Astrogorgia sp. from Saparua, Maluku: Phytochemical Content, Antimicrobial, Antioxidant, and Cytotoxicity Properties

M T Sibero\textsuperscript{1,2*}, D S Zilda\textsuperscript{3}, D Haryanti\textsuperscript{1}, Y Igarashi\textsuperscript{4}

\textsuperscript{1} Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Diponegoro, Jl. Prof. Soedarto, SH., Semarang, Central Java, Indonesia
\textsuperscript{2} Natural Product Laboratory, Integrated Laboratory for Research and Services, Universitas Diponegoro, Jl. Prof. Soedarto, SH., Semarang, Central Java, Indonesia
\textsuperscript{3} Research and Development Center for Marine and Fisheries Product Processing and Biotechnology, St. KS. Tubun Petamburan VI, Jakarta 10260, Indonesia
\textsuperscript{4} Biotechnology Research Center, Department of Biotechnology, Toyama Prefectural University, Imizu, Toyama, Japan

Email: madatriandalasibero@lecturer.undip.ac.id

Abstract. Gorgonian is one of marine invertebrates that is still underexplored as a source of bioactive compounds. This study aimed to discover the biological properties of Astrogorgia sp. and its phytochemical content. A consecutive extraction method using \textit{n}-hexane, ethyl acetate and methanol was conducted to obtain secondary metabolites from the sample. Antimicrobial assay was performed against ESBL \textit{E. coli}, MRSA, \textit{C. albicans}, and \textit{M. furfur}; cytotoxicity against P388 Murine Leukaemia Cancer Cells, antioxidant was tested using DPPH method. The consecutive extraction method gave yield (\%) as follows: 0.21 ± 0.22 from \textit{n}-hexane; 0.67 ± 0.17 from ethyl acetate; and 1.20 ± 0.50 from methanol. All fractions gave positive results on antibacterial assay against all pathogens while only gave antifungal activity against \textit{C. albicans}. Methanol fraction had the highest antioxidant activity, while \textit{n}-hexane fraction showed the best cytotoxicity.

1. Introduction
Sessile marine invertebrates produce unique chemical substances to protect themselves from predator and environmental stresses [1]–[3]. These chemical substances steal attention due to their beneficial biological activity for humans. It is proven by the FDA’s approval of several drugs that are originated from marine invertebrate such as ascidian, bryozoan, and sponge [4]. Among all reports, sponge has been emphasized as the most profiling marine invertebrate since many studies successfully isolated bioactive compounds from it [5]. Nonetheless, other invertebrates such as gorgonian become neglected. Gorgonian is a member of Alcyonacea (soft corals), which is characterized by always have eight tentacles (octocoral) in their polyps with rows of pinnules along both sides of the tentacles [6]. This animal is commonly found in almost all marine environments from shallow-water, mesophotic to the deep sea; therefore, plenty of studies were conducted to discover their biodiversity in Indonesia [6], [7]. However, the study of biological activity of Indonesia’s gorgonian is rarely reported. The latest study
was conducted by Teffu et al. [8] only reported the phytochemical content of three gorgonians from Wailiti Maumere East Nusa Tenggara.

Although Indonesia has various gorgonian species, the biological activity and bioactive compounds are also less reported than other soft corals [9]. Hence, this study was conducted to obtain secondary metabolites from Astrogorgia sp. then characterize their phytochemical content and screen their biological properties.

2. Methodology
2.1. Sample collection
Sample Astrogorgia sp. was accidentally snagged while sampling tunicates in Saparua water, Maluku, Indonesia in September 2018 [10]. The sample was identified as Astrogorgia sp. according to Tutty et al. [6]. Sample was washed using clean seawater, kept in a Ziploc plastic then brought to Tropical Marine Biotechnology Laboratory for metabolite extraction.

2.2. Metabolite extraction
Astrogorgia sp. was shade-dried at room temperature to prevent metabolite decomposition. A consecutive extraction method was conducted to extract the secondary metabolite [11]. In total 150 g of sample was resized then put into an Erlenmeyer flask. An adequate 250 mL of n-hexane was added and sat overnight on an orbital shaker (100 r.p.m.) to obtain a non-polar fraction. After that, the solvent was separated from the sample for evaporation. The sample residue was re-extracted again using ethyl acetate with the same condition to obtain the semi-polar fraction. The ethyl acetate was separated then evaporated; while the residue was re-extracted again using methanol to obtain the rest metabolites. The solvent was separated then evaporated; hence three fractions were obtained through the process. The dried fractions were stored at -20 °C for the subsequent analysis and bioassays. The yield was calculated by this simple calculation:

\[
\% \text{Yield} = \frac{\text{Extract weight} (g)}{\text{Sample} (g)} \times 100
\]

2.3. Antimicrobial assay
Antimicrobial assay was conducted against extended-spectrum β-lactamase (ESBL) E. coli, methicillin-resistant Staphylococcus aureus (MRSA), Candida albicans and Malassezia furfur. The bacteria were refreshed on Nutrient Agar, whereas the dermatophytes on Potato Dextrose Agar for 24 h before the assay. Antimicrobial assay was conducted according to Sibero et al. [12] using Kirby-Bauer method. The fractions were dissolved in dimethyl sulfoxide (DMSO) to obtain these following concentration: 2 mg/mL, 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL and 0.25 mg/mL. Nalidixic acid (50 µg/mL in DMSO) was applied as control positive for antimicrobial assay, while Cyclheximide (50 µg/mL in DMSO) for antifungal assay. A clear zone surrounding the paper-disc indicates a positive result.

2.4. Antioxidant assay
A common 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical method was conducted to determine the antioxidant activity of the fractions [13]. DPPH stock solution was prepared by dissolving 3.94 mg DPPH crystal into 10 mL of absolute methanol to reach the final concentration of 0.1 mM. The fractions were dissolved in absolute methanol to obtain the final concentration: 200, 400, 600, 800, and 1000 ppm. An adequate 160 µL of sample and 40 µL of DPPH stock were transferred into a 96-well plate then mixed gently. The plate was incubated in an incubator (37 °C) with a dark condition for 30 mins to trigger the chemical reaction. Afterward, the plate was analysed using a microplate reader with three replications at 517 nm. The DPPH scavenging effect was calculated using the following formula, while the determination of IC₅₀ value using a linear regression method.

\[
\text{DPPH Scavenging effect} = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100
\]
2.5. Cytotoxic assay
Cytotoxic effect of the fractions was conducted against P388 Murine Leukaemia Cancer Cells according to the XTT method that has been published in our previous works [14]–[17]. Five concentrations as follows 0.0002; 0.002; 0.02; 0.2 and 2 mg/mL were prepared by diluting the fractions in DMSO. To guarantee the result, Doxorubicin was used as positive control and DMSO as negative control. The IC₅₀ value was calculated using a linear regression method.

2.6. Phytochemical test
The presence of alkaloid, flavonoid, tannin, phenol hydroquinone, steroid/triterpenoid and, saponin in each fraction was detected using a phytochemical test. This qualitative assay was done according to our previous works [12], [14]–[16].

3. Result and Discussion
Metabolite extraction is a preliminary step to obtain the secondary metabolite from Astrogorgia sp. Secondary metabolite is defined as small organic compounds produced by living organisms to protect themselves from predator and environmental stress [18]. In order to obtain the target substance, a consecutive extraction method was carried out. Table 1 shows the yield from each solvent.

| Fraction      | Yield (%)   |
|---------------|-------------|
| n-Hexane      | 0.21 ± 0.22 |
| Ethyl Acetate | 0.67 ± 0.17 |
| Methanol      | 1.20 ± 0.50 |

The compound’s polarity is strongly related to the ability of the solvent to extract the targeted compounds. Data shows n-hexane gave the lowest yield then follow by ethyl acetate, whereas methanol extracted more substances and provided the highest product. Hence this result suggested that the major compounds from Astrogorgia sp. are polar compounds and dissolved in methanol. This result was similar to the previous works [19], [20] that reported methanol provided the highest extract yield from various samples. In addition, Teffu et al. [21] also stated in a consecutive extraction method using n-hexane, ethyl acetate and methanol for Rumphella and Hicksonella (gorgonian), methanol gave the highest yield, followed by ethyl acetate and n-hexane. Further, the phytochemical content of each fraction is shown in Table 2.

| Phytochemical Test       | n-Hexane | Ethyl Acetate | Methanol |
|--------------------------|----------|---------------|----------|
| Alkaloid                  | +        | +             | +        |
| Flavonoid                 | -        | -             | -        |
| Tannin                    | -        | -             | -        |
| Phenol hydroquinone       | -        | -             | +        |
| Steroid/Triterpenoid      | +        | +             | +        |
| Saponin                   | +        | -             | +        |

(+) indicates positive result; (-) indicates negative result

Phytochemical test gave various results for each fraction. Methanol fraction contained alkaloid, phenol hydroquinone, steroid/triterpenoid and saponin; ethyl acetate fraction gave positive results to alkaloid and phenol hydroquinone; while n-hexane had alkaloid, steroid/triterpenoid, and saponin. Teffu et al. [21] stated that methanol fraction from Rumphella and Hicksonella (gorgonian) possessed alkaloid, phenol hydroquinone, steroid, triterpenoid, and saponin; ethyl acetate fraction contained flavonoid, steroid, triterpenoid, and saponin; while n-hexane had alkaloid, flavonoid, phenol hydroquinone, steroid, triterpenoid, and saponin. Phytochemical content in a sample is impacted by various factors such as the suitable solvent, pretreatment, species, extraction technique, and so on [11], [12], [14]. The existence of
secondary metabolites in a sample is correlated to its biological activity. Table 3 presents the antimicrobial property of *Astrogorgia* sp.

Table 3. Antimicrobial activity of *Astrogorgia* sp. against human pathogens

| Pathogens  | ESBL *E. coli* | MRSA | *C. albicans* | *M. furfur* |
|------------|---------------|------|---------------|-------------|
| Concentration (mg/mL) | n-Hexane | Ethyl Acetate | Methanol |
| 2.0        | 8.35 ± 0.07  | 12.05 ± 0.35 | 13.55 ± 0.07 |
| 1.5        | 6.50 ± 0.14  | 10.10 ± 0.14 | 11.65 ± 0.35 |
| 1.0        | 3.35 ± 0.21  | 9.25 ± 0.21  | 10.70 ± 0.14 |
| 0.5        | 0.00 ± 0.00  | 7.45 ± 0.35  | 9.45 ± 0.07  |
| Control +  | 13.45 ± 0.35 | 12.60 ± 0.21 | 13.00 ± 0.14 |
| DMSO       | 0.00 ± 0.00  | 0.00 ± 0.00  | 0.00 ± 0.00  |

All fractions demonstrated antimicrobial activity against ESBL *E. coli*, MRSA, and *C. albicans*. The highest antimicrobial activity on ESBL *E. coli* and *C. albicans* was performed by methanol fraction (2 mg/mL); while ethyl acetate fraction gave the most potent antibacterial effect to MRSA. Antioxidant property of the fractions is shown in Figure 1.

**Antioxidant Activity of *Astrogorgia* sp.**

![Antioxidant Activity Graph](image)

**Figure 1.** Antioxidant activity of *Astrogorgia* sp. through DPPH free radical scavenging
Antioxidant assay on *Astrogorgia* sp. fractions indicated that methanol fraction gave the best property with IC$_{50}$ value of 462.12 ± 35.75 ppm, followed by ethyl acetate fraction with IC$_{50}$ value of 662.75 ± 21.57 ppm, and the weakest activity was shown by n-hexane fraction with IC$_{50}$ value of 669.30 ± 35.04 ppm. Nonetheless, the antioxidant property of ethyl acetate and n-hexane fractions was not statistically different ($p < 0.5$). Haerani *et al.* [22] stated that the IC$_{50}$ value > 200 ppm is categorized as a weak activity. Therefore, *Astrogorgia* sp. is not suggested to be the source of antioxidants.

**Figure 2.** Cytotoxicity of *Astrogorgia* sp. fractions against P388 Murine Leukaemia Cancer Cells

Figure 2 shows the n-hexane fraction of *Astrogorgia* sp. gave the lowest IC$_{50}$ with value of 78.60 ± 9.3 µg/mL, followed by methanol fraction with IC$_{50}$ value of 159.96 ± 28.22 µg/mL. In comparison, the highest IC$_{50}$ belonged to ethyl acetate fraction with IC$_{50}$ value of 854.24 ± 17.90 µg/mL. The IC$_{50}$ value means the needed concentration of a particular substance to inhibit cell growth as much as 50%, hence the lower IC$_{50}$ value indicates the stronger the anticancer activity. [23]. The n-hexane fraction had the lowest IC$_{50}$ value against P388 murine leukaemia cancer cells, therefore this fraction is noted as the most potent candidate for anticancer.

Biological properties of *Astrogorgia* sp. is strongly correlated to the phytochemical content in each fraction. Alkaloid derivative compounds were reported to have various biological activities such as anticancer, antimicrobial and, antioxidant [24], [25]. Flavonoid, tannin, and phenolic compounds are known as a strong antioxidant agent due to their ability to donor their proton to stabilize the free radical [22], [26]–[28]. Moreover, marine terpenoid, saponin, and steroid derivative compounds are known as potential antibacterial and anticancer agents [29], [30]. This information explains the biological property in *Astrogorgia* sp.fraction. The presence of alkaloid, steroid/triterpenoid, and saponin in the fractions gave potent antimicrobial and cytotoxic properties, while the absence of flavonoid and phenolic compounds led to the weak antioxidant activity. This study has discovered the potential of Indonesia’s *Astrogorgia* sp. as an antimicrobial and anticancer source. Also, it is suggested to perform bioguided-isolation method to isolate the lead compounds.

**4. Conclusion**

*Astrogorgia* sp. from Saparua, Maluku contained various secondary metabolites responsible for the biological properties such as antimicrobial and anticancer. The n-hexane fraction contained alkaloid, steroid/triterpenoid, and saponin; ethyl acetate fraction gave positive results to alkaloid and phenol hydroquinone. On the other hand, methanol fraction contained alkaloid, phenol hydroquinone, steroid/triterpenoid, and saponin. It was noted that all fractions had antibacterial activity against ESBL *E. coli* and MRSA, as well as antifungal against *C. albicans*. The n-hexane fraction showed the most potent anticancer property against P388 murine leukaemia cancer cell with IC$_{50}$ value of 78.60 ± 9.3 µg/mL.
Reference

[1] Wang C, Liu H, Shao C, Wang Y, Li L, and Guan H 2008 Shengtai Xuebao/ Acta Ecol. Sin., 28(5): 2320–2328.

[2] Pawlik J R 2012 Handbook of Marine Natural Products 677–710.

[3] Rohde S, Nietzer S, Schupp P J 2015 PLoS One 10(7): 1–19.

[4] Pereira F 2019 Expert Opin. Drug Discov., 14(8): 717–722.

[5] Carroll A R, Copp B R, Davis R A, Keyzers R A, and Prinsep M R 2020 Nat. Prod. Rep., 37(2): 175–223.

[6] Tutti Y and van Ofwegen L P 2018 Gorgonians in Indonesian waters, no. 1. PT. Media Sains Nasional.

[7] Rowley S J 2018 Coral Reefs 37(2): 609–630.

[8] Teffu Y F, Kase A G O, Trianto A, and Wijayanti D P 2020 IOP Conf. Ser. Mater. Sci. Eng.

[9] Putra M Y, Wibowo J T, and Murniaish T 2017 J. Appl. Pharm. Sci., 7(5): 219–227.

[10] Kristiana R et al. 2019 Biodiversitas 20(7): 1811–1819

[11] Nawaz H, Shad M A, Rehman N, Andaleeb H, and Ullah N 2020 Brazilian J. Pharm. Sci., 56.

[12] Sibero M T, Radjasa O K, Bondar K G, Simbolon L M I, Meilana L, Ayuningrum D 2019 AIP Conf. Proc., 080005.

[13] Sedjati S, Pringgenies D, and Fajri M 2020 Jordan J. Biol. Sci., 13(1): 55–58.

[14] Sibero M T, Siswanto A P, Pribadi R, Sabdono A, Radjasa O K, Trianto A, Frederick E H, Wijaya A P, Haryanti D, Triningsih D W, Hayuningrat S J, Igarashi Y 2020 Biodiversitas 21(5): 2180–2187.

[15] Sibero M T, Siswanto A P, Murwani R, Frederick E H, Wijaya A P, Syafitri E, Farabi K, Saito S, Igarashi Y 2020 Biodiversitas 21(9): 4147–4154.

[16] Sibero M T, Sabdono A, Pribadi R, Frederick E H, Wijaya A P, Haryanti D, Siswanto A P, Igarashi Y 2020 IOP Conf. Ser. Earth Environ. Sci.

[17] Wijaya A P, Bondar K G, Frederick E H, Igarashi Y, and Sibero M T 2020 IOP Conf. Ser. Earth Environ. Sci.

[18] Ianora et al. 2006 Estuaries and Coasts 29(4): 531–551.

[19] Truong D H, Nguyen D H, Ta N T A, Bui A V, Do T H, and Nguyen H C 2019 J. Food Qual.

[20] Abarca-Vargas R, Peña Malacara C F, and Petricevich V L 2016 Antioxidants 5(4).

[21] Teffu Y H, Suwandi R, and Nurjanah 2015 J. Pengolah. Has. Perikan. Indones. 18(1): 83–97.

[22] Haerani A, Chaerunisa A Y, and Subarnas A 2019 Indones. J. Pharm. 1(2): 57–61.

[23] He Y et al. 2016 Oncotarget 7(43): 70803–70821.

[24] Othman L, Sleiman A, and Abdel-Massih R M 2019 Front. Microbiol. 10.

[25] Bian C, Wang J, Zhou X, Wu W, and Guo R 2020 Chem. Biodivers. 17(10).

[26] Nimse S B and Pal D 2015 RSC Adv. 5(35): 27986–28006.

[27] Dasgupta N, Sengupta C, and Das S 2014 Ann. Trop. Res. 22: 1–22.

[28] Tiago O et al. 2017 African J. Agric. Res. 12(2): 71–84.

[29] Núñez-Pons L, Shilling A, Verde C, Baker B J, and Giordano D Mar. Drugs 18(8).

[30] Avila C 2020 Mar. Drugs 18(3).