Bioactivity of methanolic extract of marine tunicate *Pyura sp* against methicillin resistant *Staphylococcus aureus* (MRSA)

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Abstract. The research on potential methanol extract from marine tunicate *Pyura sp* as antibacterial *Methicillin-Resistant Staphylococcus aureus* (MRSA) has been done from February to April 2017. This research was intended to know the potential of methanol tunicates extract of proposed tunicates as an antibacterial MRSA. Tunicates were collected from Barrang Lompo waters of Sangkarang Archipelago of South Sulawesi Indonesia. Samples were dried, then the active compound was extracted used methanol solvent according to a standard procedure elsewhere. Tunicate's extract was prepared into three different concentrations: 10%, 15%, and 20%, were tested against MRSA bacteria. A test of inhibition zone was applied at 24 h and 48 h incubation time in order to determine the bioactivity of tunicate's extract towards MRSA. The result indicates that crude extracts of tunicates *P. Pyura sp* at all concentration performs antibacteria activity against MRSA. This study concluded that tunicate *Pyura sp* has bactericidal activity against MRSA and this can lead to developing a new antibiotic in the future.

1. Introduction

Sea area is known for biological and chemical resources produced by marine biotas. Nowadays, a number of research on bioactive compound isolated from marine organism increase significantly, after an inventory of a hundred new lead compound every year. Those bioactive compounds were isolated from various number of marine invertebrates [1],[2],[3]. More than a 10,000 bioactive compound had been found in marine invertebrates, including tunicates [4]. Ascidian known as tunicate belongs to chordates are sessile fauna that lives at shallow to a deep ocean. The body of tunicates is covered by an epithelial layer known as the tunic. Approximately, 3000 species of ascidian have been documented [5].

One of the tunicate group is Pyura, a solitary ascidian is widely distributed at hard substrate such as ruble or hard coral, which is common in Sangkarang Archipelago of South Sulawesi province [6]. As filter-feeding animals, this group is fragile to predator and depends on chemical defenses to a predator [7].

Marine invertebrate is known for their bioactive compound [8]. Tunicates which harbor different symbiotic bacteria are having potency on secondary metabolite such as alkaloid, flavonoid and steroid. Besides, a compound including 4-methoxypyrrole, methanol, ethanol, butanol, and hexane have been reported from tunicates [2]. Some tunicates contain bioactive compound such as dehydrotyrosyl or dopa- and top dehydrodopyl peptide in their blood cell or tissue, including
halocyanines from *Halocynthia roretzi* [9], lamellarins from *Didemnum chartaceum* [10] and ferreascidin from *Pyura sp.* [11].

Secondary metabolite has been utilized by a human being as bioactive substance in different ways including antitumor, anticancer, anti-bacterial, and anti-fungi [12]. For example, dopa- or peptides topa compound holds a unique biological characteristic and has shown antibiotic activity against bacterial [8].

A widely use and long term use of certain antibiotic has proven as one of the factors in controlling multi-resistant antibiotic [13]. *Staphylococcus aureus* bacteria are recognized as the main cause for infectious diseases, hence has also the ability to be resistant toward antibiotic[14]. Since an introduction of penicillin in 1940, resistant of *Staphylococcus aureus* toward antibiotic happened sporadically in short time after using this antibiotic for clinical purposes. Infection by *Staphylococcus aureus* which is antibiotic resistant occurs endemic or epidemic during more than 60 years. After 10 years application of penicillin to a human being, this antibiotic was recognized ineffective against *S. aureus*, due to fact that β-lactamase enzyme that produces destroyed ring β-lactam in penicillin [15]. The number of *S. aureus* infection cases increases along with the occurrence of strain that resistant to methicillin antibiotic (Methicillin-resistant *S. aureus*/MRSA)[16]. MRSA is known as global world infection, that of against MRSA in which they can be found in a hospital or elsewhere [17]. Therefore, research was conducted to find an alternative antibacterial that solves a problem on *Staphylococcus aureus* resistant antibiotic (Methicillin-resistant *Staphylococcus aureus*/MRSA).

2. Material and Methods
2.1. Tools and Materials
Materials used in this study are masker, *snorkel*, SCUBA diving, *cool box*, analytical scale, rotavapor, channel Buchner, incubator, Petri dish, measuring glass, Erlenmeyer, vial, reaction tube, stick, aluminium stub, autoclave, oven, centrifuge, refrigerator, hot plate, LAF (Laminary Air Flow), vortex, spoon, Bunsen, round ose, pin-set, reaction tube holder, micropipette, drop pipet, vernier caliper and spectrophotometer. Materials used in this study were tunicate *Pyura sp.*, *aquadest*, 70% alcohol, Methicillin-resistant *S. aureus*, filter paper, 96% methanol, Nutrient Agar (NA), Mueller-Hinton Agar (MHA), disk amoxicillin, standard MC. Farland concentration 1.5 x 10^8 CFU/mL, paper disk, aluminium foil, 0.9 % NaCl, spiritus, cotton and cling wrap.

2.2. Sample collection and preparation
Tunicata *Pyura* was collected from Baranglombo, Makassar, South Sulawesi from a depth of 2-10 meter by using SCUBA. Samples were washed several times with an aquadest in order to remove debris and salt. Samples were kept in a cool box then transported to a laboratory for further study.

2.3. Extraction procedure
*Pyura* sp was air dried, so bioactive compound was not damaged by sunlight [18]. After samples were dried, the sample was prepared for chemical extraction stage. Extraction procedure follows a common method used elsewhere. A dried *Pyura* was crushed in order to get optimal of chemical content [19], then 150 g Simplicia was soaked with 1:3 96% methanol for 3 x 24 h. This was conducted in dark condition. The solvent was removed by using rotary evaporator. Extract of tunicate *Pyura sp.* was made in a series concentration of 10%, 15%, and 20%.

2.4. Test anti-MRSA
Pure methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria originated from Integrated Laboratory Science Building, The Faculty of Mathematics and Natural Sciences, Hasanuddin University. MRSA was growth in Nutrient Agar (NA) medium, incubated at 37 °C for 24h before being used as test bacteria. Test bacteria then suspended into 0.9% sterile NaCl, bacterial
transmittance was measured 25% transmittance (equal to the density of $10^8$), at 580 nm wavelength spectrophotometer in which 0.9% sterile NaCl was applied as blank.

The antibacterial activity was performed as an inhibition zone, was applied using the disc diffusion Kirby Bauer method [20]. In this method, MHA media was enriched with $10^8$ CFU/mL test bacterial MRSA, and a disc (diameter 6 mm) contain methanol extract of Pyura sp was placed into. A clear zone that occurs outside disc as responding to inhibition to a growth of test bacterial was measured after 24 h and 48 h incubation times at 37°C. A diameter of the inhibition zone was measured using Vernier caliper.

3. Result and Discussion

3.1. Inhibition zone

The result of the inhibition zone is shown in Table 1 and Figure 1. As indicated in the Table diameter of inhibition zone at all concentrations of crude extract methanol Pyura sp increase from incubation time 24 h to 48 h. This indication for the biological activity of this extracts in inhibiting the growth of MRSA. As also described in the Table, no change in diameter of inhibition zone in controls. The highest activity occurred at concentration 20% extract that of 14.00 mm (24 h) and 14.50 mm 48h. No inhibition zone in negative control and 7 mm in the positive control (Table 1 and Figure 1).

| Concentration | The diameter of Inhibition Zone (mm) |
|---------------|-------------------------------------|
|               | 24 h   | 48 h   |
| 10%           | 11.50  | 14.00  |
| 15%           | 11.50  | 13.50  |
| 20%           | 14.00  | 14.50  |
| C (+)         | 7.00   | 7.00   |
| C (-)         | -      | -      |

Note: C (+) = positive control (Amoxicillin), C (-) = negative control

Figure 1. The diameter of the inhibition zone of crude extract of Tunicate Pyura sp against MRSA incubation (a) 24 h and (b) 48 h.
4. Discussion
The result of the inhibition zone in this study reveals that methanol extract from *Pyura sp* can inhibit the growth of *Staphylococcus aureus* resistant antibiotic from methicillin group. Data indicated an increase of inhibition zone with an increase of incubation time, this in accordance with [21], which is the higher concentration on antimicrobial, the ability to control growth or even kill microorganism. With an increase of concentration, bioactivity of an active compound is also increased. Hence its ability to inhibit the growth of MRSA is also increased.

Amoxicillin belongs to penicillin beta-lactam group, widely used to combat various infection caused by bacteria. However, MRSA has proven to resist to this antibiotic group. As shown in the positive control, the diameter of inhibition shown is 7 mm, indicating that this group is positive MRSA, as stated by NCCLS, that antibiotic is categorized resistant where inhibition zone is ≤ 9 mm.

According to [22], an antibiotic is categorized effectively where the diameter of the inhibition zone towards bacteria is ≥ 14 mm and not effective where ≤ 9 mm, respectively. The methanol extract of *Pyura sp* is having a potency to be a candidate of antibiotic towards MRSA. As previously described, many resistant cases towards the antibiotic beta-lactam group which is used to handle an infection caused by *S. aureus*. Resistance *S. aureus* to methicillin group happened due to plasmid bacteria that carry gen blaZ that codes beta-lactamase. Besides, the resistance of *S. aureus* is also influenced by the expression of Penicillin-Binding Protein 2a (PBP-2a) that efflux penicillin group out of the cell [23].

In the present study, an increase of the diameter of inhibition zone during incubation may indicate of bactericidal activity of methanol extract from tunicate *Pyura sp*. An antibacterial as an antibiotic agent was grouped based on how it works that of bactericidal and bacteriostatic. Bactericidal is an antibiotic that actively kills pathogen bacteria and bacteriostatic is only inhibiting the growth of bacteria.

The ability of a crude extract of *Pyura sp* in inhabiting test bacteria even killing MRSA is indicating that this organism is having potency in an antimicrobial compound. This is in accordance with previous findings that marine tunicates can produce various secondary metabolites as antimicrobes. Furthermore, symbiotic bacteria of tunicates can also produce secondary metabolites such as alkaloid, flavonoid, and steroid. In addition, a compound such as 4-methoxypyrrole, methanol, ethanol, butane, and hexane was found in tunicates [2].

Several tunicates contain active compounds including dehydrotyrosyl or dopa- and topa-dehydrodophyl peptide in their blood cell or other tissue, one of them is ferreascidin in *Pyura sp*. Dopa- or topa- peptide have unusual biology activities and antibiotic activities through chemical defense, enzyme inhibitor, free radical mediator, recovery bound and cross structure of exoskeleton [8].

5. Conclusion
The methanol extract of marine tunicate *Pyura sp* is potency as anti-bacteria against MRSA (methicillin-resistant *Staphylococcus aureus*) at the concentration of 10%, 15% and 20%, and categorized as bactericidal.

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