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BIOASSAY-GUIDED ISOLATION OF AN ANTIBACTERIAL COMPOUND FROM THE INDONESIAN SOFT CORAL *Sarcophyton trocheliophorum*

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Abstract

With the aim of searching for new antibacterial compound from marine soft corals, the investigation had been conducted on antibacterial activity of an extract from soft corals *Sarcophyton trocheliophorum* in the *n*-hexane, ethyl acetate, *n*-butanol, and aqueous fractions. The antibacterial activity was tested against two Gram-positive bacteria, *viz*. *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923), and two Gram-negative bacteria, *viz. Escherichia coli* (ATCC 25922) and *Vibrio cholerae* (ATCC 14035) using the agar disc diffusion assay. Among them, the n-hexane fraction was the most active against three tested bacteria, *viz. Bacillus subtilis*, *Staphylococcus aureus* and *Vibrio cholerae* at the concentration 125 µg/ml, with inhibition zone 14.2, 18.2, 13.8 mm, respectively. Isolation and purification of the active component from the n-hexane fraction led to a known cembranoid-type diterpene, sarcophytoxide. The chemical structure of the isolated compound was determined by IR, MS and NMR, as well as compared to data from the literature. Sarcophytoxide showed moderate activity against *B. subtilis*, *S. aureus* and *V. cholerae,* with a minimum inhibitory concentration (MIC) of 125, 100, 125 mg/ml, respectively.

Keywords: antibacterial, cembranoid, soft coral, *Sarcophyton trocheliophorum*

1. Introduction

Soft corals (subclass Octocorallia, order Alcyonacea) are widely distributed along tropic and subtropic oceans. These organisms are known to produce a broad array of chemical compounds, particularly sesquiterpenoid, diterpenoids and steroids. Currently, more than 20 publications have reported on the chemical compounds from Indonesian soft corals such as *Lobophytum* sp., *Cladiella* sp., *Sarcophyton* sp., and *Sinularia* sp, dominated by the cembrane-type of diterpenes (Putra & Murniasih, 2016).

Soft corals of the genus *Sarcophyton* are well recognized as a rich source of secondary metabolites including sterols, sesquiterpenoid, diterpenoids and other related metabolites (Tang, Sun, Zou & Yin, 2016; Zubair, Al-Footy, Ayyad, Al-Lihaibi & Alarif, 2015; Yao et al., 2012). Untill now, nearly 16 species of soft corals of the genus *Sarcophyton* have been chemically investigated from various geographical areas (Chen, Liang, Li, Xiao & Guo, 2016). Cembranoid-type diterpenes appeared to be characteristic constituents of the genus *Sarcophyton* and commonly described as chemical defense tools to protect soft corals against natural predators. Cembranoids are considerable interest and merit continuous attention as they exhibit a range of biological activities especially antibacteria, anti-inflammatory and cytotoxic (Ishii, Kamada & Vairappan, 2016; Lin et al., 2010; Pollastro et al., 2016).

In the frame of our ongoing screening for antibacterial compounds from Indonesian marine soft corals, there was an opportunity to analyze a specimen of the soft coral *Sarcophyton trocheliophorum*, collected from Selayar Island (South

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Sulawesi, Indonesia). The *n*-hexane, ethyl acetate, *n*-butanol, and aqueous fractions *Sarcophyton trocheliophorum* extract, have been evaluated for antibacterial activity against four pathogen bacteria *viz*. *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Vibrio cholerae* (ATCC 14035). The *n*-hexane fraction showed the highest antibacterial activity and led to the isolation of a known cembranoid-type diterpene, named sarcophytoxide as antibacterial compounds. The structure of sarcophytoxide was elucidated through extensive spectroscopic analyses including IR, NMR, MS techniques and comparison with literature data. Sarcophytoxide showed inhibitory activity against two Gram-positive bacteria, *viz. S. aureus* and *B. subtilis* and one Gram-positive bacteria *V. cholerae*.

2. Material and Methods

2.1. General Experimental Procedures and Chemical Reagents

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured by JEOL spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: dH 7.26, dC 77.0). ESIMS spectra were obtained by LC/MS (Waters Alliance 2695). Ethyl acetate, *n*-hexane, *n*-butanol, methanol, and dichloromethane were purchased from Merck (Darmstadt, Germany). All chemicals used were analytical grade. Silica gel 60 (Merck) as the stationary phase for column chromatography and plastic-backed plates coated with Si gel F254 (Merck) were used for TLC. Plates were visualized by spraying with vanillin-H_2S0_4 and warming.

2.1.1. Soft coral samples

Colonies of soft coral *Sarcophyton trocheliophorum* were collected by scuba diving in Selayar Island, South Sulawesi, Indonesia at a depth 10 m in June 2015. A voucher specimen was deposited in the Research Center for Oceanography-LIPI, under the registration number SLYR SC-2.

2.1.2. Extraction and Isolation

The colonies of *Sarcophyton trocheliophorum* (225 g) were repeatedly extracted with MeOH: CH_2Cl_2 (1:1) at room temperature to get the crude extract (5.2 g). The crude extract was partitioned based on the polarity of the solvent using *n*-hexane (non-polar), ethyl acetate (semi-polar), *n*-butanol (polar) and water. Each of fractions was subjected to preliminary phytochemical screening and antibacterial activity. The most active fraction was subjected to further purification using SiO_2 gel column chromatography eluted with a gradient system of increasing polarity from *n*-hexane to EtOAc to MeOH. Each of fractions SiO_2 gel column chromatography was evaluated for their antibacterial activity. Once fraction ST 2 showed potential antibacterial activity, then it will be further purified by SiO_2 column chromatography, eluted with *n*-Hexane: EtOAc (9:1) to afford sarcophytoxide (1.2 g).

2.2. Chemical Screening

All fractions were subjected to preliminary phytochemical screening test for the presence of secondary metabolites utilizing the standard conventional protocol described by Senguttuvan et al., 2014.

2.3. Antibacterial Assay

2.3.1. Agar diffusion test

Antibacterial activity of all fractions from crude extract of *Sarcophyton trocheliophorum* was conducted using agar disk diffusion method. Briefly, 125 µg/ml the sample was diluted in MeOH then about 20 µl was dropped into 6 mm diameter filter paper disc. The paper disc was then placed on a Mueller Hinton Agar (Himedia) in a petri dish that had been inoculated with test bacteria. Test bacteria used in this research, were two Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 14035*)*, two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633). Inhibition of bacterial growth activity appeared as a clear zone around the paper disc. The inhibition zone was observed after incubation at 37 °C for 20-24 h. 10 µg/ml ampicillin and methanol were used as a positive and solvent control, respectively. All the assays were performed in triplicate.

2.3.2. Microdilution method

The microdilution method was used to evaluate the minimum inhibitory concentration (MIC) of the bioactive compound which showed good activity (growth inhibition zone more than 9 mm) in the disc diffusion assay. Tests were performed in 96-well round bottom sterile culture plates using the Infinite® 200 PRO microplate reader (Tecan Austria GmbH). The assay plates were filled with Mueller-Hinton broth medium (MHB) in serial dilutions ranging from 6.25, 12.5, 25, 50, 100, 200, 250, 500 and 1000 mg/ml of isolated compound, ampicillin or methanol and the test microorganism (10^7 CFU/ml). The turbidity in each

Table 1. Chemical analysis of all fractions from the crude extract of *Sarcophyton trocheliophorum*

No	Chemical constituents	Fractions			
		n-Hexane	*n*-Butanol	Ethyl acetate	Aqueous
1	Alkaloids	+	-	+	+
2	Steroids	+	+	+	-
3	Flavanoids	-	-	-	-
4	Saponins	-	-	+	+
5	Terpenoids	+	+	+	-
6	Phenols	+	+	+	-
7	Tannins	-	-	-	-

well was measured at 600 nm, after 24 h incubation periods at 37 °C.

3. Results and Discussion

Chemical constituents from the crude extract of *Sarcophyton trocheliophorum* were showed in Table 1. In the present study, the chemical analysis of all fractions indicated the presence of alkaloids, steroids, triterpenoids, saponins, terpenoids, and phenols. The *n*-hexane, ethyl acetate, and *n*-butanol fractions from *Sarcophyton trocheliophorum* crude extract showed positive results for terpenoids and steroids. The presence of secondary metabolites in the fractions can provide a preliminary explanation of their antibacterial activities. Numerous articles reported the isolation of terpenoid compounds from the soft coral genus *Sarcophyton*, which displayed good biological activities such as anti-inflammatory, antibacterial and cytotoxic activity. Most of the isolated terpenoids were cembranoid-type diterpenes, which were found in high concentrations (up to 5% dry weight) in soft corals (Gross et al., 2003; Coll, 1992).

All fractions from *Sarcophyton trocheliophorum* crude extract showed antibacterial activity against Gram-positive (*B.subtilis* dan *S. aureus*) and Gram-negative (*E. coli* dan *V. chloreae*) bacteria (Figure. 1). The *n*-hexane fraction showed strong inhibition activity against *B. subtilis*, *S. aureus,* and *V. chloreae*, with inhibition zones of 14.2, 18.2, and 13.8 mm, respectively, while other fractions showed moderate activity against all tested bacteria. Differences were observed in the antibacterial activities of the fractions. These could be due to the differences in their chemical structures as well as the mechanism of activity of their bioactive constituents.

Furthermore, *n*-Hexane fraction, which shown potential antibacterial activity, has been purified by SiO_2 gel column chromatography using a gradient system of increasing polarity from *n*-hexane to EtOAc to MeOH. All fractions were analyzed by TLC, and those with similar profiles were combined to give fractions ST-1 to ST-21. Each of these fractions was evaluated for their antibacterial activity. The results showed that fraction ST-2 has a potential inhibitory activity against *S. aureus*, *B. subtilis*, and *V. cholerae*. This fraction was subsequently subjected to SiO_2 column chromatography, eluted with *n*-Hexane EtOAc (9:1), to afford a known cembranoid-type diterpene, sarcophytoxide (Figure 2). As a pure compound, this compound yielded 1.2 g as yellow crystalline needles.

The molecular formula of the compound was determined to be $C_{20}H_{30}O_2$ from the EI-MS (positive ions), with the molecular ion peak at *m/z* 325 $[M+Na]^+$ for $C_{20}H_{30}O_2Na$. The IR spectrum showed absorptions at 2930, 2851, 1664, 1446, 1383, 1035, 940 and 866 cm^{-1}. ^1H (500 MHz, $CDCl_3$, *J* in Hz): 5.54 (m, 1H, H-2), 5.22 (d, 1H, *J* =10.2 Hz, H-3), 5.09 (dd, 1H, *J* = 10.8, 5.1 Hz, H-11). 4.49 (s, 1H, H-16), 2.71 (t, 1H, *J* = 4.1, H-7), 2.6 (1H, overlapped, H-14b), 2.3 (1H, overlapped H-5), 2.2 (1H, overlapped, H-10a), 2.0 (1H, overlapped, H-9a), 1.9 (1H, overlapped, H-13), 1.9 (1H, overlapped, H-10b), 1.9 (1H, overlapped, H-6a), 1.81 (s, 3H, H-18), 1.64 (s, 3H, H-17), 1.6 (1H, overlapped, H-14b), 1.58 (s, 3H, H-20), 1.3 (1H, overlapped, H-6b), 1.26 (s, 3H, H-19) 1.0 (dt, 1H, *J* = 13.0, 2.9, H-

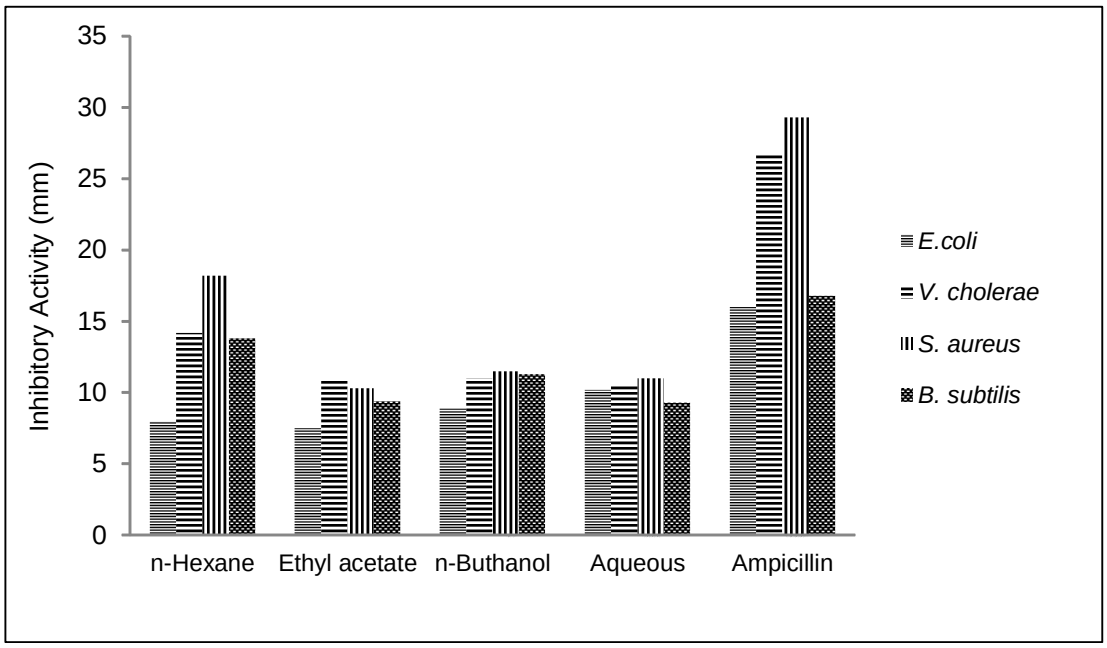

Figure 1. Antibacterial assay of all fractions from the MeOH-CH_2Cl_2 extract of *Sarcophyton trocheliophorum* using an agar disc diffusion method.

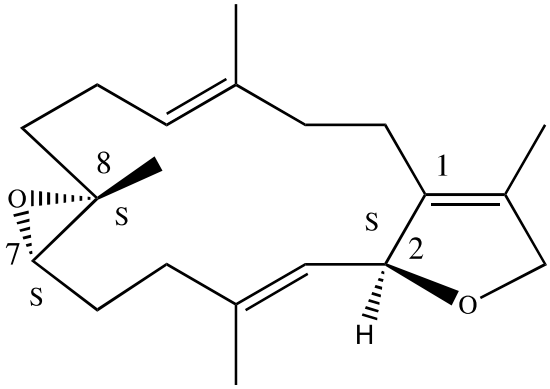

Figure 2. Sarcophytoxide.

9);[13]C (125 MHz, $CDCl_3$): 139.2 (C-4), 136.7 (C-12), 133.2 (C-1), 127.8 (C-15), 126.3 (C-3), 126.3 (C-11), 83.6 (C-2), 78.3 (C-16), 61.9 (C-7), 59.8 (C-8), 39.7 (C-9), 37.6 (C-5), 36.7 (C-13), 26.0 (C-14), 25.4 (C-6), 23.5 (C-10), 17.0 (C-19), 15.6 (C-18), 15.2 (C-20), 10.1 (C-17). Based on the physicochemical analyses, including IR, 1D NMR, MS, and their comparisons with data from the literature, the isolated compound was determined to be a known cembranoid type-diterpene, (2*S*,7*S*,8*S*)-sarcophytoxide (Bowden, Coll, Heaton, Konig & Scheuer, 1987; Nii et al., 2006; Pollastro et al., 2016).

A (2*S*,7*S*,8*S*)-sarcophytoxide was screened for antibacterial activity using agar disk diffusion. The results of the antibacterial activity of sarcophytoxide shown in Table 3 displayed potential inhibitory activity against *S. aureus, B. subtilis*, and *V. cholerae*. Further characterization of the antibacterial properties of sarcophytoxide by determining the MICs using the broth microdilution method was done (Table 3). In this work, sarcophytoxide showed moderate activity against two Gram-positive bacteria (*S. aureus* and *B. subtilis*) and actively against the Gram-negative bacteria *V. cholera*. It is known that Gram-negative bacteria are less sensitive to marine invertebrate extracts of subtropical or tropical species, while the Gram-positive bacteria were particularly sensitive (Fleury, Coll & Sammarco, 2006).

Table 3. Agar plate diffusion assay (zone inhibition in mm) and minimum inhibitory concentration of sarcophytoxide and ampicillin (MIC in mg/ml)

Microorganisms	Sarcophytoxide		Ampicillin	
	Zone of inhibition in mm	MIC in mg/ml	Zone of inhibition in mm	MIC in mg/ml
S. aureus	20.83	100	25.6	25
B. subtilus	19.58	125	26.1	25
E. coli	6.95	-	24	-
V. cholerae	19.68	125	25.3	25

4. Conclusion

Results of the present investigation showed that the *n*-hexane fraction from *Sarcophyton trocheliophorum* extract had potential antibacterial activity. Through bioassay-guided fractionation, a cembranoid-type diterpene, named (2S,7S,8S)-sarcophytoxide was isolated from this soft coral. In addition, its chemical structure was identified on the basis of its spectroscopic data and their comparisons with data from the literature. Sarcophytoxide showed moderate activity against two *S. aureus B. subtilis* and *V. cholera*. Furthermore, it is known that soft corals of the genus *Sarcophyton* has secondary metabolites such as sarcophytoxide causing allelopathic effects.

5. Acknowledgment

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