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Research Article

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EFFECTS OF METHANOLIC EXTRACT OF A SOFT SPONGE, *Haliclona* sp. ON BACTERIAL CELLS: STRUCTURAL DEGENERATION STUDY

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ABSTRACT

In-vitro antimicrobial screening of *Haliclona* sp. isolated from Malaysian coastal waters was conducted against a selected of pathogenic bacteria, yeast and fungi. It was found that the methanolic extract of this soft sponge only showed antibacterial activity but not antifungal or antiyeast activities. The MIC and MBC values obtained were 0.5 mg ml^{-1} and 1.0 mg ml^{-1} for MRSA and *Bacillus spizizenii,* respectively, whereas 1.0 mg ml^{-1} and 2.0 mg ml^{-1} for *B. subtilis, B. cereus*, *Acinetobacter anitratus* and *Erwinia* sp., respectively. The effects of the extract on MRSA and *B. subtilis* cells were also studied by means of scanning electron microcopy (SEM) and the results revealed that the extract caused internal shrinkage of the cells and finally completely collapsed the cells after prolonged exposure to it. The time-killed study suggested that the extract significantly inhibited the cell growth and possessed bacteriostatic activity at lower concentration and bactericidal activity at higher concentration.

Keywords: *Haliclona* sp., methanolic extract, antibacterial activity, minimal inhibitory concentration, SEM.

INTRODUCTION

Marine organisms are a rich source of structurally novel and biologically active secondary metabolites and to date many chemically unique compounds with different biological activities have been isolated. Sponges are simple, multicellular, sessile invertebrates with no true tissue layers or organ[1], and are the most primitive invertebrates that are frequently exposed to intense predation as well as tissue infection by microorganisms.

Sponge secondary metabolites obviously present a defensive role against predation, infections and fouling.[2-4] Marine sponges are shown to exhibit antibacterial[5,6], antifungal[6], antiplasmodial[7], antihelminthic[8], antiamoebitic[9,10], antileishmanial[11] as well as anti-inflammatory[12] and anticancer[5] activities. They are also seems to produce the largest number and diversity of secondary metabolites, even though the functions of these secondary metabolites are unknown. There are more than 5000 different compounds have been isolated from about 500 species of sponges[13], and about 800 antibiotic compounds have been isolated from them.[3,14]

Haliclona sp. which belongs to Phylum Porifera, Class Demospongia, Order Haploscleridae and Family Haliclonidae is an animal colonies, sedentary, brownish yellow in color, irregularly rounded, asymmetrical about 4-10 cm, remain attached to sea bottom by means of masses of spicules. These colonies are attached on the dead coral stones in shallow water inter-tidal areas at the depth of 3-6 m. Even though there are many studies done on this species but there are more on the screening and isolation of the compounds, but not many studies done on the bacterial cells after exposure to the extract. As there is not many report on marine soft sponges of Malaysian waters documented, we studied a soft sponge of Malaysia which can be a potential source of new antimicrobial compounds. In this study, we tested the methanolic crude extract of *Haliclona* sp., a marine soft sponge against a series of pathogenic bacteria, yeasts and fungal species. The MIC and MBC values of the extract against selected bacteria were also carried-out and the time-killed curved as well as structural degeneration of the selected bacterial cells were studied.

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MATERIALS AND METHODS

Collection of Material

The fresh sample of *Haliclona* sp. was collected from the rocks off the shoreline of Kera Island, Penang, Malaysia. The samples were collected by hand picking during low tide and filled in a container containing sea water and were transported to the laboratory. Once arrived at the laboratory, the samples were cleaned off the epiphytes and extraneous matter with running tap water using a soft brush. The samples were then rinsed repeatedly with tap water to remove the salt attached on its surface. The cleaning process had to be finished in the shortest time in order to prevent perishness of the samples. The sponge samples were dried at 45°C to a constant weight. The dried samples were grinded into fine powder form using an electrical blender (1-3 min) and kept in a sealed plastic bag to prevent deterioration.

Extraction

Twenty grams of dried powdered form *Haliclona* sp. was soaked in 400 ml of methanol (Sigma, USA) for 72 h at

room temperature (28±2°C) in a fume cabinet. The mixture was stirred from time to time twice a day during the soaking process to ensure homogeneity. The mixture was then filtered through a muslin cloth that was folded in to several layers followed by filtration using Whatman no. 1 filter paper, to remove suspended particles in the mixture. The filtrate was then concentrated to dryness under reduced pressure using a rotary evaporator at 50°C and 200 rpm before left air-dried in a fume cabinet until a dark colored paste obtained. The methanolic extract of *Haliclona* sp. was kept at 4°C until further used.

Test Microorganisms

Twelve bacterial strains (five Gram positive and seven Gram negative bacteria), two yeasts and six fungal species (Table **1**) were used in this study. The bacterial cultures were subcultured on fresh nutrient agar (NA) slants and incubated at 37°C for 24 h, whereas the yeasts and fungal cultures were subcultured on potato dextrose agar (PDA) slants and incubated at 37°C for 24-48 h for yeasts and at 30°C for 24-72 h for fungal cultures. The cultures were kept at 4°C prior to use.

Disc Diffusion Technique

The disc diffusion technique was carried as described previously by[15] with slight modifications. Briefly, 1.0 ml of bacterial suspension ($1.0x10^5$ cells ml^{-1}) was added into 15.0 ml sterilized molten NA, whilst yeast suspension ($1.0x10^3$ cells ml^{-1}) or fungal spore suspension ($1x10^6$ spores ml^{-1}) were added into 15.0 ml sterilized molten PDA. The mixtures were poured into sterile Petri plates and swirled slowly to ensure the uniformity of the mixtures. The agar plates were left to solidify at room temperature for 15-20 min. Then, the disc impregnated with 20 µl of the methanolic extract of *Haliclona* sp. corresponding to 100 mg ml^{-1} (2 mg $disc^{-1}$) were placed onto the surface of the seeded NA and PDA plates. Chloramphenicol (30 µg ml^{-1}) and ketoconazole (30 µg ml^{-1}) were used as positive control for the bacterial and yeast/fungal respectively. A disc impregnated with 20 µl of methanol solvent was used as negative control. The bacterial and yeasts plates were incubated at 37°C for 24 h and 24-48 h, respectively, whereas the fungal plates were incubated at 30°C for 24-72 h. All the experiments were carried out in triplicates. The antimicrobial activity was determined by measuring the clear inhibition zones formed around the discs.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of the extract was determined by broth dilution assay as previously described by[16,17]. Only the bacterial cultures that showed inhibition zones were proceeded for the MIC determination. A serial two fold dilutions of the extracts were set up in sterile screw cap test tubes containing 0.5 ml of the bacterial inoculums, 1.5 ml of NB and 1.0 ml of extract that gave the final extract concentrations tested within the range of 0.04 to 2.00 mg ml^{-1}. All the tubes were incubated at 37°C for 24h. The experiments were carried out in triplicate. MIC value was determined by comparing the turbidity of the tubes to negative controls (NB inoculated with bacteria only without the extract; NB alone; NB with extract alone). The lowest dilution of the tube that showed no visual turbidity was taken as the MIC value.

Determination of Minimum Bactericidal Concentration (MBC)

MBC of the extract was determined as described by[16,17], where one loopful of the inoculums from each of the non-turbid tubes was streaked on to NA plate and incubated at 37°C for 24 h. The lowest concentration at which there was no visible growth was regarded as the MBC value.

Time-Kill Study

In order to assess the antibacterial activity with MIC, 0.5x MIC and 2x MIC concentrations over time, growth profile curves were plotted. Two bacterial strains (*B. subtilis* and MRSA) were tested in this study. The 16 h bacterial cells grown on NA plates were suspended in 10 ml of sterile distilled water and the turbidity of the bacterial suspension was compared with 0.5 McFarland standard solutions ($1.0x10^8$ cell ml^{-1}) and was adjusted to $1.0x10^5$ cell ml^{-1}. Then, 1.0 ml of inocula and 1.0 ml of extract was added to aliquots of 25 ml NB in a 50 ml Erlenmeyer flask that give the final concentrations of 0.5x MIC, MIC and 2x MIC. The final concentrations of extract tested for *B. subtilis* are 0.50 mg ml^{-1} (0.5x MIC), 1.0 mg ml^{-1} (MIC) and 2.0 mg ml^{-1} (2x MIC). Meanwhile for MRSA, the final concentrations of extract tested are 0.25 mg ml^{-1} (0.5x MIC), 0.50 mg ml^{-1} (MIC) and 1.0 mg ml^{-1} (2x MIC). A flask without the addition of extract (untreated, only inocula) served as control. Finally, 1.0 ml portion was placed on NA plates and incubated at 37°C for 24 hours. The growth of bacteria was counted and expressed as colony forming unit (cfu/ml). The growth of bacteria was measured every 4 h for 48 h continuously by the above method.

Scanning Electron Microscope (SEM) Observations

SEM observations were carried out on the treated bacterial cells. One ml of the bacterial cell suspension at the concentration of $1x10^5$ cells ml^{-1} was inoculated on Nutrient agar plates and were incubated at 37°C for 24 h. Two milliliter of the extract (at a concentration of MICs, 0.5 mg ml^{-1} for MRSA and 1.0 mg ml^{-1} for *B. subtilis*) was then pipetted on to the inoculated agar and was further incubated for another 12, 24 and 36 h at the same incubation temperature. An inoculated Nutrient agar plate without the addition of extract act as control cells (0 h). A small block of bacteria (1.0 cm^3) containing agar was cut and withdrawn from the inoculated plate at 0, 12, 24 and 36 h, and was fixed for SEM observation.[18] The prepared samples were observed under the SEM (Fesem Leo Supra 50 VP, Carl Zeiss, Germany).

RESULTS AND DISCUSSION

Antimicrobial Activity of the Extract

The results of the antimicrobial activity of the methanolic extract of *Haliclona* sp. against a series of bacterial, yeasts and fungal cultures are shown in Table **1**. Out of 12 tested bacteria, only six of them (four Gram positive and two Gram negative) were inhibited by the methanolic extract of *Haliclona* sp. The Gram positive bacteria that were inhibited by the extract were Methicillin-resistant *Staphylococcous aures* (MRSA), *Bacillus spizizenni, B. subtilis* and *B. cereus*, meanwhile the Gram negative were *Erwinia* sp. and *Acinetobacter anitratus*. The remaining six bacteria together with all the yeast and fungal isolates showed no inhibitory sign. The present results revealed that the methanolic extract of *Haliclona* sp. only exhibited antibacterial activity on the prokaryotic but not on the eukaryotic cells. The extract showed better antibacterial activity against the Gram positive as compared to the Gram negative bacteria.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Table **2** shows the MIC and MBC values of the extract

against six bacterial cultures. The MIC values for MRSA and *B. spizizenii* were 0.5 mg ml^{-1}, whereas for *B. subtilis, B. cereus, A. anitratus* and *Erwinia* sp. were 1.0 mg ml^{-1}. The MBC values for MRSA and *B. spizizenii* were 1.0 mg ml^{-1}, whereas for *B. subtilis, B. cereus, A. anitratus* and *Erwinia* sp. were 2.0 mg ml^{-1}.

Table 1. The antimicrobial activity* of the methanolic crude extract of *Haliclona* sp. on bacteria, fungi and yeasts using disc diffusion agar method.

Microorganism	ME	C	K	M
Gram-positive bacteria				
Bacillus cereus	+	+++	NT	-
Bacillus spizizenni	++	+++	NT	-
Bacillus subtilis	+	+++	NT	-
Methicillin resistant *Staphylococcus aureus*	++	+++	NT	-
Gram-negative bacteria			NT	-
Acinetobacter anitratus	+	+++	NT	-
Citrobacter freundii	-	+++	NT	-
Escherichia coli	-	+++	NT	-
Erwinia sp.	+	+++	NT	-
Klebsiella pneumoniae	-	+++	NT	-
Pseudomonas aeruginosa	-	+++	NT	-
Yersinia sp.	-	+++	NT	-
Fungi				
Aspergillus niger	-	NT	++	-
Microsporum gypseum	-	NT	++	-
Penicillium sp.	-	NT	++	-
Rhizopus sp.	-	NT	++	-
Trichoderma viride	-	NT	++	-
Trichophyton rubrum	-	NT	++	-
Yeasts				
Candida albicans	-	NT	++	-
Candida utilis	-	NT	++	-

**The antimicrobial activity was determined based on the diameter of inhibition zone measured in mm. The results for the screening test were recorded according to the following scale: (+++) ≥ 18 mm, (++) = 11-17, (+) ≤ 10mm, (-) = no antimicrobial activity, NT = not tested; (ME) = Methanolic extract – 100 mg ml^{-1}, (C)= Chloramphenicol – 30 μg ml^{-1}; positive control for bacteria, (K)= Ketoconazole – 30 μg ml^{-1}; positive control for fungi and yeast, (M) = Methanol (negative control)*

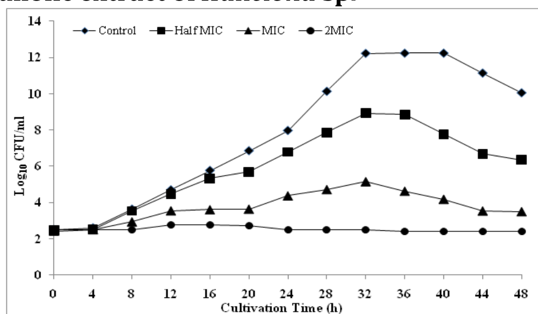
Table 2. MIC and MBC values of the methanolic crude extract of *Haliclona* sp. on test bacteria.

Bacterial strains	MIC (mg ml^{-1})	MBC (mg ml^{-1})
Gram-positive		
MRSA	0.5	1.0
B. spizizenii	0.5	1.0
B. subtilis	1.0	2.0
B. cereus	1.0	2.0
Gram-negative		
A. anitratus	1.0	2.0
Erwinia sp.	1.0	2.0

The Effects of Addition of Methanolic Extract of *Haliclona* sp. on the Growth of Bacteria

In this study, the growth of two bacterial species (MRSA and *B. subtilis*) were evaluated. Figure **1** shows the growth profile of MRSA, without the addition of the extract, the addition of extract at the 0.5x MIC (0.25 mg ml^{-1}), at the MIC (0.5 mg ml^{-1}) and at the 2x MIC (1.0 mg ml^{-1}) values.

Figure 1. Time killing profile for MRSA by the methanolic extract of *Haliclona* sp.



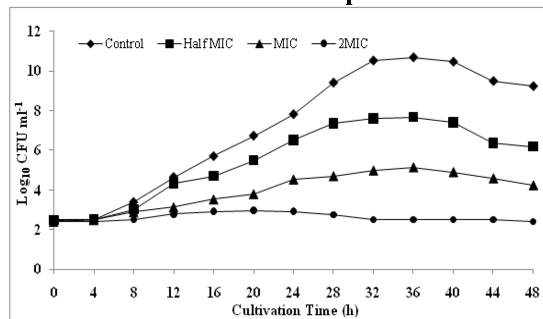
**Concentration: 0.25 mg ml^{-1} (Half MIC), 0.50 mg ml^{-1} (MIC) and 1.00 mg ml^{-1} (2MIC)*

Generally, the growth of MRSA cells without the addition of the extract exhibited a normal growth curve pattern with lag, log (exponential), stationary and death phases. After the addition of the extract at 0.5x MIC value (0.25 mg ml^{-1}), the growth pattern of the MRSA cells were almost the same as the control but with lesser colony forming unit (CFUs) number. The maximum number of CFU at the end of exponential phase (32 hours of cultivation time) for control was 1.35x10^{10} CFU ml^{-1} while for half MIC, the number was reduced to 8.4x10^{8} CFU ml^{-1}. The results also revealed that the number of MRSA cells formed was lower and the exponential period was shorter for half MIC as compared to the control. At the concentration of MIC (0.5 mg ml^{-1}), the MRSA cells growth was inhibited significantly by the extract and there was no distinct exponential phase. At the concentration of twice MIC (1.0 mg ml^{-1}), the growth curve showed almost a stagnant line with no significant growth of the cells throughout the incubation period.

In addition, the generation times of the exponential phase for the growth profiles were determined. By definition, generation time is the period of time required for a bacterium to perform one round of binary fission process to produce two identical bacterial cells. The generation time recorded for the control (without the addition of the extract) was 34.2 min whereas for the half MIC and at the MIC values were 1.13 h and 2.21 h, respectively. Thus, the addition of the methanolic extract of *Haliclona* sp. was effectively prolonged the generation time of MRSA cells.

Figure **2** shows the growth of *B. subtilis* without the addition of the methanolic extract of *Haliclona* sp. and also with the addition of the extract at the concentration of half MIC (0.50 mg ml^{-1}), at MIC (1.0 mg ml^{-1}) and at twice MIC (2.0 mg ml^{-1}).

Figure 2. Time killing profile for *B. subtilis* by the methanolic extract of *Haliclona* sp.



**Concentration: of 0.50 mg ml^{-1}(Half MIC), 1.00 mg ml^{-1} (MIC) and 2.00 mg ml^{-1} (2MIC)*

The growth pattern of *B. subtilis* was almost the same as shown by the MRSA, where both the growth of control and half MIC samples showed the normal growth patterns with distinct phases of lag, log (exponential), stationary and death. However, the number of the cells produced at half MIC was lower than the control, in which can be detected through the CFU value recorded at the end of the exponential phase. The recorded CFU for control was 1.08x10^{10} CFU ml^{-1} while CFU at 0.5x MIC value was 6.0x10^{8} CFU ml^{-1}. At the concentration of MIC (1.0 mg ml^{-1}), the growth of *B. subtilis* cells was low with no distinct growth phases detected. At the concentration of twice MIC (2.0 mg ml^{-1}), there was no sign of growth phase at all. The number of CFU was constant from the 0 hour up until the end of the cultivation time, indicating that the extract at 2.0 mg ml^{-1} inhibited the cell growth. The generation time for *B. subtilis* at control (without the addition of extract) was 38.4 min, whereas for 0.5x MIC and at the MIC were 1.09 h and 2.08 h, respectively.

Effect of the Methanolic Extract of *Haliclona* sp. on the Cell of MRSA and *B. subtilis*

The effects of the extract on structures and morphology of bacterial cells were studied by observing the treated MRSA and *B. subtilis* cells with the aid of SEM and TEM micrographs. Figure **3** shows the effect of extract at the concentration of 100 mg ml^{-1} on the MRSA cells.

Figure 3. SEM micrographs of the MRSA cells that were treated with methanolic extract of *Haliclona* sp. at 100 mg ml^{-1} but at different exposure time (a) control (untreated) cells, (b) at 12 h (c) at 24 h, (d) at 36 h of exposure to the extract.

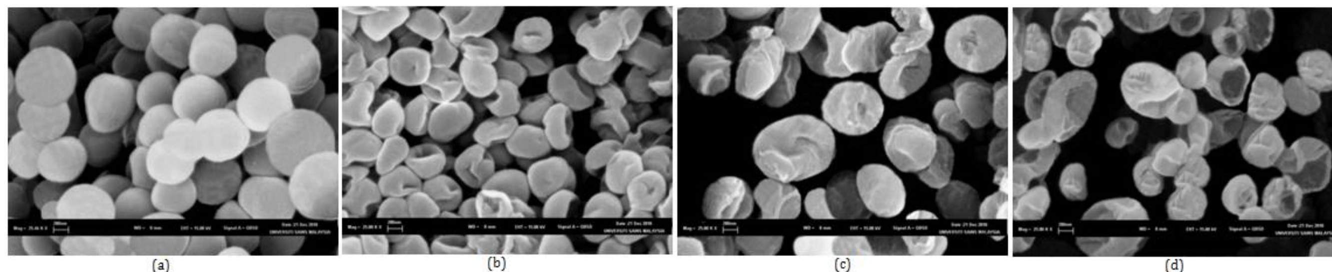
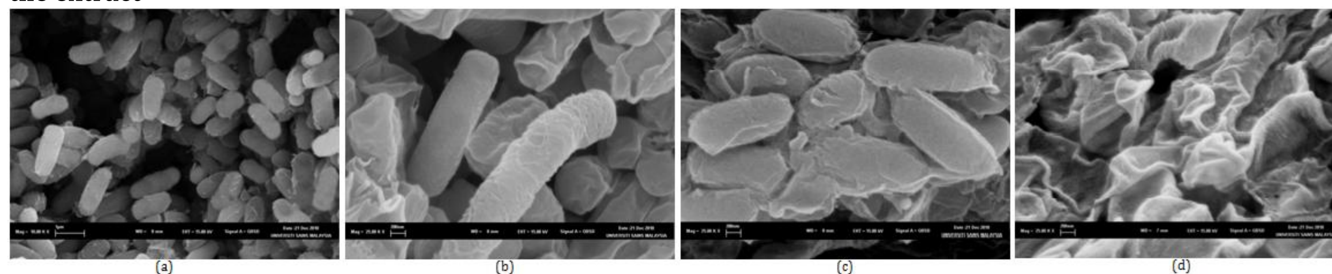


Figure **3a** shows the untreated cells with typical coccal-shaped cells. The surface of the cells was smooth without any mucous layer on them. However, progressive changes were observed over the period of exposure. At 12 h of exposure (Figure **3b**), the surface of the cells were crumpled and shrunk. This condition was then followed by the subsequent formation of cavities or pores on the surface of the cells after 24 h of exposure (Figure **3c**). These changes were suspected to be due to the lost of cellular materials from the cells and thus caused some of the cells lost their coccal-shaped and finally exhibited indistinctive shapes as compared to the control cells. After 36 h of exposure, the cells were totally damaged and collapsed (Figure **3d**) to the extent of losing their original coccal-shaped.

The observations of the effects of the methanolic extract of *Haliclona* sp. on *B. subtilis* morphologies were also carried-out (Figure **4**). The control cells (without treatment with the extract) showed typical cell of *B. subtilis* with rod-shaped cells and smooth surfaces (Figure **4a**). After 12 h of exposure (Figure **4b**) the bacterial surface became irregular and shrunken. This condition became more severe after 24 h of exposure (Figure **4c**), where majority of the cells collapsed and lost their cell shaped. After 36 h of exposure (Figure **4d**), the cells exhibited significant morphological changes with the formation of collapsed cells. At this stage, most of the cells had lost their ability to function normally and eventually unable to resume their growth. Finally, only dead cells were observed and this condition was beyond repair.

Figure 4. SEM micrographs of the *B. subtilis* cells that was treated with methanolic extract of *Haliclona* sp. at 100 mg ml^{-1} but at different exposure time (a) control (untreated) cells, (b) at 12 h (c) at 24 h, (d) at 36 h of exposure to the extract



Drug from marine resources is an area which offers an unprecedented opportunity for their pharmacological explorations and hence has received great attentions within these recent years in the natural product chemistry study. Secondary metabolites that are produced by the marine organisms might be the source of highly potential bioactive compounds that are useful in modeling the new drugs. Marine microorganisms with immense genetic and biochemical diversities are only beginning likely to become a rich source of novel chemical entities for the discovery of more effective drugs.

Our results clearly showed that the methanolic extract of *Haliclona* sp. exhibited appreciable antimicrobial activity against pathogenic bacteria, but not against any of the tested yeasts and fungi. The overall antimicrobial results against various types of bacteria infer that the extract does contain secondary metabolites that posses a wide range of antibacterial activity. The usage of antibiotic disc susceptibility tests or disc diffusion assays has the ability to rapidly identify the activity of the active metabolites that present within the extract and therefore is particularly useful in the preliminary screening for the antimicrobial activity. However, since this assay measures cell death or inhibition of cell growth, the absence of antimicrobial activity in laboratory assays does not necessarily indicate a lack of antimicrobial chemical defense. Chemicals produced by higher organisms against microorganisms may not simply kill or inhibit the growth of the target microorganisms, but can also act selectively against particular phenotypes or characteristics that are expressed by the bacteria. The results of this work were in agreement with the findings reported by several researchers. Lippert *et al.*[19] and Volk and Kock[20] reported the antibacterial activity of *Haliclona* sp. collected from cold water regions such as *H. viscosa* which was collected from Norway. An aqueous extract of *H. rosea* from Mediterranean also showed antibacterial activity against *E. coli*.[21] *H. exigua* was reported to have bis-1-oxaquinolizidine alkaloids which exhibiting diverse biological properties such as antimicrobial, antifungal, antituberculosis and cytotoxicity activities[22]. Then, Orabi *et al.*[23] reported of their success in isolating and elucidating Araguspongine C belonging to bis-1-oxaquinolizidine type of alkaloids.

Even though antifungal compounds have been isolated from *Haliclona* spp., but from this present study there was no activity observed, and this could be due to the low therapeutic efficacy on the fungi which due to their

exceptionally strong cell wall structure that consists of chitin, -α and β–glucan[24], which cannot be perturbed by the extract or the extract itself that is lacking with compounds that easily target and destroy the cell wall. However, Fahy *et al.*[25] found that *Haliclona* extract was active against a pathogenic yeast, *Candida albicans*, whereas Richelle-Maurer *et al.*[26] reported that a sphingosine derivative isolated from *Haliclona* sp., exerted activity against a common yeast strain, *Saccharomyces cerevisiae*. However, Lippert *et al.*[24] did not detect the antimicrobial activity from *H. rosea* extract. Detected or not detected of the activity is due to many factors, including two different strains of test microorganisms and/or different experimental procedures.

Many antimicrobial screening studies have shown that Gram positive bacteria are more sensitive than Gram negative bacteria and fungi. In fact, Burkholder[27] found that 35% of the 777 species of sponges collected from the Carribean Sea showed significant activity against Gram positive bacteria, besides 15% had activity against Gram negative bacteria and 10% had activity against yeast, *Candida* sp. This is in agreement with our results, where the extract mainly inhibited the Gram positive bacteria.

The susceptibility of the microorganisms on antibiotic depends on the mechanism of action of the compound and also on the differences of the cell wall structure of both groups of bacteria. The less susceptibility of the Gram negative bacteria against the extract could be due to the presence of an outer membrane surrounding the cell wall, which can hinder the access of active compound through its lipopolysaccharide layer, proteins and phospholipids that serve as outer membrane barrier.[28] This particular feature might prevent the accessibility of any outside substance in to the cell, including the antibiotics.[29] On the other hand, Gram positive bacteria is lacking of the barrier because they only have a thick layer of peptidoglycan but none of the outer membrane. Thus, these conditions somehow increase the bacteria susceptibility towards the foreign substances. Even though the mode of action of the extract against the MRSA and *B. subtilis* cells were not studied, but it can be hypothesized that the effects of the extract were exerted on the outer membrane of the cell wall which then altered the membrane structure and permeability of the cell. The changes of the membrane structure might be resulted from the breakage of the hydrogen bonds that functions in keeping the rigidity of the membrane.[30] This assumption is coincided well with the findings reported by Sasidharan *et al.*[31] who found that the extract of marine macro algae exert their inhibitory effect on the cell wall of the bacterial cells, *Pseudomonas aeruginosa* which eventually led to complete destruction of the bacterial cells. Shai[32] and Zhao *et al.*[30] reported that the mechanism of actions involved in the killing of bacterial cells is mainly focused on the interactions between antibiotic or antimicrobial compounds and the cell membrane.

Resistance to antibiotic is emerging in a wide variety of microorganisms and multiple drugs resistant microorganism pose a serious threat to the treatment of infectious disease especially in the population of hospitalized immune-compromised patients. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions have to be taken to reduce this problem by controlling the use of antibiotic, developing research to better understand the genetic mechanism of resistance and continuing studies toward the discovery of new effective antibiotics, either synthetic or natural.

CONCLUSION

With an increasing number of bacteria developing resistance to commercial antibiotic, MRSA for instance, extract from *Haliclona* sp. hold great promise for novel medicine in modern times. It is apparent that *Haliclona* sp. is an interesting source for biologically active compounds that may be applied as antibacterial agents.

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