Antibacterial Activity of Gonad Methanol Extract of the Sea Urchin *Diadema Setosum* Against *Methicillin-Resistant Staphylococcus aureus* and *Escherichia coli*

F M Sidiqi 1*, Pringgenies D 1, Setyati W A1

1 Departement of Marine Science Diponegoro University, Jalan Prof Soedarto SH Tembalang, Semarang
Corresponding author: fawazmsidiqi@gmail.com

**Abstract.** The discovery of antibacterial compounds from nature has been widely used as an effort to overcome the resistance of pathogenic bacteria. Sea urchin is a marine biota of phylum Echinoderms that has a potency as an antibacterial to the variety of pathogenic microbes. This study aims to determine the antibacterial activity of gonad extract on *Diadema setosum* against *Methicillin-Resistant Staphylococcus Aureus* bacteria and *Escherichia coli* bacteria. The Gonad of *Diadema setosum* was extracted by maseration and rotary evaporator. Phytochemical screening tests were performed on alkaloids, flavonoids, triterpenoids, steroids and phenolic compounds. The antibacterial activity test was performed by two different methods of agar diffusion method and microdilution broth method. The results of phytochemical screening tests showed that gonad methanol extract of the *Diadema setosum* contain flavonoid and saponin compounds. The result of the antibacterial activity test of the diffusion method showed that gonad methanol extract did not have antibacterial activity with no inhibition zone formed at all test concentrations. The result of antibacterial activity test of broth microdilution method showed that *Minimum Inhibitory Concentration* (MIC) value extract to MRSA and *E. coli* bacteria were 62.5 mg/ml, meanwhile *Minimum Bactericidal Concentration* (MBC) value was not found until even at the highest concentration.

**1. Introduction**

Sea urchin (Echinoidea) is one of the marine biota parts of Echinodermata phylum. There are some types of sea urchins were found in the Java, Bali, and Sumatra. *Diadema setosum* sea urchins are also found in Pulau Panjang, Jepara. Gonads (eggs) are reproductive organs in sea urchins that fulfill more than a half of the chest cavity on the apical side. The international market has a very huge enthusiasm on gonad because gonad contains high protein content[1], high essential amino acid content, ability to increase hemoglobin, body weight and neurons or brain nerve cells[2].

Antibacterial compounds can inhibit and also kill pathogenic bacteria at low doses[4][3]. The publication of bioactive compounds from marine biota is increasing due to the unique character of secondary marine biota metabolites[4].

*Diadema setosum* including marine biota are reported to have antibacterial compounds against various pathogenic bacteria[8]. The potential of sea urchins as an antibacterial against various pathogenic microbes has not been utilized optimally, even some regions in Indonesia consider sea urchins as pests. Some parts of gonad sea urchins often consumed in raw conditions. This study was conducted to determine the antibacterial activity of gonad extract of *Diadema setosum* on *Methicillin-Resistant Staphylococcus aureus* (MRSA) and *Eschericia coli* bacteria.
2. Research Method

The material used in this study was gonad \textit{Diadema setosum} from Pulau Panjang waters, Jepara, Central Java. MRSA and \textit{E. coli} pathogenic bacteria were obtained from bacterial isolates in the Laboratory of Tropical Marine Biotechnology, Diponegoro University.

2.1. Preparation of extracts

Gonad of \textit{D. setosum} was extracted by maceration process using methanol for 3 x 24 hours in a tightly closed bottle and stored at room temperature (27 °C), and protected from direct light to prevent reactions that are catalyzed by light or discoloration. The crude extract was filtered using filter paper and evaporated with a rotary evaporator at 37-40ºC, until a thick extract was formed and no solvent smell has occurred. The extract was put into a vial and stored in a refrigerator at 4 oC [5].

2.2. Bacterial preparation

Bacterial planting test was carried out by stamping techniques. As much as one unit of the test bacteria from the stock was taken, then scraped with a zig-zag pattern over the petri dish which Nutrient agar (NA) media had previously added. The media which had been inoculated with the test bacteria were then incubated at 30°C for 24 hours [6]. The bacteria which had incubated were cultured all night (18–24 h) at 37°C on nutrient broth for the preparation of cell suspensions. The bacteria cell suspensions were homogenized and adjusted to 0.5 McFarland standards (1.5 × 10^8 CFU/mL).

2.3. Antibiocellular susceptibility assays

Antibacterial activity test was carried out using the disc diffusion method (Kirby-Bauer test) with two different concentrations [7]. Tests were carried out with two variations of gonad extract concentration of \textit{D. setosum}, which were 200 ppm, 400 ppm, 600 ppm, 800 ppm (Kirby-Bauer test) and 12.5 mg / mL, 25 mg / mL and 37.5 mg / mL [8]. The positive control used was Chloramphenicol 1000 ppm, while for negative control Dimethyl Sulfoxide (DMSO) was used as a solvent in the extract dilution process. Observations were made by measuring the amount of formed inhibitory zones around the paper disc after incubation for 24 hours and 48 hours.

2.4. Antibiocellular susceptibility assays of the microdilution broth method

Testing of the antibacterial activity of the broth-microdilution method was carried out with a half dilution principle of the previous concentration (w / 2v). The concentrations of \textit{D. setosum} gonad methanol extract were 1000 mg / ml, 500 mg / ml, 250 mg / ml, 125 mg / ml and 62.5 mg / ml and observed the inhibition activity using the Total plate count (TPC) method [9]. Positive control on this test was the bacterial suspension equivalent to 0,5 McFarland standards while the negative control was nutrient broth as a bacterial suspension media.

2.5. Phytochemical screening

The extract of \textit{D. setosum} was screened for the presence of different classes of secondary metabolites including alkaloids, flavonoids, saponins, steroids, triterpenoids, phenolic, using the method of Suzery and Kusri [10].

3. Results and Discussion

Extraction results showed that the weight of gonad extract of \textit{D. setosum} after extraction process was 6.1 grams or 3.78% of the total weight of the gonad used, which was 161 grams. Percentage of methanol gonad extract of sea urchin (Echinoidea) was 4.31% or the highest compared to methanol extracts of thorns and shells [11]. The high sea urchin gonadal yield is thought to be influenced by the amount of content of the soluble compounds in methanol. Methanol solvents were able to extract components derived from alkaloids, phenolics, carotenoids, tannins, sugars, amino acids and
glycosides, besides that methanol solvents also had less polar properties compared to water, thus methanol solvents were able to destroy cell wall and causes the components in the cell to disintegrate and dissolve in methanol solvents[12].

Phytochemical screening obtained showed that methanol gonad extract of D. setosum contained active compounds from flavonoids and saponin (Table 1). Saponin compounds have the potential as antibacterial, because they are polyphenol compounds that can inhibit bacteria by damaging the permeability of bacteria cell membranes [13]. Saponins are exclusively present in echinoderms. The biological function of saponins in echinoderms is related to the system of self-defense against marine fungi, predators and parasites [14]. Flavonoids are one of the polar phenol compounds that dissolve in polar solvents such as methanol, ethanol, butanol and acetone. Flavonoids include secondary metabolites which have high antibacterial compounds [15].

Steroid and triterpenoid test results of gonad extract of sea urchin D. setosum were different compared to phytochemical tests [11]. The difference in results can be due to the detection ability of the phytochemical test which is not able to detect steroids and triterpenoids which are small in the sample [16]. The difference in steroid and triterpenoid test results may also due to different environmental conditions and the age of the gonad of sea urchins that are used as research material is immature, where the gonad of D. setosum used is still in the pre-mature phase [1].

Table 1. Results of phytochemical screening of gonad methanol extract of sea urchin D. setosum

| Compounds     | Gonad D. setosum Methanol Extract |
|---------------|-----------------------------------|
| Alkaloids     | -                                 |
| Flavonoids    | +                                 |
| Saponin       | +                                 |
| Steroids      | -                                 |
| Triterpenoid  | -                                 |
| Phenolic      | -                                 |

(+)= Detected (-)= Not detected

The diffusion antibacterial test results showed that none of D. setosum methanol gonad extract concentrations indicated to have antibacterial activity, characterized by inhibitory zones which were not formed at all test concentrations (Tables 2 and 3). The results obtained differ from the other research who reported that methyl gonad extract of sea urchins had antibacterial activity against various pathogenic bacteria [8] [17] [11]. This difference can be caused by several reasons including the drying process of gonad sea urchin extract after evaporation [18], the size and type of gonads extracted [1], test bacterial resistance [19], and the extract concentration is too low [20].

The sea urchin gonad D. setosum used in this study is in a different phase from each other, where almost all gonads are still in the pre-mature or early mature phase. The different use of gonad of D. setosum is caused by the gonads inside the shell that do not appear to be visible from the outside when sampling and also the characteristics of the gonads that need to be investigated by using additional tools because gonadal maturity can not only be determined by the morphology of sea urchins. physical size, weight and volume [1]. Research conducted by Tee et al reported that different drying method of gonad urchin extract (Echinoidea) after being evaporated with a rotary evaporator affected the antibacterial activity of the extract [18]. The drying process to remove the water content in this study used a desiccator, which has the possibility of remaining water in the extract during the drying process. The type of test bacteria were used also affects the results obtained. MRSA bacteria were used as test bacteria in this study are S. aureus bacteria that have experienced resistance to methicillin [19]. Another factor that caused the absence of antibacterial activity in diffusion testing was that the low concentration of gonad methanol extract of sea urchin was not able to inhibit the
growth of test bacteria. The concentration of antibacterial agents were used greatly affects the ability of its inhibitory effect on the growth of test bacteria [20].

Table 2. Results of screening of gonad methanol extract of sea urchin *D. setosum* on *E. coli* and MRSA type 1 dilution

| Concentration | MRSA | E. coli |
|---------------|------|---------|
|               | D1 U1 | D2 U2 | D1 U1 | D2 U2 |
| 200 ppm       | -     | -     | -     | -     |
| 400 ppm       | -     | -     | -     | -     |
| 600 ppm       | -     | -     | -     | -     |
| 800 ppm       | -     | -     | -     | -     |
| Cloramphenicol| -     | -     | +     | +     |
| DMSO          | -     | -     | -     | -     |

D1 = Observation 1x24 hours  
D2 = Observation 2x24 Hours  
U1 = Deuteronomy 1  
U2 = Deuteronomy 2  
(+ ) = Forming a Inhibition zone  
( - ) = Does not form a Inhibition zone

Table 3. Results of screening of gonad methanol extract of *D. setosum* against *E. coli* and MRSA type 2 dilution

| Concentration | MRSA | E. coli |
|---------------|------|---------|
|               | D1 U1 | D2 U2 | D1 U1 | D2 U2 |
| 12,5 mg/ml    | -     | -     | -     | -     |
| 25 mg/ml      | -     | -     | -     | -     |
| 37,5 mg/ml    | -     | -     | -     | -     |
| Cloramphenicol| -     | -     | +     | +     |
| DMSO          | -     | -     | -     | -     |

Description:  
D1 = Observation 1x24 hours  
D2 = Observation 2x24 Hours  
U1 = Deuteronomy 1  
U2 = Deuteronomy 2  
(+ ) = Forming a Inhibition zone  
( - ) = Does not form a Inhibition zone

The results of the broth microdilution antibacterial activity test showed that generally methanol gonad extract of *D. setosum* can inhibit the growth of bacteria against MRSA bacteria (Table 4) and *E. coli* (Table 5) with increasing inhibitory power at higher concentrations. The higher concentration of antibacterial ingredients, the stronger the antibacterial activity [20]. The test results also showed that methanol gonad extract of *D. setosum* had higher antibacterial activity against MRSA bacteria than *E. coli* bacteria.

The difference in activity due to Gram-positive bacteria (MRSA) is more easily inhibited by gonad methanol extract of sea urchins than Gram-negative bacteria (*E. coli*). Jawetz et al stated that Gram-positive bacteria had a simpler cell wall structure compared to Gram-negative bacteria, namely single-layered with low lipid content (1-4%) so that the active ingredient easily entered the cell, whereas Gram-negative bacteria negative has a three-layered cell wall structure consisting of an outer layer of lipoprotein, the middle layer of lipopolysaccharide which is a barrier to the entry of active ingredients, and an inner layer of high lipid-containing peptidoglycan (11-12%) [21].
Table 4. Results of TPC calculation of antibacterial activity of gonad methanol extract of *D. setosum* against MRSA bacteria with broth microdilution method

| Extract Concentration (mg/ml) | 24 Hours Average ± SD $\times 10^9$ (CFU/ml) | 48 Hours Average ± SD $\times 10^9$ (CFU/ml) |
|-------------------------------|---------------------------------------------|---------------------------------------------|
| 1000                          | 1.2 ± 0.42                                  | 3.3 ± 4.24                                  |
| 500                           | 28 ± 12.02                                  | 0.15 ± 0.21                                 |
| 250                           | 51 ± 22.63                                  | 16 ± 7.78                                   |
| 125                           | 44 ± 23.33                                  | 46 ± 35.36                                  |
| 62.5                          | 73 ± 9.90                                   | 70 ± 2.83                                   |
| Control +                     | 77 ± 7.78                                   | 75 ± 4.24                                   |
| Control -                     | -                                           | -                                           |

SD = Standard Deviation

Testing with MRSA bacteria showed that in the first 24 hours there was a decreasing of more than 60% of the colonies growth at a concentration of 500 mg/ml while at an extract concentration of 1000 mg/ml, there was a growth decline of 98.4%. The broth microdilution test results also proved that at a concentration of 423 mg/ml, methanol extract of sea urchin *Diadema setosum* can inhibit 50% growth of MRSA bacterial colonies (Figure 1). The value of 50% is used based on the principle of calculating IC50 (inhibitory concentration 50%), this value states the amount of the concentration of the extract which can reduce colony growth by 50%. IC50 value is obtained by linear regression equation ($y = a + bx$), by plotting the extract concentration and percent inhibition values on the x and y axes (Figure 1). Big and small IC50 values show effectiveness in inhibiting bacterial growth, where there is a small IC 50 value, the effectiveness is getting better and it will be occurred for the opposite result [11].

![MRSA first 24 Hours Inhibition](image)

**Figure 1.** Inhibition of gonad extract of *D. setosum* against MRSA bacteria in the first 24 hours
Table 5. Results of TPC calculation of antibacterial activity of gonad methanol extract of *D. setosum* against *E. coli* bacteria with broth microdilution method

| Extract Concentration (mg/ml) | 24 Hours | 48 Hours |
|------------------------------|----------|----------|
|                              | Average ± SD x10^9 (CFU/ml) | Average ± SD x10^9 (CFU/ml) |
| 1000                         | 38 ± 4.24 | 49 ± 24.04 |
| 500                          | 36 ± 10.61 | 64 ± 7.07 |
| 250                          | 59 ± 20.51 | 60 ± 12.02 |
| 125                          | 56 ± 12.73 | 72 ± 36.06 |
| 62.5                         | 74 ± 4.95  | 75 ± 9.19  |
| Kontrol +                    | 85 ± 9.19  | 87 ± 15.56 |
| Kontrol -                    |          |          |

SD = Standard Deviation

The minimum value of Inhibitory Concentration (MIC) of gonad methanol extract of sea urchin *D. setosum* against MRSA and *E. coli* based on the test results was 62.5 mg/ml. The determination of MIC value was based on the first 24-hour observation, because the growth of *E. coli* and *S. aureus* bacteria began at 3 to 24 hours [22]. Tests with *E. coli* bacteria showed that up to the highest concentration (1000 mg/ml) the growth of the colony was only able to be inhibited to 56% compared to growth in positive control both at 1x24 hours and 2x24 hours (Table 5). Minimum Bactericidal Concentration (MBC) values for test bacteria were not found, both for MRSA and *E. coli*, this was due to the discovery of the growth of bacterial colonies at the highest concentration of 1000 mg/ml. Strength of antibacterial activity extracts on antibacterial testing with the Broth Microdilution method has 4 categories, where MIC less than 100μg/ml is declared to have good anti-microbial activity; 100 to 500μg/ml of antimicrobial activity is moderate; and 500-1000 μg/ml of weak anti-microbial activity; more than 1000 μg/ml of the extract is considered inactive [23].

4. Conclusion
The gonad methanol extract of *D. setosum* did not have antibacterial activity with no inhibition zone formed at all test concentrations. The result of antibacterial activity test of broth microdilution method showed that *Minimum Inhibitory Concentration* (MIC) value extract to MRSA and *E. coli* bacteria were 62.5 mg/ml, meanwhile *Minimum Bactericidal Concentration* (MBC) value was not found until even at the highest concentration.

References
[1] Darsono P 1986 *Jurnal Oseana* 11 151-162
[2] Pringgenies D, Aizaturroifah A, Indriatmoko, Sejati S and Haryo D 2016 *Asia Journal of Pharmaceutics* 10 100-107
[3] Madigan MT, MArtinko JM, Stahl DA and Clark DP 2011 *Biology of microorganisms 13th ed.* (London: Pearson) p 1043
[4] Supriya J and Yogesh C 2010 *Int. J. Res Ayur Pharm* 1 55-62.
[5] Shangkarlal S, Prabu K and Natarajan E 2011 *American-Eurasian Journal of Scientific Research* 6 178-181.
[6] Setyati WA dand Subagiyo 2012 *Jurnal Ilmu Kelautan* 17 164-168
[7] Willey JM, Linda MS and Christoper JW 2008 *Microbiology 7th Edition* (Prescott : McGraw-Hill) p 1088
[8] Marimuthu K P, Gunaselvam, Rahman A, Xavier R, Arockiaraj J, Subramanian S, Yousoff FM
and Arshad A 2015 *European Review for Medical and Pharmacological Sciences* 19 1895-99

[9] Mulyani Y, Sukandar EY and Adyana IK 2012 *Jurnal Ilmu-ilmu Hayati dan Fisik* 14 22-30

[10] Suzery M and Kusrini D 2004 *Buku Ajar Pemisahan dan Analisis Bahan Alam* (Semarang :Undip Press) p 131

[11] Akerina, Tati Nurhayati and Ruddy Suwandy 2015 *JPHPI*. 18 61-73

[12] Lapornik B, Prošek and Wondra AG 2005 *Journal of Food Engineering* 71 214-222

[13] Sikkema LA, de Bont JA and Poolman B 1995*Microbiological reviews* 59 201-222.

[14] Pranoto EP, Widodo FM and Delianis P 2012 *Jurnal Pengolahan Dan Bioteknologi Hasil Perikanan* 1 1-8

[15] Darsana IGO, Besung INK and Mahatmi H 2012 *Indonesia Medicus Veterinus* 1 337-351.

[16] Artini PE, Astuti KW and Warditiani NK 2013 *J. Farmasi FMIPA Univ. Udayana* 2 1-7.

[17] Abubakar, Wangi C, Uku J and Ndirangu S 2012 *African Journal of Pharmacology and Therapeutics* 1 1923

[18] Tee LK, Daneshwar P and Vishwakalyan B 2017 *J. of Applied Biology & Biotechnology* 5 9-13

[19] Pfaller MA 2012 *The American Journal of Medicine* 125 S3-S13.

[20] Ariyanti NK, Darmayasa IBG and Sudirga SK 2012 *Jurnal Biologi* 16 1-4.

[21] Jawetz E, Melnick JL and Adelberg EA 2001 *Mikrobiologi Kedokteran translated by Nani W* (Jakarta : Salemba Medika) p 196-198

[22] Eng R, Padberg FT, Smith SM, Tan EN and Cherrubin CE 1991 *J. of Antimicrobial Agents and Chemotherapy* 35 1824-1828.

[23] Holetz FB, Pessini GL, Sanches NR, Cortez DAG, Nakamura CV and Filho BPD 2002 *Memorias do Instituto Oswaldo Cruz* 97 1027-31