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Antibacterial Activity of Marine Sponge (*Stylotella sp.*)

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Abstract: Sponges are marine biota that makes up coral reefs that live on the bottom of the waters and have an important role in marine ecosystems. Sponges have bioactive components that have not been widely used by the community. This study aimed to analyze the content of secondary metabolites and the antibacterial activity of the sponge Stylotella sp. antibacterial activity test against Gram-negative (-) *Escherichia coli* and Gram-positive (+) *Staphylococcus aureus* was carried out using the good diffusion method. The results of antibacterial testing with various concentrations of 50, 100, and 150 ppm of the isolates showed that the zone of inhibition against E. coli was 11.3; 12; and 13 mm while for *S. aureus* was 12.3; 13; and 14 mm. The results of antibacterial testing with a concentration of 600 ppm of sponge extract showed an inhibition zone on *E. coli* and *S. aureus* in the amount of 13.3 and 14 mm, respectively. The test results showed that the sponge extract and isolate were in the intermediate category and were resistant to *E. Coli* bacteria. and *S. Aureus*

Keywords: Antibacterial; *Escherichia coli*; Sponge extract; *Staphylococcus aureus*

Introduction

Infectious diseases are still the main cause of high mortality rates, especially in developing countries such as Indonesia. This disease is caused by a bacterial attack that attacks humans. Based on data in 2022, there are around 1.27 million people die each year due to drug-resistant infectious diseases (Kemenkes RI, 2020). Treatment for infectious diseases is the use of antibiotics. The high cases of this infectious disease resulted in the high use of antibiotics. Inappropriate use of antibiotics can cause bacterial resistance to antibiotics (Denk-Lobnig & Wood, 2023). Therefore, the search for new antibacterial compounds needs to be done by prioritizing compounds sourced from natural ingredients, one of which is by utilizing the diversity of marine life (Muharni et al., 2015).

More or less 10,000 pharmacologically bioactive compounds have been derived from marine invertebrates such as tunicates, soft corals, sea hares, nudibranchs, bryozoans, sea slugs, sponges, and other marine organisms (Kiran et al., 2014). The sponge and their associated microbes (bacteria, fungus, and actinomycetes) produce many pharmacologically and chemically diverse compounds like peptides, alkaloids, steroids, terpenoids, sesquiterpenes, macrocyclic lactones, polyketides, phosphatidylcholines, triterpenoids, glycoproteins, biopolymers, macrolides, acetogenins, polyacetylenes and tannins (Kumar & Adki, 2018). One type of sponge that has the potential for bioactive compounds is *Stylotella sp.* The secondary metabolites that have been isolated from the sponge *Stylotella sp*. and showed antibacterial activity, as previously studied by (Yoghiapiscessa et al., 2016) getting high antibacterial activity against bacterial species *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherecia.coli*, *Candida albicans* and *Staphylococcus aureus* (Sun et al., 2015).

Another group of secondary metabolites that have also been isolated from the genus *Stylotella aurantium* is from the alkaloid, hymenin, and z-axinohydantoin, and their activity was tested against cancer cells (Ruocco et al., 2017). In addition, *Stylotella aurantium* sponge was also isolated by producing a compound Palauamin which has anticancer activity against several cancer cells such as leukemia cancer cells with an IC_{50} of 0.1 g/mL and lung cancer cells with an IC_{50} of 0.2 g/ mL (Musman et al., 2001).

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Hence, the present study was carried out to find out the antibacterial potential of the secondary metabolites of *Stylotella sp*. from the Sumbawa coast. Due to there being no previous reports of both of them being of the same type from that place (Qi et al., 2017).

Method

Samples of *Stylotella sp* were collected in February 2022 from Maluk Coast Sumbawa West Nusa Tenggara Indonesia. Sponge *Stylotella sp* (500 g) was extracted at room temperature with acetone (3x 24 h) to give a dark green extract (85 g). A portion of the extract weighing 20 g was subjected to vacuum liquid chromatography (VLC) using 150 g of silica gel as the stationary phase. Elution was performed using a series of n-hexane: EtOAc solvent mixtures with increasing polarity ratios, namely 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, and 1:4. TLC analysis was used to guide the combination of fractions, resulting in the identification of 10 fractions denoted as F1 to F10. The terpenoid components were found to be present in fractions F3, F6, and F7. Fraction F7 (3 g) was re-fractionated using the same method and eluted with $CHCl_3$-EtOAc (9:1, 4:1, 7:3. 3:2, 1:1, 2:3, 3:7, and 1:4) to give 4 sub-fractions F71-F74. Sub-fraction F73 (450 mg), purified by CPC eluted with *n*-hexane-EtOAc 8:2, yielded compound 1 (240 mg). Fraction F6 (860 mg) was also fractionated by VLC (eluents *n*-hexane-EtOAc of increasing polarity 9:1, 4:1, 7:3. 3:2, 1:1, 2:3, 3:7, and 1:4) to afford 6 subfractions F61-F66. Purification of F64 with CPC (eluents n-hexaneEtOAc 8:2) gave compound 2 (18 mg). Compound 3 (20 mg) was obtained by employing the identical purification technique on F3 (640 mg) using an eluent consisting of a mixture of n-hexane and CHCl3 in a ratio of 7:3 (S. S. Nair et al., 2013).

FT-IR spectra were measured with a Bruker Alpha instrument and ^{1}H and ^{13}C-NMR spectra with an Agilent DD2 system operating at 500 (^{1}H) and 125 (^{13}C) MHz using tetramethyl silane (TMS) as a reference standard. High-resolution mass spectra were obtained with an ESI-TOF Waters LCT Premier XE mass spectrometer. Vacuum liquid chromatography (VLC) and centrifugal planar chromatography (CPC) were carried out using Merck silica gel 60 GF254 art. 7731 and 7749, respectively. For thin-layer chromatography (TLC) analysis, precoated silica gel plates (Merck Kieselgel 60 GF254, 0.25 mm thickness) were used.

Result and Discussion

Identification of secondary metabolite structure was carried out using an NMR spectrophotometer (^{1}H-NMR and ^{13}C-NMR) for compounds isolated from isolates F2.d.11. Based on ^{1}H-NMR spectral data, two proton signals appear at a chemical shift of 7.77 ppm (2H, dd, *J*= 3.3 Hz), and H 7.65 ppm (2H, dd, *J*=3.35 Hz) which indicates the presence of protons from aromatic compounds. The *J* coupling provides important information about the position of the functional group in a compound (Velema et al., 2013). These two proton signals are characteristic of ortho-coupled aromatic protons. Each proton appears as a doublet-doublet signal, so it is suspected that the two protons are cleaved by two neighboring protons with different chemical environments. Besides appearing in the aromatic region, a proton signal also appeared in the δ_H 4.42 (4H, m) ppm region indicating the presence of methylene protons. At δ_H 4.42 ppm this is a typical signal for the CH_2 group which is bound to the heteroatom, namely the O atom in the form of an ester and has a neighboring proton in the form of an aliphatic chain. The methylene proton also appears in the δ_H 2.89-2.85 (s, t, 2H, 2H J=2.2 Hz) ppm region and in the H shift 1.73-0.87 (8H, m) and appears as an aliphatic which is a signal for two methyl groups (CH_3) coupled by neighboring protons. However, protons in the δ_H 4.42 ppm region are more shielded (unprotected) than δ_H 1.73-0.87 ppm. The methylene group in this region is directly bonded to the O atom (Rajamanikyam et al., 2017).

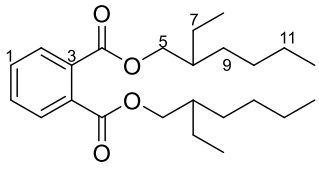

Figure 1. The structure of bis-(2-ethyl hexyl) phthalate

The ^{13}C-NMR spectrum data for isolating showed the presence of 12 carbon signals. Based on the ^{13}C-NMR spectrum data, the appearance of 2 methyl carbons at chemical shift (δ_C 11.3 and 14.3) ppm, 4 methylene carbon signals (δ_C 30.15; 29.69; 24.48; 23.64) ppm, 1 methine alcohol carbon signal (δ_C 68.29) ppm, 3 aromatic carbon signal (δ_C 133.4; 132.0; 129.6) ppm and 1 ester carbon signal (-COO-) at δ_C 168.0 ppm. The data results from the ^{1}H-NMR and ^{13}C-NMR spectra mutually reinforce the possibility that the compound formed is a bis-(2-ethyl hexyl) phthalate framework. Based on the spectroscopic data that has been identified in isolate F2.d.11, it is suspected that it is a bis-(2-ethyl hexyl) phthalate compound. This compound was previously found in the sponge Halicondria sp. sea origin in China (Xiu et al., 2012).

The antibacterial activity of the sponge extract and bis (-2-ethyl hexyl) phthalate compound was carried out by measuring the diameter of the inhibition zone for the growth of *E. coli* and *S. aureus* bacteria at various concentrations (Rasyid, 2008). The diameter of the

inhibition zone is the activity of a compound in the tested bacterial media, the larger the clear zone, the greater the antibacterial activity. Based on the data, it can be seen that the presence of antibacterial activity of bis (-2-ethyl hexyl) phthalate with concentrations of (50, 100, and 150) ppm had different diameters of inhibition zones. The highest inhibition zone produced by isolates against *E. coli* and *S. aureus*, respectively, was at a concentration of 150 ppm with an average of 13 and 14 mm. Based on these results, it can be seen that the diameter of the inhibition zone is influenced by concentration. The increase in compound concentration is directly proportional to the ability to inhibit bacterial growth of *E. coli* and *S. aureus*. The largest inhibition zone against bacteria *E. coli* and *S. aureus* were 13.3 and 14 mm, respectively. Based on CLSI data, (2012) the antibacterial inhibition of extracts and compounds was classified as resistant and intermediate because the diameter formed was 11-14 mm (Presson et al., 2021).

Table 1. NMR data of F2d.11 in $CDCl_3$

No.	(δ)^{1}H-NMR, (*J*) Hz	((δ) ^{13}C- NMR (*J*) Hz	(δ)*^{1}H-NMR (*J*) Hz	(δ)*^{13}C-NMR (*J*) Hz
1	7.77 (2H, dd, 3.3)	133.4	7.70 (2H, m)	132.4
2	7.65 (2H, dd, 3.35)	132.0	7.50 (2H, m)	130.9
3	-	129.6	-	128.8
4	-	168.0	-	168.8
5	4.24 (4H, m)	68.2	4.25 (2H, d, 5)	68.1
6	2.89 (2H, s)	39.6	-	38.7
7	2.85 (2H, 2.2)	31.6	-	30.3
8	-	29.6	-	28.9
9	-	24.4	-	22.9
10	-	23.6	-	23.7
11	1.73 -132 (15H, m)	14.3	1.80-1.20 (18H, m)	14.0
12	0.96- 0.90 (9H, m)	11.3	1.0-0,75 (12H, m)	10.9

Sun et al. (2007)

The inhibition zone produced by *Stylotella sp*. extracts larger than bis(-2-ethyl hexyl) phthalate. The difference in inhibition zones was caused by the secondary metabolites contained in the sponge extract having a synergistic effect according to each mechanism of action in inhibiting the antibacterial activity of the compounds present in the extract. The synergistic effect is the ability of several compounds such as flavonoids, saponins, steroids, and triterpenoids to combine to fight bacteria (Tang & Zheng, 2018). The synergistic effect of these compounds causes the extract to be more active as an antibacterial than the bis(-2-ethyl hexyl) phthalate. Other factors that affect the diameter of the inhibition zone of the extract such as the level of sensitivity of the test organism, the rate of diffusion of the antibacterial compound, and the concentration of the antibacterial compound (Dharmayani et al., 2016).

The results showed that the inhibition zone produced by bis (-2-ethyl hexyl) phthalate against *S. aureus* was higher than *E. coli*, this was related to the chemical structure of the bis (-2-ethyl hexyl) phthalate compound. The ester functional group is known to improve the biological activity of drugs and this has been linked with various chemical properties of this functional (Matshwele et al., 2022). This bis (-2-ethyl hexyl) phthalate compound also has a benzene ring which makes it able to penetrate the lipid layer in the bacterial wall (Dharmayani et al., 2020). This causes the cell walls of Gram-positive bacteria to be more easily damaged than gram-negative bacteria by bis(-2-ethyl hexyl) phthalate. In Gram-negative bacteria, outer membranes are typically rich in lipopolysaccharides, which are formed of a conserved lipid and variable O-antigen polysaccharide chain (3) (R. Nair et al., 2017). In both Gram-positive and Gram-negative bacteria, capsular polysaccharides (CPS) form a thick outer layer around bacterial cells, while exopolysaccharides are released in the surrounding medium (4). Finally, in Gram-positive bacteria, wall teichoicacids (WTAs) or cell wall polysaccharides (CWPSs) are covalently anchored onto peptidoglycan and partly embedded inside the peptidoglycan layer(Brennan et al., 2008).

The difference in the zone of inhibition between *S. aureus* and *E. coli* was also caused by the polarity of the bis (-2-ethyl hexyl) phthalate compound. Antimicrobial activity which is more hydrophobic (nonpolar) has effectiveness in inhibiting the growth of gram-negative bacteria because the outer membrane contains lipopolysaccharide (Dharmayani, 2019). The ability of nonpolar compounds to inhibit bacteria is thought to be because nonpolar compounds can cause cell membrane composition and cell membrane dissolution, so cell membranes are damaged (Peng et al., 2019).

The test results for bis (-2-ethyl hexyl) phthalate showed that the compound is active in inhibiting *E. coli* and *S. aureus* bacteria as indicated by the formation of an

inhibition zone on the test medium(Wangkanusa et al., 2016). In addition, gram-positive bacteria have a thicker layer of peptidoglycan than gram-negative bacteria. This thicker peptidoglycan layer causes the cell wall permeability of gram-positive bacteria to be lower so it is difficult for antibacterial compounds to penetrate the cell membrane (Carroll et al., 2016).

Conclusion

Bioactivity test of the sponge extract *Stylotella sp*. showed antibacterial activity in inhibiting *E. coli* and *S. aureus* bacteria. In addition, the compound bis (-2-ethyl hexyl) phthalate isolated showed antibacterial activity in inhibiting the growth of E. coli bacteria with the largest inhibition zone of 13 mm and S. aureus bacteria with the largest inhibition zone of 14 mm which was included in the intermediate category.

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Author Contributions
Ni Komang Tri Dharmayani, the primary author, made significant contributions to research design, execution, and the composition of research articles. The second author, Isnaini, provided guidance from the research stage to the writing phase of the articles. Maria Ulfa, the third author, assisted in implementing the research and preparing the necessary instruments for data collection. Sudirman, the fourth author, aided in the collection of data. Emmy Yuanita, the fifth author, played a role in guiding the article's writing process. The sixth author, Baiq Nila Sari Ningsih, contributed to the analysis and interpretation of the research findings. All authors have thoroughly reviewed and consented to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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