether and ethyl acetate extracts of *Halimeda discoidea* on *A. anitratus, B. subtilis, B. cereus, Erwinia sp., Yersinia sp.* and *MRSA*. Their findings are in contrast with our findings. Furthermore, many researchers reported their findings in antibacterial activity of marine algae were in methanol extracts[8,12,25]. In this study it was found that some bacterial strains did not respond to some organic extracts whereas other strains showed some activity. Such unusual response could be attributed to masking antibacterial activity by the presence of some inhibitory compounds in the extract. Some researches concerning the effectiveness of extraction methods highlight that methanol extraction yields higher antimicrobial activity than n-hexane[26,27] whilst others reported that chloroform is better than methanol and benzene[28]. It is clear that extraction by organic solvents always provide a higher efficiency for antimicrobial activities as compared to water extracts[29,30]. Moreover, the effects of *C. sertularioides* methanol extract on the growth profile of *B. subtilis* exhibited a dose- and time-dependent pattern. The increase in extract concentration together with longer treatment period was found to be directly proportional to the reduction in the number of viable cells.

Koprivnjak et al[31] suggested that the bacterial cell wall is the main target of the algal extract to attack the cells. This is due to the presence of polyanionic bioactive compounds in most of the plant extract. Generally, the integrity of the cell wall depends on Ca^{2+} and Mg^{2+} ions which ionically link the polysaccharide side-chains. Thus, the interaction between these polyanionic groups and the divalent cations in the bacterial cell wall disrupted the integrity of the cell wall and subsequently causes cell lysis. Blondell et al[32] suggested the interaction between the bioactive compounds with the sialic acid or lipid layer in the microbial cell wall that cause lysis of the cells by affecting the lipid packing in the cell wall. Gram positive bacteria were more susceptible to the *C. sertularioides* extracts compared to Gram negative bacteria. A possible explanation for these observations may be attributed to the significant differences in the outer layers of Gram negative and Gram positive bacteria. Gram negative bacteria possess an outer membrane and a unique periplasmic space which is not found in Gram positive bacteria[33]. The resistance of Gram negative bacteria against antibacterial agent is usually related to the hydrophilic surface of their outer membrane that is rich in lipopolysaccharide material, which presenting a barrier to the penetration of antibacterial compounds. The membrane is also associated with the enzyme in the periplasmic space which are capable of breaking down the antibacterial compounds introduced from out side[34]. The Gram positive bacteria do not have such outer membrane and cell wall[35]. The results suggested that the cells without a normal and sturdy cell wall structure would finally burst and the complete invagination of the cell wall indicated the lost of cellular materials as well as organelle from the cells' cytoplasm[36,37].

Conclusions

The results revealed that the differential antibacterial activities of the *C. sertularioides* extracts may be attributed to the presence of different antibacterial compounds which are easily extracted with organic solvents. However, the active compounds responsible for the antibacterial activities need to be evaluated. Therefore, it is suggested that further works may be carry out on the isolation and identification of the antimicrobial compounds in *C. sertularioides* for its pharmaceutical application.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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