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Biological Activities and Metabolite Profiling of *Polycarpa aurata* (Tunicate, Ascidian) from Barrang Caddi, Spermonde Archipelago, Indonesia

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Abstract

The U.S. Food and Drug Administration (FDA) has legalized several bioactive compounds from marine organisms, and two of them were isolated from tunicate (ascidians). However, the bioactive compounds from marine tunicate are less reported than other marine organisms. This study was conducted to screen biological activities and secondary metabolite of Indonesia's marine tunicate *Polycarpa aurata* from Barrang Caddi, South Sulawesi. Sample was extracted using methanol for 24 h by maceration. The bioactive compounds were characterized using phytochemical tests and HPLC-DAD. Antibacterial activity was performed against multidrug-resistant (MDR) Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus*, *Salmonella typhi*, together with a non-MDR *Escherichia coli*, while antifungal against *C. albicans* and *T. rubrum*. Antioxidant activity was analyzed using DPPH method; and cytotoxicity against P388 murine leukemia cells using XTT method. The crude extract inhibited all pathogenic bacteria in its lowest concentration (0.5 mg) but did not show antifungal effect, toxicity at 0.08 mg/mL; antioxidant 534.60 ppm. The result of phytochemical test gave positive result for alkaloid and steroid/triterpenoid. In addition, HPLC chromatograms indicated 6 major peaks.

Keywords: antibacterial, cytotoxic, Leukemia, MDR bacteria

1. Introduction

In exploring novel medicine to treat various infections and diseases, marine natural product (MNP) has seized the world's attention through its incredible bioactive compounds (Blunt et al., 2018; Pereira, 2019). Their secondary metabolites exhibit potential biological activities such as antibacterial, anticancer, antitumor, antiviral, immunostimulant, etc (Blunt et al., 2018; Carroll et al., 2019; Hanif et al., 2019). Furthermore, Carroll et al., (2020) stated that bacteria, fungi, sponge, cnidarian, and algae were highlighted as the most productive source of new MNPs. For instance, 8 MNPs have been approved by European Medicines Agency (EMEA), Japanese Ministry of Health and Australia's Therapeutic Goods Administration, and U.S. Food and Drug Administration (FDA) (Pereira, 2019). In addition, several other MNPs such as Plinabulin, Plocabulin, and Salinosporamide A are still under clinical trial before legalized and approved by authorized institutions (Jiménez, 2018; Pereira, 2019).

Prior reports stated discovering new compounds from marine tunicates (ascidian) is less reported than sponge and cnidarian (Blunt et al., 2018; Carroll et al., 2019; Carroll et al., 2020). In 2018, there were only 27 new MNPs from tunicate (Carroll et al., 2019), while in 2019, only 12 new MNPs were recorded (Carroll et al., 2020). Despite the low number of new MNP from tunicate, the metabolites from this sessile animal exhibited outstanding biological activities (Arumugam et al., 2017; Leisch et al., 2019). Moreover, two tunicate-derived MNPs have been approved by internationally authorized institutions for drugs. Besides, other tunicate-derived compounds such as Didemnin B from *Tridemnum solidum* are examined in Phase II and Phase III for clinical trial as anticancer (Arumugam et al., 2017; Jiménez, 2018; Leisch et al., 2019). Most of the studies on biological activity from tunicate-derived MNPs are focused on anticancer; however, other properties are pretty neglected (Palanisamy et al., 2017). Hence, screening of biological properties from marine tunicate is essential.

Although Indonesia harbors various tunic species, a survey by Hanif et al. (2019) indicated a lack of MNP data from this animal. Palanisamy et al. (2017) stated that among all reported MNPs from marine tunicate, only 12% of them were isolated from family Stylidae. *Polycarpa aurata* is one of marine tunicate from family Stylidae that is scattered in the center and eastern part of Indonesia nevertheless, only a few studies reported its biological

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activity (Litaay, 2018; Litaay et al., 2018; Ayuningrum et al., 2019; Sumilat et al., 2019). Therefore, the study of biological properties of Indonesia's *P. aurata* is interesting to be conducted. This current study aimed to obtain a crude extract of *P. aurata* collected in Barrang Caddi, analyse the bioactive compounds, and study the biological properties such as antibacterial, antifungal, anticancer, and antioxidant.

2. Materials and Methods

2.1. Sampling

Sampling was conducted in Barrang Caddi waters, Spermonde Archipelago, South Sulawesi, Indonesia (Figure 1). *P. aurata* was collected by SCUBA diving. Samples were put into ziplock plastics, then transferred into a cold box to retain the quality then sent to Laboratory of Tropical Marine Biotechnology (TMB), Universitas Diponegoro, Semarang. The sample was identified by comparing the morphology to a previous report by Ayuningrum et al. (2019).

2.2. Metabolite Extraction

Samples were cleaned using flowing marine and freshwater. The internal organs were discarded while the other part (flesh) was resized and homogenized using a blender machine. The extraction was conducted using methanol (2:1 %v/w) then agitated using an orbital shaker for 24 h (Sibero et al., 2019[1]). The metabolites were concentrated using a rotary evaporator. Afterward, ethyl acetate was added into the unevaporated part then agitated for 1h and separated using separatory funnel. The ethyl acetate layer was collected, then evaporated to get the crude extract then kept at -20 before use for further analysis (Sibero et al., 2020[1]).

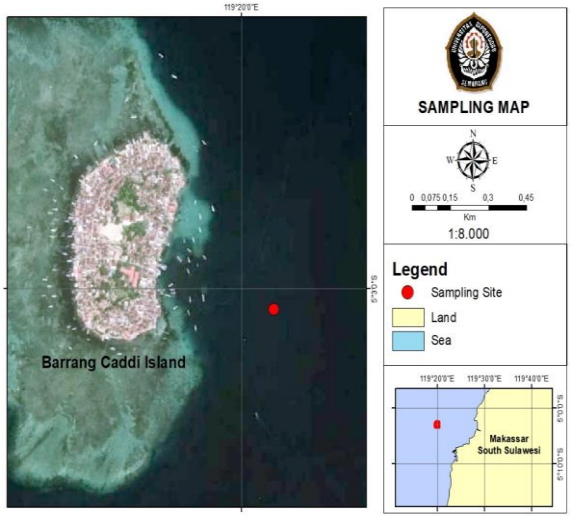

Figure 1. Sampling site in Barrang Caddi, Spermonde Archipelago, South Sulawesi, Indonesia

2.3. Bioassay

2.3.1. Antimicrobial activity

Antimicrobial activity consisted of an antibacterial assay against multidrug-resistant (MDR) *B. cereus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi* and non-MDR *E. coli* obtained from Dr. Kariadi Hospital, Semarang, Indonesia; while antifungal assay against *C. albicans* and *T. rubrum* were from Toyama Prefectural University, Japan. The assay was conducted using the Kirby-Bauer disc diffusion method with a modification that has been published in our previous report (Sibero et al., 2019[1]; Sibero et al., 2020[1]). The crude extract was diluted in dimethyl sulfoxide (DMSO) with 4 concentrations (0.5; 1.0; 1.5 and 2.0 mg/mL) then 15 µL of each concentration was injected into paper disc (Advantec, Ø 6mm). Amoxicillin + clavulanic acid 30 µg/disc (Oxoid) was carried out as positive control for antibacterial and Cyclohexamide (Wako, Japan) 30 µg/mL was prepared as positive control for antifungal. The presence of a clear zone around the paper disc indicated the presence of antimicrobial.

2.3.2. Antioxidant activity

Antioxidant activity was determined using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical method according to Sejati et al. (2020). A total of 3.94 mg DPPH crystal was dissolved into 10 mL absolute methanol (Merck) to prepare a DPPH stock solution with a concentration 0.1 mM. Extract was dissolved in methanol to reach the concentration of 200, 400, 600, 800 and 1000 ppm. In total 160 µL of each concentration was transferred into a 96-wells plate, then 40 µL of DPPH stock was added to reach 200 µL/well. Then it was kept for 30 mins in an incubator (37 ºC) without any light exposure. Afterward, the absorbance was measured at 517 nm using a microplate reader with three replications. The IC_{50} values for antioxidant activity were calculated by linear regression method.

$$DPPH\ Scavenging\ effect = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

2.3.3. Cytotoxicity

The cytotoxic potential of *P. aurata* methanol extract was tested against P388 murine leukemia cells. This step was done according to our previous works with XTT cell proliferation assay (Sibero et al., 2020[1]; Sibero et al., 2020[2]). The crude extract was diluted in DMSO to reach concentration of 0.0002; 0.002; 0.02; and 2 mg/mL. Doxorubicin was used as positive control while DMSO as negative control. The cell viability was counted using a microplate reader at 540 nm. The results of experiments were plotted on single-logarithmic charts to deduce IC_{50} values.

2.4. Metabolite Profiling

2.4.1. Phytochemical test

The presence of bioactive compounds in the crude extract such as alkaloid, flavonoid, glycoside, saponin, steroid and triterpenoid were detected by phytochemical test, following our previous study (Sibero et al., 2019[1]; Sibero et al., 2020[1]; Sibero et al., 2020[2]).

2.4.2. High Performance-Liquid Chromatography with Diode Array Detector (HPLC-DAD)

Metabolite profile of *P. aurata* crude extract was carried out using HPLC-DAD (Sibero et al., 2020[1]; Sibero et al., 2020[3]). The crude extract was prepared in DMSO to reach concentration of 1 mg/mL, filtered using cosmonice filter pore size 0.45 µm (Nacalai Tesque, Inc) then injected into HPLC-DAD (Agilent 1100 Series). Acetonitrile (CH_3CN) and 0.1% formic acid buffer were applied as the

eluent while COSMOSIL 3C18-AR-II (4.6ID × 100 mm) from Nacalai Tesque was set as the column. The sample was analyzed with the following condition: 0-40 % of acetonitrile for 0- 25 min, 40-85 % for 25-28 min, 85 % for 28-30 min, and 85-90 % for 30-35 min with flow rate was 1.2 mL/min and pressure 160 bar. Metabolite in the crude extract was compared to the database in the computer.

3. Result

3.1. Sample identification

Barrang Caddis is one of the small islands in the Spermonde Archipelago, South Sulawesi Indonesia with abundance of marine resources. In this location, samples were collected at 5-10 m depth. The samples had an urn shape and two siphons at the top with yellow color in the inner part. Blue and white colors dominated the body while blue line color separated the compartment in the tunic (Figure 2). Samples were found attached on coral as solitary tunicate and co-presence with other organisms.

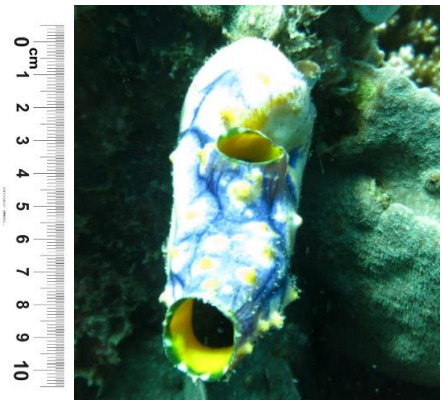

Figure 2. Morphology of *Polycarpa aurata* from Barrang Caddi, Spermonde Archipelago

3.2. Antibacterial activity

The samples were extracted using methanol to obtain the secondary metabolites then tested their biological activities. The antimicrobial activity of *P. aurata* is presented in Table 1, while Figure 3 shows the inhibition zone of each extract concentration.

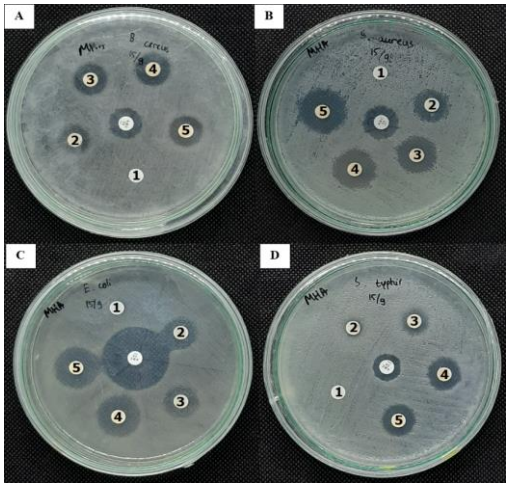

Figure 3. Result of antibacterial activity against pathogenic bacteria

(A. *Bacillus cereus*, B. MRSA, C. *Escherichia coli*, D. *Salmonella typhi*)

((1) DMSO, (2) 0.5 mg/mL, (3) 1.0 mg/mL, (4) 1.5 mg/mL, (5) 2.0 mg/mL)

Table 1. Antimicrobial activity of *P. aurata* crude extract against pathogens after 24 h incubation

Pathogen	Concentration (mg/mL)	Diameter of Inhibition Zone (mm)
Bacillus cereus	DMSO	0.00 ± 0.00[a]
	0.5	3.90 ± 1.50[b]
	1.0	5.90 ± 1.20[c]
	1.5	6.15 ± 0.21[c]
	2.0	6.65 ± 0.49[d]
	Amoxicillin + clavulanic acid 30 µg/disc	6.20 ± 0.70[c]
MRSA	DMSO	0.00 ± 0.00[a]
	0.5	7.05 ± 0.07[b]
	1.0	9.75 ± 0.35[c]
	1.5	9.95 ± 0.91[c]
	2.0	11.5 ± 0.75[d]
	Amoxicillin + clavulanic acid 30 µg/disc	7.80 ± 1.13[c]
Escherichia coli	DMSO	0.00 ± 0.00[a]
	0.5	6.30 ± 0.98[b]
	1.0	7.55 ± 1.48[b]
	1.5	11.65 ± 0.49[c]
	2.0	12.60 ± 1.41[c]
	Amoxicillin + clavulanic acid 30 µg/disc	21.65 ± 0.49[d]
Salmonella typhi	DMSO	0.00 ± 0.00[a]
	0.5	2.25 ± 0.77[b]
	1.0	5.75 ± 0.21[c]
	1.5	7.60 ± 1.41[d]
	2.0	7.10 ± 0.84[d]
	Amoxicillin + clavulanic acid 30 µg/disc	5.80 ± 0.28[c]
Candida albicans	DMSO	0.00 ± 0.00
	0.5	0.00 ± 0.00
	1.0	0.00 ± 0.00
	1.5	0.00 ± 0.00
	2.0	0.00 ± 0.00
	Nystatin 30 µg/mL	18.00 ± 0.00
Trichophyton rubrum	DMSO	0.00 ± 0.00
	0.5	0.00 ± 0.00
	1.0	0.00 ± 0.00
	1.5	0.00 ± 0.00
	2.0	0.00 ± 0.00
	Nystatin 30 µg/mL	18.00 ± 0.00

((Data were average ± standard deviation. Different notations indicate significant difference at $P < 0.05$)

The data (Table 1) shows that the crude extract of *P. aurata* inhibited all pathogenic bacteria; however, it did not show antifungal activity against *C. albicans* and *T. rubrum*. It was highlighted that the crude extract gave the best antibacterial activity against *E. coli*, followed by MRSA, then *S. typhi* while the narrowest antibacterial was shown against *B. cereus*. It was also noted that the enhancement of crude extract concentration was in line with increasing the inhibition zone diameter.

3.3. Antioxidant activity

Another important biological activity is antioxidants. The result of antioxidant activity of the crude extract is shown in Table 2 and Figure 4.

Table 2. Antioxidant activity of *P. aurata* crude extract

Concentration (ppm)	Inhibition (%)	IC_{50} (ppm)
200	21.63 ± 2,85	
400	44,23 ± 2.05	
600	58.42 ± 1.32	534.60 ppm
800	67.95 ± 1.19	
1000	80.93 ± 1.32	

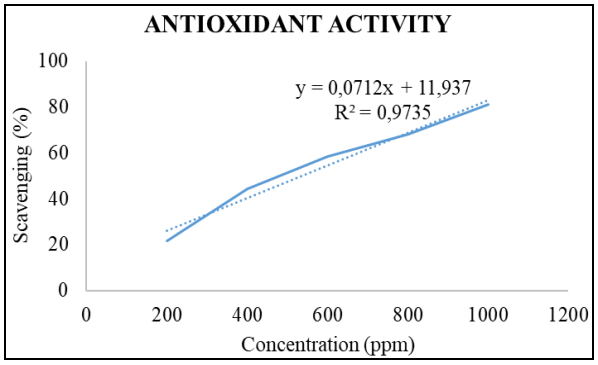

Figure 4. IC_{50} value of *P. aurata* crude extract using DPPH method

The ability of *P. aurata* crude extract on inhibiting DPPH was applied to screen the antioxidant activity. The result in Table 2 and Figure 4 shows that this extract had IC_{50} value of 534.60 ppm. It means that the crude extract scavenged 50% of DPPH activity at that particular concentration.

3.4. Cytotoxicity

The ability of *P. aurata* crude extract against P388 murine leukemia cancer cells is presented in Figure 5. It was noted that the crude extract exhibited anticancer potential with IC_{50} value of 0.08 mg/mL or equal to 80 µg/mL.

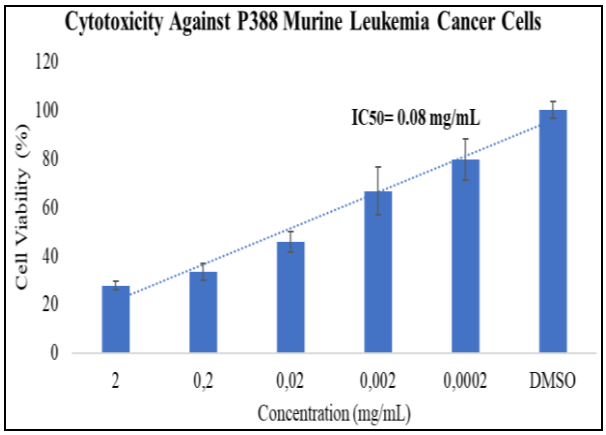

Figure 5. Cytotoxicity of *P. aurata* crude extract against P388 murine leukemia cancer cell

3.5. Metabolite profile

The presence of antibacterial, antioxidant, and cytotoxic properties leads to an understanding that *P. aurata* from Barrang Caddi is a potential source of bioactive compounds. Hence, profiling the secondary metabolites was conducted in this study. A fundamental analysis to detect the presence of bioactive compounds was performed using the phytochemical test. The result of this test is presented by Table 3.

Table 3. Phytochemical content of *P. aurata* crude extract

Target	Indicator	Result
Alkaloid	Formation of yellow to orange feculence after addition of Dragendorff reagent	Detected
Flavonoid	Formation of yellow to orange color in amyl alcohol layer	Not Detected
Glycoside	Formation of a brown ring between the layer	Not Detected
Saponin	Formation of a stable foam after 30 min and addition of 2N HCl	Not Detected
Steroid/Triter penoid	Formation of green color at the upper layer and maroon to brown color at the lower layer	Detected

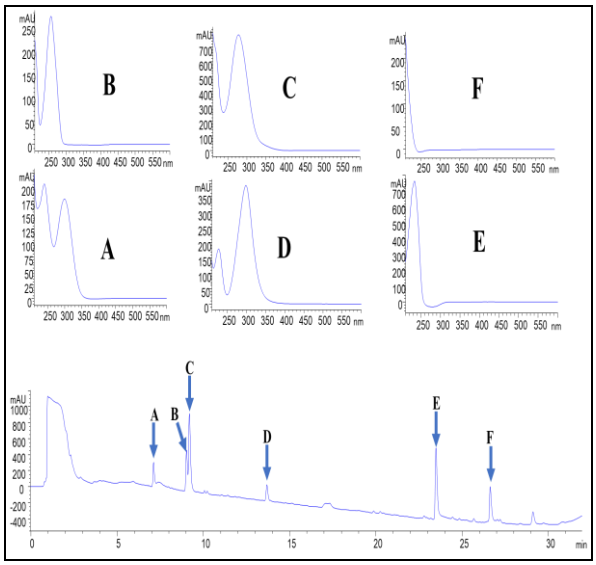

Figure 6. Metabolite profile of *P. aurata* crude extract using HPLC

According to the phytochemical test, the crude extract of *P. aurata* contained alkaloid and steroid/triterpenoid. Further, the HPLC chromatogram of the crude extract is presented by Figure 6. The HPLC analysis discovered 6 major peaks from *P. aurata* at retention time of 7.07 min (peak A), 8.97 min (peak B), 9.21 min (peak C), 13.71 min (peak D), 23.48 min (peak E) and 26.63 min (peak F).

4. Discussion

Morphology and molecular studies by Ayuningrum et al. (2019) successfully explained that the diverse color variations in *P. aurata* are probably induced by multiple allele inheritance with codominance. The existence of marine tunicate *P. aurata* in Barrang Caddi has been reported before, moreover, this species was noted as a cosmopolitan tunicate in Spermonde archipelago, South Sulawesi (Litaay, 2018). In addition, several tunic color variations of *P. aurata* are blue color with blue lines, orange color, yellow color with a blue line and white spots and blue lines (Ayuningrum et al., 2019). This species was also found in Indonesia, such as Bali, North Sulawesi, South Sulawesi, and Maluku (Wainwright et al., 2013; Litaay, 2018; Ayuningrum et al., 2019; Casertano et al., 2019; Sumilat et al., 2019). Even though this species has been studied widely, there are only a few reports about its biological activity, especially as antimicrobial and antioxidant agents.

The methanol crude extract was noted to possess alkaloid and steroid/triterpenoid (Table 3). Alkaloid derivatives compounds were commonly isolated from *P. aurata* such as N,N-Didesmethylgrossularine-1 (DDMG-1); polycarpaurines A-C; polyaurines A-B; and polycarpathiamines A-B (Wang et al., 2007; Pham et al., 2013; Casertano et al., 2019; Sumilat et al., 2019). Palanisamy et al. (2017) noted that most of the reported compounds from tunicates were alkaloid, consisting of indole 48%, pyrocridine 18%, β-carboline 8%, and saturosporine 5%. In addition, steroid only possesses 2% of the total tunicate-derived MNPs. Other chemical classes from marine tunicate were peptide 4%, alkene 3%, alkyl sulfate 3%, polyketide 2%, polysaccharide 2%, esters 1% and spiroketal 1%. In addition, HPLC chromatogram (Figure 6) endorsed the presence of secondary metabolites in *P. aurata*. The HPLC chromatogram and U.V. spectrum could be used to isolate the lead compounds from the sample. Previous studies successfully isolated new compounds from marine organisms through HPLC-UV guided isolation method (Sibero et al., 2019; Karim et al., 2020; Zhang et al., 2020). Furthermore, bioguided fractionation could also be combined with HPLC to obtain active fractionations based on the retention time (Sibero et al., 2020). Therefore, a further study is suggested to isolate the lead compounds. The presence of bioactive compounds in *P. aurata* crude extract is expected to influence its biological activities.

It was highlighted that the crude extract only inhibited the bacterial growth; however, it did not show any antifungal effect to *C. albicans* and *T. rubrum.* The ability of the crude extract on inhibiting gram positive pathogens (*B. cereus*, MRSA) and gram negative pathogens (*E. coli* and *S. typhi*) indicated the broad-spectrum antibacterial property (Kaur et al., 2011). Our data shows that the crude extract could inhibit MDR pathogens such as *B. cereus*, MRSA, and *S. typhi*. This result was expected to strengthen *P. aurata* as a prospective source of new antibiotics to overcome multidrug-resistance (MDR) bacterial infection. Although there are several studies about *P. aurata* crude extract's antimicrobial property nonetheless, there is no study about the lead compound of antibacterial activity within the past 5 years (Casertano et al. 2020). Palanisamy et al. (2017) stated that among all tunicate-derived MNPs, only 12% had antibacterial activity, and 3% had antifungal effect. Antifungal compounds combat the pathogens by inhibiting and or disrupt ergosterol, glucan, chitin, nucleic acids, and protein synthesis (Kathiravan et al., 2012). Some compounds reported to have antifungal activity are arylamidine, azoles, echinocandin, enfumafungin pyrrole, and quinazoline derivatives (Kathiravan et al., 2012; Castelli et al., 2016). These compound derivatives are barely isolated from *Polycarpa* spp. In addition, Casertano et al. (2020) stated that most antimicrobial compounds from ascidian are sulfur-containing compounds, meroterpenes, alkaloids, peptides, furanones derivatives. Therefore, only a few antifungal compounds were discovered from ascidian (Palanisamy et al., 2017).

The screening of antioxidant activity gave IC_{50} value of 534.60 ppm. A substance will be considered as a powerful antioxidant agent if it has IC_{50} values < 50 ppm, strong activity if the IC_{50} value is 50-100 ppm, moderate activity if the IC_{50} value is 100-150 ppm, and weak if the IC_{50} value is > 150 ppm (Haerani et al., 2019). Hence, our crude extract was not suggested as a candidate for a new antioxidant agent. Study of antioxidant property of marine tunicate is less popular than other biological activity. The latest update stated that only 2% of all reported compounds had antioxidant properties (Palanisamy et al., 2017). The presence of hydroxyl (HO) functional group in a compound is positively correlated to antioxidant activity; therefore, most phenolic-derivate compounds such as flavonoid have strong antioxidant activity (Chandra et al., 2020). Flavonoid was not detected in the crude extract; hence, it generally explains the infectivity of *P. aurata* as an antioxidant agent.

The anticancer property of *P. aurata* was evaluated by cytotoxicity test against P388 murine leukemia cancer cells. There are 4 categories of cytotoxicity according to U.S. National Cancer Institute, namely non-toxic if the IC_{50} value is > 500 µg/mL, weak cytotoxic if the IC_{50} value is 201-500 µg/mL, moderate cytotoxic if the IC_{50} is 21-200 µg/mL and very toxic if it has IC_{50} value < 20 µg/mL (Sajjadi et al., 2015; Alabsi et al., 2016; Amaani and Dwira, 2018; Widiyastuti et al., 2019). This bioassay gave IC_{50} value of 0.08 mg/mL or equal to 80 µg/mL, it means the *P. aurata* crude extract had moderate cytotoxicity against P388 murine leukemia cancer cell. It is suggested that the presence of alkaloid compounds in the crude extract has correlation to the anticancer activity. Plenty of alkaloid-derivative compounds were widely reported as the anticancer agent from marine tunicate (Cooper and Yao, 2012; Palanisamy et al., 2017; Arumugam et al., 2020). Previous studies successfully discovered several cytotoxic compounds from *P. aurata* such as polycarpaurines A-C and polycarpathiamines (Wang et al., 2007; Pham et al., 2013). Interestingly, isolation of new cytotoxic compounds from *P. aurata* is less reported; therefore, this preliminary study gives an additional data on its prospect as an anticancer agent (Cooper and Yao, 2012; Arumugam et al., 2017, 2020; Khalifa et al., 2019).

5. Conclusion

Marine *P. aurata* from Indonesia exhibited antibacterial activity against MDR *B. cereus*, MRSA, *S. typhi* and non-MDR *E. coli*, while the antifungal assay indicated the absence of bioactivity. The crude extract showed very weak antioxidant activity with 534.60 ppm and a moderate cytotoxic against P388 murine leukemia cancer cell with IC_{50} value of 0.08 µg/mL. Phytochemical test indicated the presence of alkaloid and steroid/triterpenoid. The HPLC chromatogram gave six major peaks in the crude extract.

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