Journal of Sustainability Science and Management 2010 Volume 5 (1): 116-124

ANTI-BACTERIAL ACTIVITY OF THREE SPECIES OF SEA URCHIN EXTRACTS FROM PULAU BIDONG, TERENGGANU

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Abstract: Sea urchins have been known to be among the main threats to the sea-bed community, especially to the reefs. Sea urchins are important grazers in most marine benthic sublittoral communities, where the species composition and sea urchin population directly indicates the health status of the sea-bed community in terms of pollution aspects. Sea urchin study has been conducted to ascertain the anti-bacterial compound in three respective sea urchin species such as *Diadema setosum*, *D. savignyi* and *Echinomatrix calamaris* collected from the sea-bed of Pulau Bidong, Terengganu. This study consisted of utilising three kinds of solvent for extraction method such as methanol, ethanol and phosphate buffer solution (PBS) for water-soluble substance extraction. Inner tissues and outer layers of each sea urchin species were subjected to be extracted. Negative results occurred for PBS extraction method in all extract samples. However, for inner-tissues extraction method, ethanol and methanol solvent exhibits positive results for inhibitory effects against several test strains of Gram-positive bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and Gram-negative bacteria such as *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Escherichia coli*. Methanol solvent solely exhibits positive inhibitory effects against two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus cereus* for outer-layer extraction method of three sea urchins species. Methanol and ethanol solvent-extraction methods for inner tissues of two sea urchin species, *D. setosum* and *D. savignyi* gave promising results against the test strains. These results revealed that the sea urchins' extracts of Pulau Bidong, Terengganu have great potential in developing anti-bacterial compounds.

KEYWORDS: Anti-bacterial activity, sea urchin, Pulau Bidong

Introduction

Many researches have been published involving sea urchins in the past few decades. The sensitivity of sea urchins' eggs have given ways for researchers around the world to measure the toxicity of sewage within the treatment process and has enhanced the suitability of sea urchin application to assess the environmental impact of marine fishcage farming by carrying out larval toxicity bioassay using the sea urchin, *Paracetrotus lividus* (Dinnel and Stober, 1987). Many toxicity bioassays have been done using several types of sea urchin for regulatory and monitoring purposes, and this marine organism is assumed to be a good indicator of ecological damage to benthic infaunal communities (Marin *et al.*, 2007). Different approaches have been taken for screening the pharmaceutical and medical values. Anti-bacterial activity in the green sea urchin, *Strongylocentrotus droebachiensis* was carried out and a potential source for the discovery of novel antibiotics has been derived (Haug *et al.*, 2002). For this reason, several species of sea urchins found on the sea-bed of Pulau Bidong, Terengganu were collected for preliminary study to ascertain any potential of anti-bacterial compounds.

Materials and Methods

Sample Preparation. Sea urchins were collected along the sea-bed of Pulau Bidong, Terengganu (05°37.29'N 103°03.36E) during the night. The animals were sorted according to their species and the spines of the sea urchins were cut off and kept in labelled plastic bags. Samples were then kept in -30 °C for further used in the laboratory. The exo-skeletons were separated from the inner parts which were then homogenised for extraction.

Sample Extraction. Extractions were carried out using three types of solvent-extraction methods such as methanol, ethanol and Phosphate-buffer solutions (PBS). Extraction using methanol was performed according to Shimada (1969). 50 ml of concentrated methanol was added to 25g of homogenized wet tissue. The extracts were incubated for 72 hours at room temperature and centrifuged at 4000 rpm for five minutes. The supernatant was then filtered through a 0.45µm GF/F (/Whatman, England). A rotary evaporator was used to concentrate the extracts at 40 °C. Ethanol extraction was carried out with the improvised method of Shimada (1969), as mentioned above. Extraction using PBS was carried out according to Yasumoto *et al.*, (1967), with the same amount of samples as mentioned above. Methanol was replaced with PBS as a solution for extraction. The extracts were centrifuged at 4000 rpm for five minutes and the supernatants were collected for further centrifugation at 6000 rpm for ten minutes due to high viscosity. Then, the supernatants were transferred and concentrated using rotary evaporator at 40 °C. The concentrated extracts were filtered through a 0.45 µm GF/F (Whatman, England) prior to analysis.

Anti-bacterial bioassay. Eight test strains of Gram-positive and Gram-negative bacteria were used in this study, such as *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis, Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Klebsiella pneumonia,* respectively. Anti-bacterial activities were carried out using Kirby-Bauer anti-microbial method (Bauer *et al,*. 1966). Anti-bacterial activities were determined for each extract using the disc-diffusion test technique. The discs of extracts were prepared as mentioned by Khosravi and Behzadi (2006) with some modification. Sterile 6 mm paper discs (Whatman, England) were impregnated with 10 µl of the sea urchin stock extracts (50 mg/ml) according to species and extraction solvents. The discs were air-dried before being applied on the surface of agar plates which had already been swabbed with test strains of bacteria. The bacteria concentration used was 10^8 $CFUml^{-1}$ as the bacteria suspensions were compared to 0.5 McFarland standards (Kronvall and Holst, 2000). The plates were incubated at 35 °C for 48 hours. The positive controls used were Gentamicin and Penicillin (Oxoid, England) as well as methanol, ethanol (Merck, Germany) and PBS as negative controls. Anti-bacterial activity was evaluated by measuring the diameter of inhibition zone (mm) against the test strains.

Results

The three species of sea urchins collected from Pulau Bidong, Terengganu were identified as *Diadema savignyi, D. setosum* and *Echinothrix calamaris* based on their morphology (Coppard and Campbell, 2006). Anti-bacterial activities of these extracts were qualitatively assessed by the absence or presence of inhibition zone. Observation of inhibitory effect for inner-tissues extracts were recorded in Table 1 and as well as the outer layers in Table 2.

The results for control factors observed were recorded in Table 3. Results revealed that anti-bacterial activities of PBS extracts for both internal tissues and exo-skeleton for all three species of sea urchins showed no inhibitory effects against all tested pathogenic strains. Extracts of inner tissues from *E. calamaris* were able to inhibit the growth of *S. aureus* and did not show any positive

effect on other strains of bacteria. *D. setosum* showed positive results on two Gram-negative and one Gram-positive bacteria, *P. aeruginosa*, *E. coli* and *S. aureus* respectively. *D. savignyi* inhibited three Gram-negative bacteria and one Gram-positive bacterium, which are *S. aureus*, *P. aeruginosa*, *E. coli* and *S. typhimurium*. In comparisons of three species of sea urchins extracts, *E. calamaris* did not reveal any positive results on all eight bacteria test strains.

Results of extracts from outer layer of all three sea urchins using different solvents were different from the inner tissues. Extraction of all three sea urchins' outer layers, which are skeletons, using methanol extraction revealed inhibitory effects against *B. cereus*, and *D. setosum;* outer layer extract also gave positive result against *S. aureus.* There were no positive effects on other tested bacteria. Extraction solvents used as negative controls showed negative result toward all eight bacteria, with no inhibition zone determined. Positive control revealed zones inhibition range from 8 to 30 mm**.** All tested bacteria revealed positive result except for Gram-negative, *P. aeruginosa* and Gram-positive *S. aureus* which did not showed any inhibitory effect toward Penicillin and Gentamicin respectively.

Discussion

Inoculums preparations were the most important step in any susceptibility test. More than one colony was isolated to minimise the possibility of testing a colony that might have been derived from a susceptible mutant (Connie and George, 1995). The elimination of the organic solvents used in the process of extraction such as methanol and ethanol, by evaporating with the aid of rotary evaporator avoided false positive results. The species and strains of microorganisms studied are one of the parameters that should be considered when running the anti-bacterial screening test. The analyses were conducted three times to obtain significant results as the experiment conducted could be influenced by many different external factors such as inoculum concentration, extract concentration and media thickness. Extraction of inner part showed more promising activity compared to extraction from outer layer. Results indicated that different parts of the body gave different activities. This might be due to diffirent distribution of bacteria residue in the marine organisms. Besides that, results obtained from outer layer showed inhibition only against Gram-negative bacteria. Extraction using PBS and ethanol for outer layer also did not reveal any positive effect. Inner tissues, in contrast showed higher potential of antimicrobial activity against five out of eight bacteria strains tested (Table 1). Bacteria that showed sensitivities to extract were *E. coli*, *P. aeruginosa*, *S. typhimurium, B. cereus*, *S. aureus* and *E. faecalis*. This indicated the methanolic and ethanolic extracts have broad-spectrum activities with both group of bacteria.

Results from the extraction made by PBS for all the three species from both the outer layer and inner tissues exhibited no inhibition results. Both ethanol and methanol showed significantly good results against Gram-positive bacterium *S. aureus* with a mean inhibition diameter of 12.67 ± 1.53 mm (Table 1).

Gram-positive bacteria were proven to be more sensitive towards the extracts compared to Gram-negative bacteria (Grierson and Afolayan, 1999; Safaeian *et al.*, 2009). The different sensitivities among those two groups of bacteria could be related to the morphological difference between those microbes. Gram-negative bacteria contain outer membranes carrying lipopolysaccharide components, which makes the cell wall impermeable to lipophilic solution while Gram-positive bacteria have only an outer peptidoglycan layer which is not an effective permeability barrier (Arias *et al.*, 2004). This indicated the capability of methanol and ethanol extracts of *D. setosum* and *D. savignyi* to inhibit bacteria, and therefore prove the presence of antimicrobial compounds. No activities were observed against *K. pneumonia* and *B. subtilis,*. This may be due to

the ability of *K. pneumonia* and *B. subtilis* to form highly-resistant resting stages called endospores, which caused them to be least sensitive against the extracts if compared with the other test strains (Tilmann *et al.*, 2003).

The difference in term of zones of inhibition possesed by each of the bacteria, show that not all agents have the same effect, and the technique selected must be appropriate for a specific situation as suggested by Cappuccino and Sherman (2001). There are four major sites in the bacterial cell that are sufficiently different from the human cell that they serve as the basis for the action of clinically-effective drugs; cell wall, ribosome, nucleic acids and cell membranes. Besides that, there are four major mechanisms that mediate bacterial resistance to drugs. First, bacteria produce enzymes that inactive the drug. Second, bacteria synthesise modified targets against which the drugs have no effects. Third, bacteria decrease their permeability such that an effective intracellular concentration of the drug is not achieved and fourth, bacteria actively export drugs using a 'multidrug resistances pump' (MDR pump) (Levinson *et al.*, 2000). These can differ the activities from different treatments used to each of the specific bacteria. The differences in susceptibility among bacteria also may be explained by the different cell-wall composition and the inheritance genes on plasmids of the antimicrobial compounds which can be easily transferred among the bacteria types tested (Karamen *et al.*, 2003). There is abundant evidence that crude organic extracts of marine invertebrates exhibit anti-bacterial activity against medically-important bacteria (Faulkner, 2000). The invertebrates are potentially vulnerable to microbial infection, which may have led to the evolution of chemical defense. In many invertebrate species, several kinds of immune-related humoral activities have been reported by Philippe (1998), but it can vary between each species. The presence of anti-bacterial effects from *D. setosum* and *D. savignyi* could be clearly observed and there were no activities from *E. calamaris*, with their effect different by extraction solvents and even tested bacteria. This suggests that the anti-bacterial activity could be due to different classes of compounds. This could be attributed to both physical environmental and biological factors, extremes of temperature, nutrient deficiency, perpetual threat from predators, microbial pathogen and competition for limited resources faced by organisms. Potential roles of these chemical defenses include protection against invasion, settlement by other invertebrates and predation (Dyrynda *et al.*, 1995).

Overall, sea urchins' extract that revealed positive effects showed weak antimicrobial activity compared with positive control. The level of activities that were measured in the disc-diffusion bioassay is dependent on both the rate of diffusion of extract onto agar and the potency of the extract itself. An extremely potent extract with a slow diffusion bioassay rate will appear to have a low level of activity in the bioassay (Esther, 1997). Since the condition of evaluation for extract effectiveness was similar for all the bacteria used, the difference in bacterial response was possible due to the nature of the latter extracts. It might be possible that the non-active compounds in the extract interfered with the active ones so inhibition activities were not present, so the diameter of zones inhibition observed compared to positive control are low.

Conclusion

Inner tissues extracts of methanol and ethanol exhibited promising results of anti-bacterial activities against several types of Gram-positive and Gram-negative bacteria such as *S. aureus, E. faecalis, S. typhimurium*, *P. aeruginosa* and *E. coli*. As for the extraction method for outer layers, methanol solvent was suitable as it showed an inhibition zone against Gram-positive bacteria, *B. cereus* and *S. aureus*. These results revealed that the sea urchins' extracts of Pulau Bidong, Terengganu have great potential in developing anti-bacterial compounds in the future.

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Table 1.1: Inhibition effect of three sea urchins' inner-tissue extracts in different solvents.

Bacteria	**Inhibition zone diameter of test discs (mm)**								
	PBS			**Ethanol**			**Methanol**		
	D. savignyi	***D. setosum***	***E. calamaris***	***D. savignyi***	***D. setosum***	***E. calamaris***	***D. savignyi***	***D. setosum***	***E. calamaris***
Gram-negative bacteria									
K. pneumonia	-	-	-	-	-	-	-	-	-
S. typhimurium	-	-	-	**8.67 ± 0.58**	**10.00 ± 0.00**	-	**9.33 ± 0.58**	-	-
P. aeruginosa	-	-	-	-	**8.33 ± 1.53**	-	**8.67 ± 1.53**	**9.67 ± 1.15**	-
E. coli	-	-	-	**7.33 ± 0.58**	**7.67 ± 1.15**	-	**8.33 ± 0.58**	**8.33 ± 0.58**	-
Gram-positive bacteria									
B. cereus	-	-	-	-	-	-	-	-	-
S. aureus	-	-	-	**12.67 ± 1.53**	**7.67 ± 1.15**	-	**9.00 ± 0.00**	**9.33 ± 0.58**	**8 ± 1.00**
E. faecalis	-	-	-	**9 .00 ± 0.00**	**7.67 ± 1.15**	-	-	-	-
B. subtilis	-	-	-	-	-	-	-	-	-

Abbreviations: -, no anti-bacterial activity; ±, standard deviation, as the data shown were mean value of three replicates; PBS, phosphate buffer solution.

Table 2: Inhibition effects of three sea urchins' outer-layer extracts in different solvents.

Bacteria	**Inhibition zone diameter of test discs (mm)**								
	PBS			**Ethanol**			**Methanol**		
	D. savignyi	***D. setosum***	***E. calamaris***	***D. savignyi***	***D. setosum***	***E. calamaris***	***D. savignyi***	***D. setosum***	***E. calamaris***
Gram-negative bacteria									
K. pneumonia	-	-	-	-	-	-	-	-	-
S. typhimurium	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-	-	-
Gram-positive bacteria									
B. cereus	-	-	-	-	-	-	**7.67 ± 1.15**	**11.00 ± 1.00**	**9.67 ± 0.58**
S. aureus	-	-	-	-	-	-	-	**8.33 ± 1.53**	-
E. faecalis	-	-	-	-	-	-	-	-	-
B. subtilis	-	-	-	-	-	-	-	-	-

Abbreviations: -, no anti-bacterial activity; ±, standard deviation, as the data shown were mean value of three replicates; PBS, phosphate buffer solution.

Table 3: Inhibition effects of positive control (anti-biotic discs) and negative control (extraction solvents) on bacteria test strains

Bacteria	Inhibition zone diameter (mm)				
	Positive Control		Negative Control		
	Gentamicin (10 µg)	Penicillin (10 µg)	Methanol (10 µL)	Ethanol (10 µL)	PBS (10 µL)
Gram-negative bacteria					
K. pneumonia	**20**	**8**	**-**	**-**	**-**
S. typhimurium	**22**	**19**	**-**	**-**	**-**
P. aeruginosa	**21**	**-**	**-**	**-**	**-**
E. coli	**29**	**13**	**-**	**-**	**-**
Gram-positive bacteria					
B. cereus	**18**	**15**	**-**	**-**	**-**
S. aureus	**-**	**19**	**-**	**-**	**-**
E. faecalis	**20**	**23**	**-**	**-**	**-**
B subtilis	**30**	**13**	**-**	**-**	**-**

Abbreviations: -, no anti-bacterial activity; PBS, phosphate buffer solution.