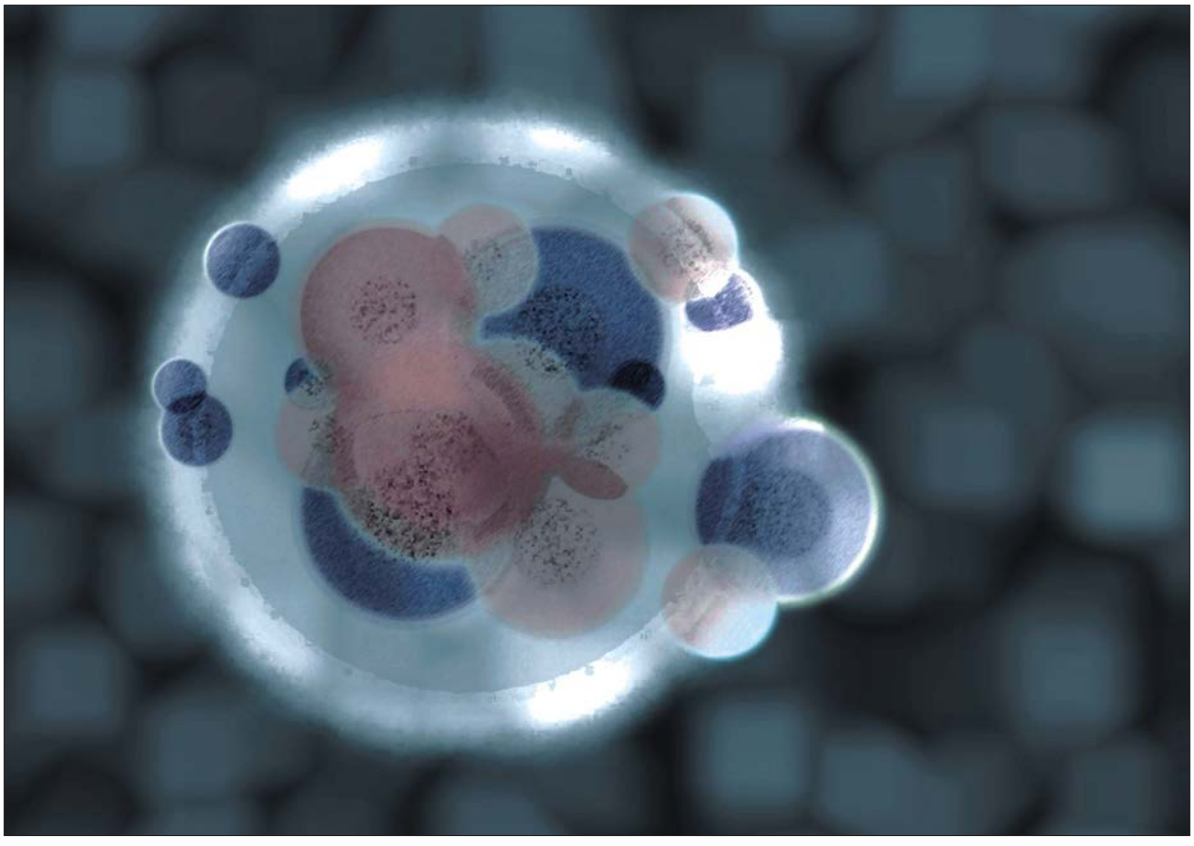

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In vitro Antibacterial Activity of Methanol Extract of A Sponge, *Geodia* sp. Against Oxytetracycline-Resistant *Vibrio harveyi* and its Toxicity

[1]A. Isnansetyo, [2]Trijoko, [3]E.P. Setyowati and [1]H.H. Anshory
[1]Fish Diseases Laboratory, Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Jl. Flora, Bulaksumur, Yogyakarta 55281, Indonesia
[2]Laboratory of Animal Taxonomy, Faculty of Biology, Gadjah Mada University, Jl. Teknika Selatan, Yogyakarta 55281, Indonesia
[3]Laboratory of Biological Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia

Abstract: In this study, the extract was tested for *in vitro* activity against oxytetracycline-resistant *V. harveyi*. Toxicity of the methanolic extract was evaluated by Brain Shrimp Lethality test. *Geodia* sp. was characterized by three spicula types (oxeas, trianes and oxyaster euaster), encrusting growth formation, hispid surface features and skeletal structure of paratangential ectosome. The methanolic extract of *Geodia* sp. exhibited anti-oxytetracycline-resistant *V. harveyi* activity with MIC of 31.25 μg mL^{-1}. The extract also was able to inhibit the growth of oxytetracycline-resistant *V. harveyi* in broth culture at concentrations of 1 and 2×MICs and able to kill almost *V. harveyi* cells at 4×MIC. Interestingly, the extract did not show any toxic effect in *Artemia salina* up to 125 μg mL^{-1}. It is the first report for the antibacterial activity of methanolic extract of *Geodia* sp. against oxytetracycline-resistant *V. harveyi*, a pathogenic bacterium in marine aquaculture. This results suggest that *Geodia* sp. might be used as a source of alternative compound to control marine bacterial pathogen especially oxytetracycline-resistant *V. harveyi*.

Key words: Anti-*V. harveyi* activity, Brain Shrimp Lethality test, fish disease, marine invertebrate, minimum inhibitory concentration

INTRODUCTION

Vibrio harveyi is a significant pathogenic bacterium in marine aquaculture (Austin and Zhang, 2006) and recognized as the main causative agent of luminous vibriosis, which often results in mass mortality in cultured marine animals. The bacterium infects almost all cultured marine animals such as crustacean, mollusk and fish. Crustacean, including shrimp, crab, lobsters and *Artemia* are very susceptible to this opportunistic pathogenic bacterium (Jivaranichpaisal *et al*., 1994; Karuna Sagar *et al*., 1994; Liu *et al*., 1996; Robertson *et al*., 1998; Diggles *et al*., 2000; Soto-Rodriguez *et al*., 2003; Bourne *et al*., 2007). *V. harveyi* is also well known as a bacterial pathogen in almost all cultured marine fish species (Kraxberger-Beatty *et al*., 1990; Saeed, 1995; Hispano *et al*., 1997; Company *et al*., 1999; Zhang and Austin, 2000; Tendencia, 2002; Pujalte *et al*., 2003a, b; Zorrilla *et al*., 2003; Liu *et al*., 2003; Sivaram *et al*., 2004; Gauger *et al*., 2006; Oh *et al*., 2006). This bacterium is also determined as a causative agent for the disease in seahorse *Hippocampus* sp. (Alcaide *et al*., 2001; Tendencia, 2004), sea cucumber *Holothuria scabra* (Becket *et al*., 2004), abalone *Holiotis discus hannai* (Sawabe *et al*., 2007) and stony corals (Luna *et al*., 2007).

Oxytetracycline that is effective against a broad range of both gram positive and negative bacteria is usually used as feed additive to control a natural infection in aquaculture (Saeed, 1995). The use of this antibiotic causes the development of resistance in *Vibrio* spesies including *V. harveyi*. The high incidence of resistance to oxytetracycline has been found in *Vibrio* in larvae and post-larvae of *Macrobrachium rosenbergii* (Hameed *et al*., 2003) and fish intestine (Nonaka *et al*., 2000). *Vibrio* sp. isolated from diseased fish are also reported as the bacteria harboring oxytetracycline resistance gene determinant, *tet* 34 which show high resistant to the antibiotic with MICs 125-500 μg mL^{-1} (Nonaka *et al*., 2002; Kim *et al*., 2003). *Vibrio* sp. have been determined as the main reservoir of another oxytetracycline resistance gene, *tet*(M) (Nonaka *et al*., 2007).

Corresponding Author: Alim Isnansetyo, Fish Diseases Laboratory, Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Jl. Flora, Bulaksumur, Yogyakarta 55281, Indonesia
Tel: +62-274-551218 Fax: +62-274-551218

In particular, *V. harveyi* has been found to be able to develop the resistance to oxytetracycline with the increase in the MIC up to 250 times (Nakayama *et al*., 2006). This bacterium has been reported to be multi-antibiotic resistant to almost all available antibiotics (Tjahjadi *et al*., 1994; Ottaviani *et al*., 2001; Nakayama *et al*., 2006). The emergences of antibiotic-resistant bacteria in aquaculture suggest that the development of alternative counter measures to control aquatic bacterial diseases is urgent.

We conducted screening to explore the antibacterial activity from sponge to develop alternative practices for diseases control in aquaculture and found that *Geodia* sp. has potential antibacterial activity. *Geodia* sp. have been reported as producers of several bioactive substances (Tinto *et al*., 1998; Sjögren, 2006; Rangel *et al*., 2006; Uy *et al*., 2002, 2003; Ohta *et al*., 2006; Rangel *et al*., 2005), but the *in vitro* activity of *Geodia* sp. extract against bacterial disease in aquaculture is little known. This study aimed to evaluate *in vitro* antibacterial activity of *Geodia* sp. extract against the most important pathogenic bacterium in marine aquaculture, oxytetracycline resistant-*V. harveyi*, as well as its toxicity.

MATERIALS AND METHODS

***V. harveyi* strain and medium:** *V. harveyi* was kindly given by Brachishwater Aquaculture Development Center, Jepara, Central Java, Indonesia. The bacterium was cultured in Zobell medium (pH 7.5) {polypepton (Nihon Seiyaku, Japan), 5 g L^{-1}; yeast extract (Oxoid), 1 g L^{-1} dissolved in filtered 75% of 30 ppt seawater}.

Resistance test of *V. harveyi* to oxytetracycline: Resistance of *V. harveyi* to oxytetracycline was examined by Minimum Inhibitory Concentration (MIC) test using agar dilution method (Clinical Laboratory Standards Institute, 2006). Zobell agar medium was supplemented with various concentrations of oxytetracycline (Zalmweg Raamsdonksveer, Nentherland) and used for culture of *V. harveyi*.

Sample of *Geodia* sp.: Sample of *Geodia* sp. was collected from intertidal zone of Wediombo coast, Gunungkidul, Yogyakarta, Indonesia in January-September 2005 and May 2006. Identification of the sponge based on spicula by bleaching digestion, skeletal structure by simple clearing method, surface structure and growth formation (Hooper, 2000).

Extraction of *Geodia* sp.: *Geodia* sp. sample was washed by freshwater and sliced. Then the sample was extracted with methanol (MeOH) at the ratio sponge and methanol of 1:4 (w/v). The extraction was done by mean of a homogenizer for 15 min and then the sample was centrifuged at 4,500 g for 20 min to obtain the supernatant as MeOH extract. The extraction was carried out twice in the same volume of MeOH. The extract was concentrated by a rotary evaporator at 40°C.

Anti-oxytetracycline resistant *V. harveyi* activity test and determination of the Minimum Inhibitory Concentration (MIC): Anti-*V. harveyi* activity of the MeOH extract was evaluated by paper disk diffusion method using double layer agar of Zobell medium as previously described by Horikawa *et al*. (1999), Miller *et al*. (2003) and Isnansetyo and Kamei (2005) after 20-fold concentrated. Sterile paper disks (ø 8 mm, Advantec, Tokyo) were impregnated with 50 μL of the MeOH extract and dried at 30°C. ZoBell medium with 0.7% agar kept in a water bath at 48°C was inoculated with an overnight culture of *V. harveyi* to give an initial bacterial density of 10^6 cells mL^{-1} and overlaid onto Zobell agar medium plate. Before inoculation, the bacterial density in the inoculum was estimated by a spectrophotometer (UV-VIS spectrophotometer, UV-1650PC, Shimadzu) at 625 nm with McFarland standard. The MeOH extract-impregnated paper disks were placed on the plates and incubated at 30°C for 24 h. The Minimum Inhibitory Concentration (MIC) of the MeOH extract was determined by the same method used for the anti-*V. harveyi* activity test with serial dilution of the MeOH extract concentrations.

Bactericidal assay: The time-kill experiment was conducted by the method described by Aeschlimann and Rybak (1998) and Entenza *et al*. (1998). The experiment was conducted in 25 mL-Erlenmeyer flasks containing 15 mL fresh Zobell medium inoculated with an overnight *V. harveyi* to give an initial bacterial density of 10^6 cells mL^{-1}. The inoculation was carried out immediately just after addition of the MeOH extract at the final concentrations of 1, 2 and 4×MIC in duplicates. The flasks were further incubated at 30°C agitating with shaker. The bacterial cells density at various incubation times was estimated by a spectrophotometer (UV-VIS spectrophotometer, UV-1650PC, Shimadzu) at 625 nm. Viable bacterial cells were estimated by plating on TCBS agar medium (Oxoid).

Brine shrimp lethality test (BST): Brine Shrimp Lethality Test (BST) was used to evaluate toxicity of MeOH extract of *Geodia* sp. based on the procedure previously described by Bailey *et al*. (2005) and Libralato *et al*. (2007). A conical container was used to hatch the brine shrimp cysts (A Quality Cysts, Great Salt Lake, Inve) in 30 ppt filtered seawater with enough aeration. Test was performed in a multiwell test plate

(6×4 wells) with 30 ppt of filtered seawater. Ten *Artemia* nauplii instar II were exposed with the MeOH extract at the concentrations of 0 (control treatment), 1, 2, 4 and 8×MIC in duplicates. After 24 h exposure, the mortality of *Artemia* was observed.

RESULTS AND DISCUSSION

The resistance test of *V. harveyi* to oxytetracycline showed that the bacterium used in this study has high resistance to the antibiotic with MIC 140 μg mL^{-1}. The resistance of marine *Vibrio* to oxytetracycline have been reported by Nonaka *et al.* (2002) and Kim *et al.* (2003) with MIC 125-500 μg mL^{-1}. Nakayama *et al.* (2006) reported that the MIC of oxytetracycline against oxytetracycline-resistant *V. harveyi* is 250 times higher than the MIC of the same antibiotic against the sensitive one.

Geodia sp. is a sponge characterized by oxeas of monaxonid megascleres and trianes of tetraxonid megascleres of spicula, while its microsclere was oxyaster euaster, with encrusting growth forms, hispid surface features and paratangential ectosome of skeletal structure (Hooper, 2000). The sponge sample was found at intertidal zone.

The extract of *Geodia* sp. exhibited high anti-oxytetracycline-resistant *V. harveyi* activity indicated by wide and clear inhibition zone at various concentrations (Fig. 1a, b). Furthermore, MIC of the methanolic extract of *Geodia* sp. was 31.25 μg mL^{-1} indicated that the extract was very potent against oxytetracycline-resistant *V. harveyi*, the causative agent of luminescence disease. *Geodia* sp. have been reported as the source of several bioactive substances including an anti cancer, *Geodiamolide* H (Tinto *et al.*, 1998), exiguamide that inhibits the cell fate specification of sea urchin embryogenesis (Uy *et al.*, 2002, 2003), macrocyclic polyketide lactam tetramic acid, an anti-nematode substance (Yan, 2004), barettin with antifouling activity (Sjögren *et al.* 2006), exiguolide that inhibits the fertilization of sea urchin gametes (Ohta *et al.*, 2006), crude extract of *G. corticostylifera* with antibacterial, antifungal, cytotoxic, haemolytic and neurotoxic activities and the cyclic peptide geodiamolides A, B, H and I from this sponge with anti-cancer activity (Rangel *et al.*, 2006). The mouse acute toxicity, neurotoxic and haemolytic activities were also reported from the extract of *Geodia* sp. (Rangel *et al.*, 2005). The geodiamolide H, a peptide from *G. corticostylifera* inhibits migration and invasion of Hs578T cells derived from breast cancer through modifications in actin cytoskeleton (Freitas *et al.*, 2008). Encarnacion *et al.* (2000) described antibacterial activity of ethanol extract from *Geodia* sp. against a gram positive

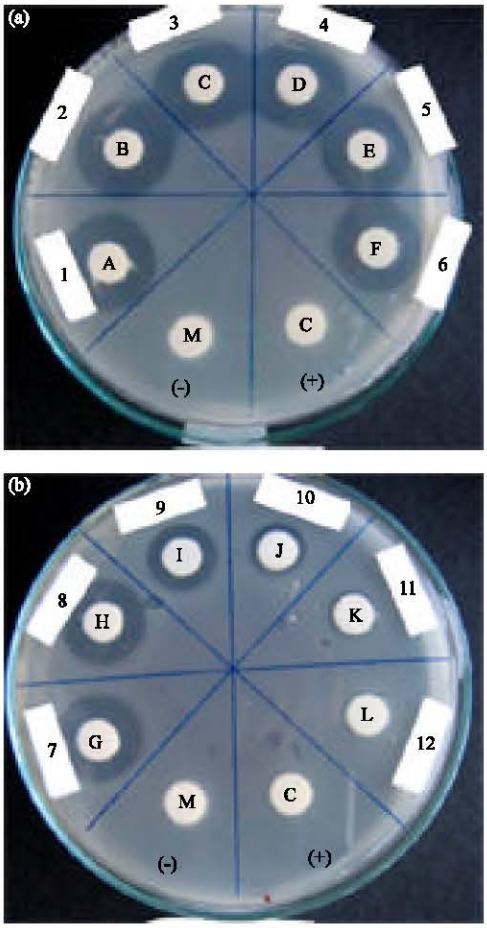

Fig. 1: (a, b) Anti-oxytetracycline-resistant *V. harveyi* activity of the MeOH extract of *Geodia* sp. A: 4,000 μg $disk^{-1}$; B: 2,000 μg $disk^{-1}$; C: 1,000 μg $disk^{-1}$; D: 500 μg $disk^{-1}$; E: 250 μg $disk^{-1}$; F: 125 μg $disk^{-1}$; G: 62.5 μg $disk^{-1}$; H: 31.25 μg $disk^{-1}$; I: 15.63 μg $disk^{-1}$; J: 7.81 μg $disk^{-1}$; K: 3.91 μg $disk^{-1}$; M: Negative control (methanol); C: Oxytetracycline at 10 μg $disk^{-1}$

bacterium, *Mycobacterium avium*, but did not prove against other bacterial strains including *Vibrio*. The anti-oxytetracycline-resistant *V. harveyi* activity of *Geodia* sp. extract has not been reported yet so far. The result of this study suggests that *Geodia* sp. may have the important ecological role in controlling the outbreak of luminescence disease caused by *V. harveyi* in marine aquaculture. This finding also shows that *Geodia* sp. is the potential source of antibacterial substance for the alternative counter measure against marine bacterial diseases, especially *V. harveyi*.

The use of antibiotic in veterinary and aquaculture contributes the increase in the resistant of pathogenic

bacteria to commercial available antibiotics (Alderman and Hasting, 1998; Teuber, 2001). The resistance of *V. harveyi* to antibiotics might be caused by the use of various antibiotics in Indonesian aquaculture especially in 1980's. *V. harveyi* has been reported to be multi-antibiotic resistant to ampicillin, amoxicillin, carbenicillin, cephalothin, colistin sulphate, kanamycin, lincomycin, neomycin, novobiocin, nitrofurantoin, penicillin, polymyxin, rifampicin, streptomycin, sulphamethoxazole, tetracycline and trimethoprim (Tjahjadi *et al*., 1994; Ottaviani *et al*., 2001). The resistance development of *Vibrio* is encoded by R-plasmid which is transferable to others bacterial cells (Aoki, 1992). The oxytetracycline resistance determinants, *tet* 34 (Nonaka *et al*., 2002; Kim *et al*., 2003) and *tet* M (Nonaka *et al*., 2007) in *Vibrio* have been determined. Two genetic determinants of tetracycline resistance in *V. harveyi* have been also found by Teo *et al*. (2002). The resistance determinants are easily transferred to other bacterial cells including bacterial pathogen in animal and human, which cause serious impacts in global environment and human health. In addition, another mechanism of resistance to β-lactam antibiotics has been found in *V. harveyi* harboring β-lactamase genes, blaVHW-1 and blaVHH-1 (Teo *et al*., 2000).

Bactericidal activity of the methanolic extract of *Geodia* sp. was tested by time course study. The optical density of *V. harveyi* treated with the extract at the concentrations of 1, 2 and 4×MICs decreased slightly. In contrast, the optical density of the bacterium in the control treatment increased constantly after 3 h incubation (Fig. 2). This result also showed that the extract has stable activity from the early incubation to 24 h incubation period. The absorbance of bacterial suspension did not increase in the broth medium added with the extract at 1×MIC (31.25 μg mL^{-1}) for 24 h

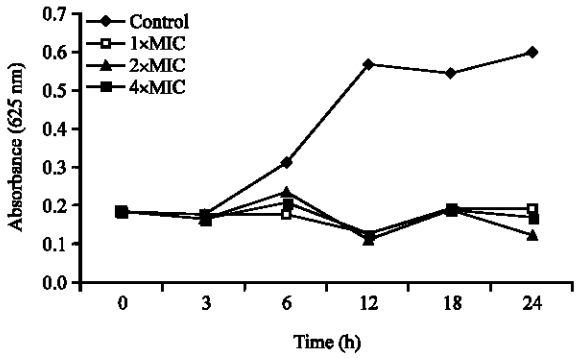

Fig. 2: Bactericidal activity of the methanolic extract of *Geodia* sp. against oxytetracycline-resistant *V. harveyi*

incubation suggesting the MIC obtained from paper disk diffusion on double layer agar had the same inhibitory effect to *V. harveyi* in broth culture. Although, there was no difference in the optical density of *V. harveyi* treated with the extract at 1, 2 and 4×MICs, the viable bacterial density of *V. harveyi* after 24 h incubation in each treatment estimated by pour plate method was different. The viable bacterial density in control treatment, at the concentrations of 1, 2 and 4×MICs were 2.39×10^{10}, 2.01×10^{8}, 2.01×10^{5} and 9.2×10^{3} cells mL^{-1}, respectively. This result shows that the methanolic extract of *Geodia* sp. was able to kill 99.1 and 79.9% bacterial cells of oxytetracycline-resistant *V. harveyi* at the concentration of 4 and 2×MICs, revealing that the extract was bactericidal at 4×MIC, but bacteriostatic at 2×MIC.

In this study, the viable bacterial density of *V. harveyi* could not be estimated merely by a spectrophotometer as there was no difference in the optical density, but considerably different in viable cells densities in each treatment. This time course study also indicated that the extract did not lyse the cells of *V. harveyi* as the antibacterial mode of action since the absorbance of the bacterium did not decrease as the decrease of the viable bacterium density. This finding suggests that the mechanism of antibacterial activity of the mathanolic extract of *Geodia* sp. is not lysis the bacterial cells of *V. harveyi*. This mechanism is likely resembled to methdilazine (Chattopadhyay *et al*., 1998), MC21 (Isnansetyo and Kamei, 2003) and microcin E492 (MccE492) (Destoumieux-Garzón *et al*., 2003) which cause bacterial cell death without cellular lysis. The further investigation on the antibacterial mechanism should be conducted after purifying the antibacterial substance from the extract.

Brain shrimp lethality test showed that the methanolic extract of *Geodia* sp. had low toxicity with no mortality at 0, 31.25 μg mL^{-1} (1×MIC), 62.5 μg mL^{-1} (2×MIC) and 125 μg mL^{-1} (4×MIC) and caused 20% mortality at 250 μg mL^{-1} (8×MIC). This result revealed that the extract might be applicable to control *V. harveyi* by immersion because the extract did not cause any mortality to *Artemia* nauplii at concentration up to 4×MIC. Comparing to oxytetracycline, the toxicity of the extract is higher because the $LC_{50\text{-}24}$ and $LC_{50\text{-}48}$ of oxytetracycline to *A. parthenogenetica* are 871 and 806 μg mL^{-1}, respectively (Ferreira *et al*., 2007). The toxic effect of the methanol extract of *Geodia* sp. might be not only caused by principle constituent of antibacterial substances in the extract but also by others substances in the extract. Therefore, purification and chemical elucidation as well as evaluation of *in vivo* activity are necessary for further study. Although, the methanolic

extract of *Geodia* sp. showed non toxic to *Artemia* at 4×MIC, the toxicity of the extract to cultured aquatic organisms should be determined before application.

The results of this study can be summarized that the MeOH extract of *Geodia* sp. exhibited potent antibacterial activity against oxytetracycline-resistant *V. harveyi* with MIC 31.25 μg mL^{-1}. The extract showed bacteriostatic activity at low concentration and bacterisidal activity at the concentration of 4×MIC. This extract might be applied to control the disease caused by the bacterium as the extract showed no toxicity up to 125 μg mL^{-1} (4×MIC) based on brain shrimp lethality test.

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