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Evaluating the Antibacterial Activity and *in vivo* Assay of Methanolic Extract of *Stichopus badionotus*

^{1,3}N.S. Mariana, ^{1,3}M.A. Norfarrah, ¹K.A.N.I. Nik, ^{2,3}F.M. Yusoff and ³A. Arshad

¹Faculty of Medicine and Health Sciences,

²Faculty of Science,

³Institute of Bioscience, Universiti Putra Malaysia 43400 Serdang, Selangor, Malaysia

Abstract: This study investigated the antibacterial activity of the methanolic extract of the animal to justify its use in traditional medicine. Antimicrobial activity was assayed by disc diffusion method and broth macro dilution method. From the result it appeared that the methanolic extract of *Stichopus badionotus* displayed antibacterial activities against *Staphylococcus aureus*, three non resistant strains and three multiple resistant strains. The Minimum Inhibitory Concentration (MIC) of the extract against non resistant strain values were 3.75 mg mL⁻¹ and for resistant strain values 7.50 mg mL⁻¹. Further more, this extract tested on rats in wound infection model justified faster healing rates compared to antibiotics. These results indicate that the traditional use of these holothurians for the treatment of *S. aureus* infection mainly on resistant strains should be elucidate to bring out the potential antibacterial agent.

Key words: Antibacterial agent, antibacterial screening, marine natural product

INTRODUCTION

Marine organisms are new alternative sources for antibiotics searches that are invaluable bio prospecting resources for features that have values for commercial development such as pharmaceutical uses. In Malaysia, marine organism such as sea cucumbers, commonly known as gamat, are traditional remedies for healing various internal and external wounds. However, the scientific evidence to support the practice is not properly documented. The research interest in sea cucumbers as a source of pharmacological agents was initiated by experiments on traditional remedy for asthma, hypertension, rheumatism, sinus, cuts and burns, those practice should be analyze scientifically. The compound produce by the sea cucumber can be exploited in drug discovery combating multi resistant bacterial especially *S. aureus* infection diseases. Diseases caused by *S. aureus* become more deadly as the bacteria acquire new virulence factors, develop resistance to modern drug therapy, thus requiring new sources of antimicrobial agents. Clinicians are continually being challenge by infection of *S. aureus*, a major caused of community acquired and health care associated infection around the world. Methicillin resistance first appeared among nosocomial isolates of *S. aureus* in 1961, since that

Methicillin Resistant *S. aureus* (MRSA) has become wide spread in hospitals and intensive care unit around the world (Zetola *et al.*, 2005). Centers for Diseases Control and Prevention's (CDC) National Nosocomial Infection Surveillance System (NNIS) reported among the patients in intensive care units, the proportion of *S. aureus* nosocomial infection that were MRSA surpass 50% in 1999 (Fridkin *et al.*, 2001). MRSA is now one of the most common causes of bacterial nosocomial infection, accounting for 40 to 70% of *S. aureus* infection in intensive care units (Zetola *et al.*, 2005). The high cure rates claimed by folk on wound healing using sea cucumber, stimulated interest in investigating the antibacterial agents in the methanolic extract of sea cucumber and to determine the application on experiments of rats wound infection model.

MATERIALS AND METHODS

Animals material: The samples of *Stichopus badionotus* were collected from Teluk Kemang, Port Dickson, Malaysia in Mei 2007. Animals were identified at the Universiti Putra Malaysia, Institute of Bioscience, in Marine Science Laboratory.

Extraction: The animal was first washed thoroughly and visceral organs were removed before dried in oven at

40°C for 2 weeks and dried blend to fine powder. The fine powder weight 50 g was soaked in 1 L methanol and left stirred for 48 h. Extract was filtered through Whatman No.1 filter paper and the filtrate evaporated at 40°C using rotary evaporator.

Microorganisms: The microorganism used in this study was *S. aureus*, resistant and non resistant strains. This bacterium was purchased from American Type Culture Collection, clinical samples were collected from hospitals; University Malaya Medical Center, Hospital Tunku Ampuan Afzan, Kuantan and Hospital Miri, Sarawak, Malaysia. The bacterial isolates were grown at 37°C and maintained on Nutrient Agar (Oxoid, UK) plate and glycerol stock culture.

Antibacterial activity: The susceptibility test was performed by disc diffusion method as recommended by National Committee for Clinical Laboratory Standard (1993) with slight modifications. Stock solutions of the extract were prepared in water at concentration of 300 mg mL⁻¹. The inocula of microorganism were prepared from exponential phase old broth cultures. The absorbance was read at 550 nm and adjusted with the same sterile medium used to grow the bacterium to match 0.5 McFarland Standard Solution. Mueller Hinton Agar (MHA) (Oxoid, UK) plate was lawned with bacterial suspension evenly. Disc of 6 mm diameter previously impregnated with 10 µL of stock solution of extracts were placed aseptically on the solid plates and then incubated at 37°C for 24 h. The susceptibility was recorded by measuring the clear zone of growth inhibition on agar surface around the discs. All the experiments were carried out in triplicates, vancomycin 30 µg, penicillin 10 µg and water were used as control.

Determination of the Minimum Inhibitory Concentration (MIC): MIC was determined by broth macro dilution with slight modification from the one described by Tamakou *et al.* (2008). The two fold serial dilutions in concentration of the extract (25 – 0.78 mg mL⁻¹) were prepared in Mueller Hinton Broth (MHB) (Oxoid, UK). In general, five tubes were prepared by dispensing 490 µL of MHB and 500 µL of test extract and the dilution was done in two fold. After the dilution was completed, inocula of 10 µL bacterial were dispensed in each tubes. The content of each tube was mixed thoroughly with pipette and incubated in a shaker incubator at 37°C for 8 h. The suspensions were then plated out on MHA (Merck) and incubated again for 24 h at 37°C. The MIC was the lowest concentration of the test substance that inhibited the bacterial growth. All the experiments were performed in triplicates. Vancomycin and penicillin at the concentration

ranging between 40-1.75 µg mL⁻¹ served as positive controls for antibacterial activities, respectively.

Wound infection model: Sprague Dawley was obtained and maintained in the animal house of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The animals were acclimatized before experimental sessions. The studies were approved by the local ethical committee. Total of 18 animals were used in this experiment, three replicate per treatments. The rats were first injured using scalpel and infected with MRSA with inoculums sizes 100 µL of 0.5 McFarland. After 24 h incubation the treatments were given accordingly depending on MICs of antibiotics for a single prior treatments done in prior. The treatment given twice a day by swabbing the treatment drugs was prepared accordingly on the wound. After the treatment, pictures were taken and the wound sizes measured and documented.

Statistical analysis: The clear zones diameters of disc diffusion test and the minimal inhibitory concentration of the antibacterial agents were expressed as the Mean±SD.

RESULTS

Antibiotic sensitivity of Methicillin Resistant *S. aureus* (MRSA) strains showed resistance to penicillin, while Methicillin Sensitive *S. aureus* (MSSA) strains were sensitive to all antibiotics tested. The disc of methanol extract from the tissues of *Stichopus badionotus* showed clear zones on eight isolates of MRSA and MSSA. The strong activity were found on MSSA strains ranging from 4.0 to 6.0 mm, while on MRSA the clear zones range between 5.0 to 6.0 mm (Table 1). The MIC of antibiotics of vancomycin against MRSA was 2.5 µg mL⁻¹ while against MSSA was 1.75 µg mL⁻¹. The MIC of antibiotic of penicillin against MRSA was 5 µg mL⁻¹ while against MSSA was 2.5 µg mL⁻¹. MIC of methanolic extract of *Stichopus badionotus* against MRSA strains were 7.50 mg mL⁻¹, higher than the extract against the MSSA strains MIC was 3.50 mg mL⁻¹ (Table 2).

Experiments continued with *in vivo* assay whereby the establishments of staphylococcal infection were confirmed after 24 h incubation by observation of pus formation on the wound exposed to MRSA and MSSA and bacterial count. Comparison between the MSSA and MRSA infection wound treated with antibiotics penicillin, ampicillin and methanolic extract of *Stichopus badionotus* very thorough observation that the extract reduce and heals the wound faster than control antibiotics (Fig. 1) followed by ampicillin and penicillin using minimal inhibitory concentration.

Table 1: The antibacterial activity of methanol extracts of *Stichopus badionotus* determined by disc diffusion method

Name of the strains	Test substances (mm)			
	Methanolic extract	Solvent	Vancomycin (30 µg)	Penicillin (10 µg)
<i>Staphylococcus aureus</i> N8	6.0	-	5.5	-
<i>Staphylococcus aureus</i> 20	4.0	-	4.0	2.0
<i>Staphylococcus aureus</i> str5	5.5	-	6.0	0.5
<i>Staphylococcus aureus</i> str9	5.5	-	5.0	-
<i>Staphylococcus aureus</i> ATCC 29247	5.5	-	6.0	7.5
<i>Staphylococcus aureus</i> ATCC 700698	6.0	-	6.0	-

Table 2: Minimal inhibitory concentration of *Stichopus badionotus* methanolic extract against resistant and non resistant strains of *Staphylococcus aureus*

Name of the strains	Test substance	
	Colony count	MIC (mg mL ⁻¹)
<i>Staphylococcus aureus</i> N8	51	3.75
<i>Staphylococcus aureus</i> 20	276	3.75
<i>Staphylococcus aureus</i> str5	186	7.50
<i>Staphylococcus aureus</i> str9	213	7.50
<i>Staphylococcus aureus</i> ATCC 29247	45	3.75
<i>Staphylococcus aureus</i> ATCC 700698	170	7.50

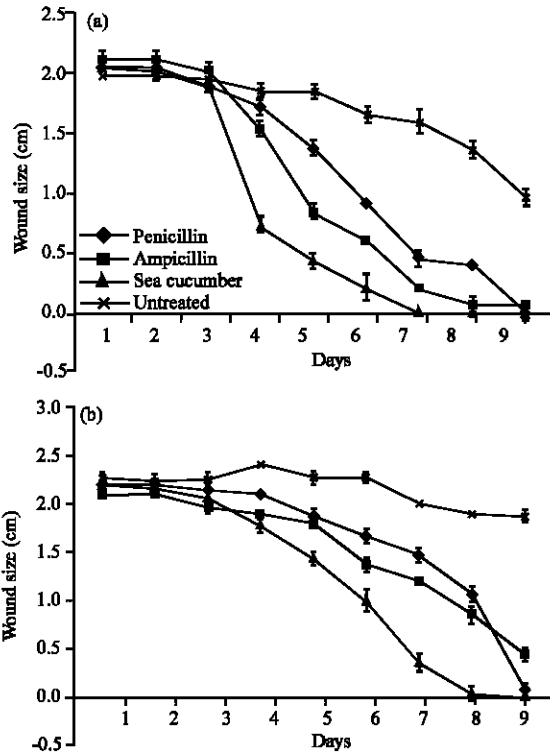


Fig. 1: MSSA and MRSA wound infection size treated with antibacterial agents at MIC. (a) Antibiotics and extract treatment on MSSA wound infection model and (b) antibiotics and extract treatment on MRSA wound infection model

DISCUSSION

MRSA has become a global concern as the bacterial resistance to a given antibiotic increase. Marine

resources of antimicrobial properties are promising resources as these organism possess active metabolites that can be exploited for discovery of new antibacterial agents. From the results, it can be said that *Stichopus badionotus* can be explored further as they are able to resist bacterial growth of MRSA and MSSA (Mariana *et al.*, 2009). MRSA is a major cause of community acquired and health care associated infections around the world. *S. aureus* infection is a major cause of skin, soft tissues, respiratory, bone, joint and endovascular disorders. These variety of infections due to *S. aureus* cause bacteremia, endocarditis, sepsis, metastatic infection, furunculosis and toxic shock syndrome give an indication of the high virulence property of this pathogen. The evolution of antibiotic resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from alternative sources including from natural marine products. MRSA is a virulent organism that causes substantial infection related morbidity and mortality in hospitalized patients. The rate of MRSA infection is increasing and treatment is not only expensive but available drug of choice is toxic to human host. New alternative sources of antimicrobial could potentially be found in marine resources which would be a feasible alternative for the country as Malaysia is known for the megadiversity resources.

Interesting finding noted that sea cucumber *Stichopus badionotus* extract showed strong inhibition against MRSA and MSSA. The differences between the reaction of methanolic extract of sea cucumber on MRSA and MSSA as far as antibacterial activities are concerned. The extract was more active on MSSA than the MRSA as indicated by bigger inhibition zones on disc diffusion plates. MRSA strains were more virulent and passed resistant genes that make them better survivor than MSSA strain.

The establishment of wound infection model for superficial skin infection caused by MRSA and MSSA which were done, are relevant model for localized skin infection in human (Bunce *et al.*, 1992). The cut through epidermis and dermis layer of the animal's skin in this model allowed MRSA and MSSA to colonize the skin and to elicit infections on the wound. Through *in vivo* study, the extract used was more effective than penicillin and ampicillin as indicated by the shorter day of healing time and smaller wound size.

CONCLUSION

The antibacterial screening of *Stichopus badiotus* on MRSA and MSSA revealed that these particular species possess antibacterial property coupled with wound healing ability that need further study. This potential unexploited local animal can be exploited as alternative drug of choice in treatment of MRSA wound infection in human.

REFERENCES

- Bunce, C., L. Wheeler, G. Reed, J. Musser and N. Barg, 1992. Murine model of cutaneous infection with gram-positive cocci. *Infect. Immun.*, 60: 2636-2640.
- Fridkin, S.K., J.R. Edwards, J.M. Courval, H. Hill and F.C. Tenover *et al.*, 2001. The effect of vancomycin and third-generation cephalosporins on prevalence of vancomycin-resistant enterococci in 126 U.S. adult intensive care units. *Ann. Int. Med.*, 135: 175-183.
- Mariana, N.S., M.A. Norfarrah, F.M. Yusoff and A. Arshad, 2009. Selective *in vitro* activity of marine extract on genes encoding membrane synthesis of methicillin resistance *Staphylococcus aureus*. *Biotechnology*, 8: 180-183.
- Tamakou, J.D., J.R. Kuate, G.S.S. Njateng, D.J.M. Simo and A.J. Njouendou *et al.*, 2008. Antimicrobial activity of dichloromethane-methanol (1:1 v/v) extract from the stem bark of *Coula edulis* Bali (Olacaceae). *Res. J. Microbiol.*, 3: 414-422.
- Zetola, N., J.S. Francis, E.L. Nuermberger and W.I. Bishai, 2005. Community-acquired methicillin-resistant *Staphylococcus aureus*: An emerging threat. *Lancet*, 5: 275-286.