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EVALUATION OF ANTIBACTERIAL ASSAYS FOR SCREENING OF MARINE INVERTEBRATE EXTRACTS

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Abstract

Marine environment continuously produces pharmacologically active compounds. To screen marine natural products as a source of potential antibiotics, it is very important to select an accurate and efficient antibacterial assay. This study aims to find out the best assay for screening of antibacterial activity of marine invertebrate extracts by comparing the performance of 3 methods, i.e. the colorimetric resazurin microtiter assay (REMA), disc diffusion assay and spectrophotometric microdilution assay (SMA). Five marine invertebrate extracts, i.e. *Stylissa* sp., *Theonella* sp., *Lobophytum* sp., *Sarcophyton* sp., and *Aaptos* sp., were tested using those 3 different methods. The best method obtained was then further tested for its performance to screen 126 marine invertebrate extracts against 2 bacterial strains i.e. *Escherichia coli* (ATCC®25922™) and *Staphylococcus aureus* (ATCC®25923™). The study showed that, resazurin microtiter assay (REMA) was simpler, produced quicker result and able to generate reliable data for antimicrobial activity screening compared to the other methods. Moreover, REMA assay is regarded as a suitable assay platform to be implemented for screening of marine invertebrate extracts antibacterial activity since it requires only a small amount of extracts. Based on the MIC values, amongst 126 marine invertebrate extracts screened, 59; 36; 25 and 6 extracts tested against *E. coli* and 69; 36; 18; and 3 extracts tested against *S. aureus* were respectively categorized as not active, moderately potential, potential and very potential sample worth to be analyzed further. The ability of the REMA to generate accurate MIC value, which is comparable to the existing antibiotic drug MIC value, will empower researchers to decide whether the extracts worth to be examined further or not.

Keywords: antibacterial activity, marine invertebrate extract, REMA, resazurin indicator

1. Introduction

Natural products have been an important source for the development of drugs, including antibacterial drugs (Martins, Vieira, Gaspar, & Santos, 2014). However, in the late of 20th century, many pharmaceutical companies abandoned their natural product discovery programs (Shen, 2015) which resulted in substantial decline of new leads for drug development pipeline (Li & Vederas, 2009). In terms of antibiotic, only seven new active substances that had been approved for bacterial infection therapy in the last 10 years (Brown, Lister, & May-Dracka, 2014).

Since the discovery of the first antibiotic, penicillin, by Alexander Fleming in 1940's, thousands of structurally different antibacterial compounds were reported (Huang & Lin, 2017). Up till now, antibiotics play an important role in fighting various types of microbial infections. However, reports on incidents of bacterial resistance against existing antibiotics are significantly increased (Allison, Higginson, & Martin, 2017; Dickey, Cheung, & Otto, 2017). The high incidence of bacterial resistance forced many researchers in the world to revisit nature as a source of novel chemical with antibacterial activity (Moloney, 2016).

Nature, including marine environment, continuously produces pharmacologically active compounds. To discover marine natural products as a source of potential antibiotics, it is very important to select a standardized, reliable, simple, fast yet inexpensive antibacterial assay. More importantly, due to the small amount of marine natural product extracts, particularly for purified compound, we have

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to consider the most efficient method of antibacterial assay for screening the extracts.

Several bioassays such as well-diffusion, disk-diffusion, and agar or broth dilution are commonly used. Moreover, several colorimetric redox-indicator methods for antibacterial assay have also been reported (Al-Bakri & Afifi, 2006; Balouiri, Sadiki, & Ibnsouda, 2016; Monteiro et al., 2012; Sarker, Nahar, & Kumarasamy, 2007). One of the most recommended colorimetric indicators is resazurin that has been reported to be excellent indicator for antibacterial screening for phytochemicals (Karuppusamy & Rajasekaran, 2009; Sarker et al., 2007), for tuberculosis drug susceptibility testing (Katawera, Siedner, & Boum, 2014), for determination of minimum inhibitory concentration of biosurfactants (Elshikh et al., 2016) and many other reports.

The aim of this study is to find out the best assay for screening of antibacterial activity of marine invertebrate extracts by comparing the performance of 3 methods, i.e. the colorimetric resazurin microtiter assay (REMA), disc diffusion assay and spectrophotometric microdilution assay.

2. Material and Methods

Five marine invertebrate extracts, i.e. *Stylissa* sp., *Theonella* sp., *Lobophytum* sp., *Sarcophyton* sp., and *Aaptos* sp., were tested using 3 different antibacterial assay methods, i.e. the colorimetric resazurin microtiter assay (REMA), disc diffusion assay and spectrophotometric microdilution assay (SMA). The best method obtained was then further tested for its performance to screen 126 marine invertebrate extracts against 2 bacterial strains i.e. *Escherichia coli* (ATCC®25922™) and *Staphylococcus aureus* (ATCC®25923™).

2.1. Sample Collection

We collected 126 marine invertebrate specimens i.e. sponges, soft corals and ascidians from Kapoposang Island, Spermonde Archipelago, Indonesia. The specimens were collected from the Kapoposang marine park reefs (118°54' 00 E – 119° 10' 00'' E and 04°37'00'' S–04°52' 00'' S) at the depth range of 5-10 m on March 2015. Some of the specimens were identified based on Coral Finder Indo Pacific book (Kelley & Society, 2012). 20 g of each specimen was extracted with 10 mL of ethanol. The samples were then kept in a cool box containing crushed ice for safe transport to the laboratory. The macerated specimens were then stored in -20 °C until further analysis. The specimen voucher of each sample was lodged in the Biotechnology Laboratory of the Research Development Center for Marine and Fisheries Product Processing and Biotechnology, Jakarta, Indonesia.

2.2. Marine Invertebrate Extract Preparation

The ethanolic extract of each marine invertebrate sample (macerated specimen) was centrifuged (Beckman Coulter) at 5.000 g for 10 minutes to remove the unwanted debris. Five hundred (500) µL of the supernatant was then concentrated in a vacuum concentrator (Chemoscience). The completely dried ethanolic extract was then used for the bioactivity study. The crude ethanolic extract was dissolved in DMSO at concentration of 100 mg/mL stock and stored in -20 °C. Each of the extracts was tested in triplicates.

2.3. Antibacterial Assay

2.3.1. Medium preparation

Mueller Hinton (MH) broth and agar medium (Oxoid) was prepared as packaging instruction. For MH broth, 21 g of the medium dissolved with 1 L of distilled water. Thus, the 3.3 x strength of MH broth medium for the SMA and REMA assay was prepared by dissolving 69.3 g of the medium in 1 L of distilled water. The medium was then sterilized by autoclaving (Hirayama) at 121 °C for 15 minutes.

2.3.2. Bacterial culture preparation

A single colony of *Escherichia coli* (ATCC®25922™) and *Staphylococcus aureus* (ATCC®25923™) was transferred into a 100 mL of Mueller Hinton broth medium aseptically. The bacteria were then incubated overnight in incubator (Memmert) at 37 °C. Before starting the antibacterial assay, the microbial inoculum was prepared by adjusting the microbial suspension to 0.5 McFarland scale to obtain a concentration of 2×10^8 cfu/mL.

2.3.3. Resazurin solution preparation

The resazurin (Sigma-Aldrich) stock solution was prepared as 10 g/L in sterile water and saved frozen at -20 °C. Before starting the REMA assay, the resazurin stock solution was thawed and the resazurin working solution was prepared by diluting the resazurin stock 1:10 in sterile water.

2.3.4. REMA assay

The REMA assay was conducted according to Sarker et al. (2007) with some modifications. The

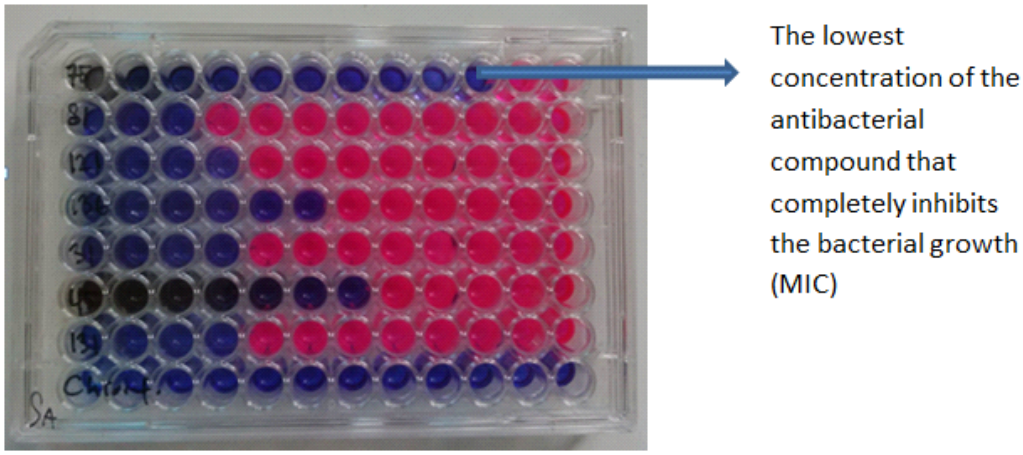

Figure 1. Broth microdilution method using resazurin as indicator. Viable cells will metabolically reduce the blue color-resazurin into pink color-resorufin.

resazurin microtiter assay was aseptically performed in 96-well plates. Serially, two-fold dilution (50 µL) of each extracts was prepared in the wells. The first well was filled with the highest concentration of the tested sample, which was 20 mg/mL. That highest concentration was then serially two-fold diluted with sterile water or normal saline (50 µL). Each of samples was serially diluted in 1 row of microplate (11 times of two-fold dilution). Ten µL of resazurin indicator solution was added into each well. Thirty µL of 3.3 x strength of MH medium was then added to get the final volume of the MH medium in each well was 1x. Finally, 10 µL of bacterial suspension (2 x 10^8 cfu/mL) was added to each of the wells. With the lid attached, the plates were then incubated in incubator at 37 °C for 24 hours. The color change was then visually assessed. If the resazurin color (blue) is not changed into pink or colorless indicating that the bacteria was dead, however if the color changed from blue to pink indicating that the bacteria was still alive. Thus, the lowest concentration at which the resazurin color did not change was recorded as the minimum inhibitory concentration (MIC). In each plate, a negative control (all solutions without test compound) and a broad spectrum antibiotic, i.e. chloramphenicol, as a positive control were prepared. The test was performed in triplicates.

2.3.5. Broth microdilution assay/Spectrophotometer microdilution assay (SMA)

The broth microdilution assay or spectrophotometer microdilution assay (SMA) was aseptically performed in 96-well plates according to Wiegand, Hilpert, and Hancock (2008) with modification. Serially, two-fold dilution (50 µL) of each extracts was prepared in the wells. The first well was filled with the highest concentration of the tested sample, which was 20 mg/mL. That highest concentration was then serially two-fold diluted with sterile water or normal saline (50 µL). Each of samples was serially diluted in 1 row of microplate (11 times of two-fold dilution). Ten µL of sterile water was added into each well. Thirty µL of 3.3 x strength of MH medium was then added to get the final volume of the MH medium in each well was 1x. Finally, 10 µL of bacterial suspension (10^8 cfu/mL) was added to each of the wells. The absorbance at 625 nm wavelength was measured (Thermo Scientific) pre- and post-incubation at 37 °C for 24 hours. The MIC values were determined as the lowest concentration where no increment of post- and pre-incubation absorbance readings (absorbance increment that lower than 0.05). Chloramphenicol was used as a positive control. To ensure that the media was capable of supporting the microbial growth, a negative control (comprise of medium and test organism without test sample) was prepared. The sterility control well which comprise of medium without test sample and test organism was also prepared. The test was performed in triplicates.

2.3.6. Agar disc diffusion assay

The agar disc diffusion assay was conducted according to Hudzicki (2009) with some modification. The bacterial inoculums were standardized to 0.5 McFarland standard. The standardized inoculum was then swabbed into the sterile MHA with sterile cotton swabs. Six mm sterile paper discs were impregnated with 20 µL of various concentrations (10000; 5000; and 2500 mg/L) of marine invertebrate extracts. The paper discs were then aseptically and distinctively placed on the inoculated MHA plates. The plates were

then incubated at 37 °C for 18 hours. The inhibition zones (clear zones) that were formed were measured. Chloramphenicol was used as a positive control. The test was performed in triplicates.

3. Results and Discussion

The diffusions based methods are the most known and commonly used antibacterial method since it is simple and inexpensive. The presence of inhibition zones in the disc diffusion assay of *Stylissa* sp., *Theonella* sp., and *Aaptos* sp. showed that those extracts elicited antibacterial activity (Table 1). However, this method has some weaknesses. First, since it is not possible to quantify the amount of the antibacterial agent diffused into the agar medium, the diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC). The MIC value is the lowest concentration of the antibacterial compound that completely inhibits the bacterial growth (Jorgensen & Ferraro, 2009). Secondly, one of the critical steps in this assay which is measuring the clear zone diameters using a ruler or caliper with an unaided eye may result in misreading the result. Therefore, this diffusion based method is apparently less suitable to be used as a screening method for antibacterial activity of marine invertebrate extracts.

The other methods, the dilution based antibacterial methods, are also simple and low cost antibacterial assays. The assays can be used to estimate the concentration of antibacterial compound tested in the medium and produce quantitative result (Jorgensen & Ferraro, 2009). Thus, dilution based methods are considered as the most appropriate antibacterial assays for the MIC values determination.

There are several dilution based antibacterial assays that are commonly used by many laboratories. Those methods are either using agar or broth as the medium and in terms of assay volume; there are macro- or micro dilutions methods. The macro-dilution is done in tubes containing minimum volume of 2 mL, while microdilution is a miniaturization of the macro-dilution using 96-wells plate platform for the assay. Since we have to consider the small amount of marine natural product extracts, particularly for purified compounds, micro-dilution method is considered more suitable assay platform to be implemented in the marine natural products screening program. In addition to that, the microdilution method needs fewer quantities of reagents and tools compare to the macro-dilution. For that reason, the method is more economical compare to other methods.

The MIC end point of the micro-dilution method was obtained by examining the lowest concentration

Table 1. Antibacterial activities of ethanol marine invertebrate extracts by means of disc diffusion assay against *Escherichia coli* (ATCC®25922™)

Marine Invertebrate Extracts	Test Concentration per disc (mg/L)	Inhibition Zones (mm)
Stylissa sp.	10000	7.43 ± 0.49
	5000	0
	2500	0
Theonella sp.	10000	7.67 ± 0.06
	5000	7.03 ± 0.86
	2500	0
Lobophytum sp.	10000	0
	5000	0
	2500	0
Sarcophyton sp.	10000	0
	5000	0
	2500	0
Aaptos sp.	10000	6.77 ± 0.72
	5000	6.53 ± 0.38
	2500	0
Chloramphenicol	1000	25.6 ± 0.75

of the antibacterial compound tested that prevents the appearance of the bacteria growth under a defined time period of incubation. The growth of the bacteria is marked by the turbidity which could be visible by an unaided eye. However, the turbidity of the growth bacteria may be distorted by the color of the extracts (marine natural products) tested. Therefore, we need to measure the bacterial growth using spectrophotometer at 600 or 625 nm or we can use dye reagent as an indicator to facilitate the reading of the micro-dilution antibacterial assay result. Several dye reagents have already been used for development of the colorimetric methods, including 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2Htetrazolium-hydroxide} (XTT) (Tunney & Ramage, 2004), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Malekinejad, Bazargani-Gilani, Tukmechi, & Ebrahimi, 2012) and resazurin (Sarker et al., 2007).

In this present study, two ways of broth microdilution methods were used to test the antibacterial activity of 5 marine invertebrate extracts, i.e SMA & REMA. According to Andrews (2001), the expected MIC value of chloramphenicol against *E. coli*

Table 2. Minimum inhibitory concentration (MIC) values of 5 marine invertebrate extracts tested with (SMA) and resazurin-colorimetric microtiter assay (REMA) against *E. coli* (ATCC®25922™)

Marine Invertebrate Extracts	MIC values (mg/L)	
	Spectrophotometric Microdilution Assay (SMA)	Colorimetric Resazurin Microtiter Assay (REMA)
Stylissa sp.	1250	625
Theonella sp.	312.5	156.25
Lobophytum sp.	No activity observed	2500
Sarcophyton sp.	5000	5000
Aaptos sp.	625	416.6
Chloramphenicol	3.9	3.9

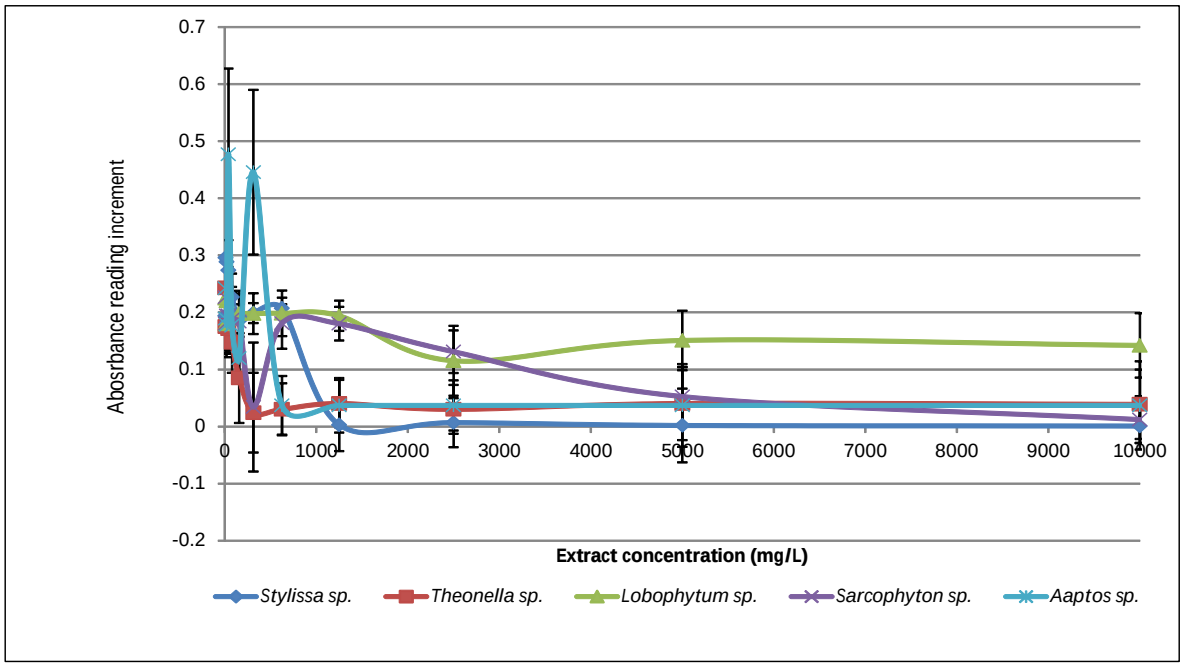

Figure 2. Absorbance reading (at 625 nm wavelength) increment of *E. coli* (ATCC®25922™) treated with 5 marine invertebrate extracts at different concentrations. The MIC values were determined as the lowest concentration where no increment of post- and pre-incubation absorbance readings (absorbance increment that lower than 0.05).

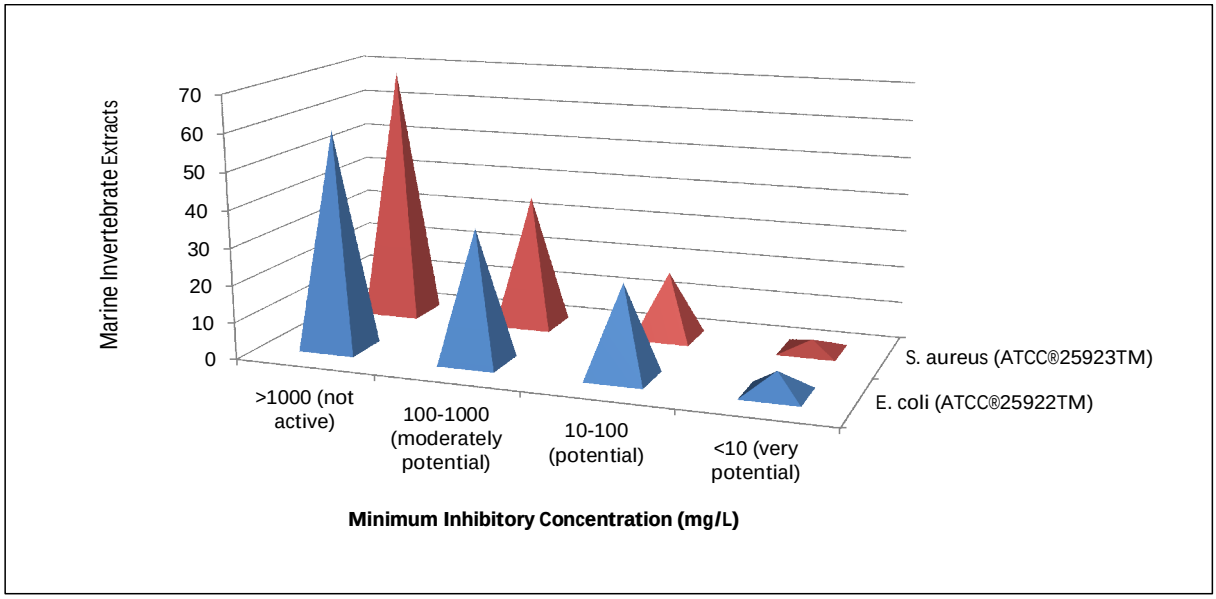

Figure 3. Minimum inhibitory concentration (MIC) obtained for 126 marine invertebrate extracts tested against *E. coli* (ATCC®25922™) and *S. aureus* (ATCC®25923™) using colorimetric resazurin microtiter assay.

is 4 mg/L. The result showed that both assays produced chloramphenicol MIC value against E.coli of 3,9 mg/L. It suggested that both assay are sensitive to test the antimicrobial activity (Table 2).

Resazurin is a blue colored redox dye that is non-toxic to cells and stable in the culture medium. Viable cells will metabolically reduce (by oxidoreductases) the blue color-resazurin into pink color-resorufin and finally into colorless hydroresorufin (Balouiri et al., 2016). The REMA assay has an advantage in terms of the result can be acquired right away after the incubation with an unaided eye, compared to the SMA. Hence, REMA assay was considered simpler and produced quicker result to effectively screen the antibacterial activity of marine natural products. REMA assay is very convenient for high throughput antibacterial screening of marine natural products.

The result of this study is also in accordance with previous studies. Resazurin has been reported to be an excellent indicator for tuberculosis drug susceptibility testing (Katawera et al., 2014), also for determination of minimum inhibitory concentration of biosurfactants (Elshikh et al., 2016). Resazurin has also been used for decades to discover yeast and bacterial contamination of milk products (Sarker et al., 2007). The effectiveness of the resazurin-based antibacterial assay to screen methanolic extracts of Scottish plants (Sarker et al., 2007), medicinal plants of Western Ghats (Karuppusamy & Rajasekaran, 2009), larval extract of fly (Teh, Nazni, Nurulhusna, Norazah, & Lee, 2017) has also been reported.

We tested the effectiveness of REMA to screen antibacterial activity of 126 marine invertebrate extracts against 2 bacterial strains, *i.e. E. coli* (ATCC®25922™) and *S. aureus* (ATCC®25923™). Our study showed that REMA assay was able to screen the antibacterial activity of the marine natural product extracts quite easily. Extracts with potential antibacterial compound killed the tested bacterial, indicated by the unchanged color of resazurin in the well. On the contrary, extracts without antibacterial activity kept the bacteria viable. The viable bacteria was then metabolically reduced the blue resazurin into the pink resorufin. The color changed is easily visualized through unaided eye. Thus, this antibacterial assay is very simple, relatively cheap and able to produce data within short time (less than 48 hours).

The ability of the REMA assay to generate accurate MIC value, which is comparable to the MIC value of the existing antibiotic drug, will empower researchers to decide whether the extracts worth to be examined further or not. In this study, we used chloramphenicol, a broad spectrum antibiotic, as a positive control. The MIC value of chloramphenicol against *E. coli* and *S. aureus* is 3.9 mg/L. Therefore, in terms of antimicrobial potential, we categorized an extract with MIC value of >1000 mg/L, 100-1000 mg/L, 10-100 mg/L and <10 mg/L respectively as not active, moderately potential, potential and very potential samples worth pursuing further analysis. The data (Figure 2) showed that amongst 126 marine invertebrate extracts screened, 59; 36; 25 and 6 extracts were respectively categorized

as not active, moderately potential, potential and very potential sample against *E. coli*, a Gram negative bacteria. While against *S. aureus*, a Gram positive bacteria, 69; 36; 18; and 3 extracts were respectively categorized as not active, moderately potential, potential and very potential sample.

4. Conclusion

Compared to disc diffusion assay and spectrophotometric microdilution assay, resazurin microtiter plate-based assay was simpler and produced quicker result for antibacterial activity screening. More importantly, the assay was also able to generate a reliable result and only required a small amount of extracts. Thus, the assay was considered as more suitable assay platform to be implemented in the marine natural products, especially marine invertebrate extracts screening program. The ability of the REMA assay to generate accurate MIC value, which is comparable to the existing antibiotic drug MIC value will empower researchers to decide whether the extracts worth to be examined further or not.

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