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Antimicrobial and Cytotoxic Properties of the Ascidians *Lissoclinum patella*, *Oxycoryna fascicularis, Didemnum molle* and *Botryllus schlosseri*

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ABSTRACT

The aim of this research was to investigate antimicrobial and cytotoxic activity of Indonesian ascidians. Extracts prepared from the Indonesian ascidians namely *Lissoclinum patella*, *Oxycoryna fascicularis, Didemnum molle* and *Botryllus schlosseri* were assessed for anti-microbial and cytotoxic properties. Antibacterial activity of the extracts was tested against two Gram-positive bacteria, *viz. Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923, and three Gram-negative bacteria, *viz. Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 14035 and *Pseudomonas aeruginosa* ATCC 101454 using the disk diffusion test. Antifungal activity was also tested against *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The minimum inhibitory concentrations (MICs) of potential ascidian extracts were determined by the microdilution technique. Cytotoxicity of the extracts was assessed using the brine shrimp lethality bioassay. By comparing the inhibition zones in the disk diffusion test, the most active anti-bacterial activity against Gram-positive bacteria (*S. aureus* and *B. subtilis*) was found in the crude extracts of *Oxycoryna fascicularis* and *Didemnum molle*. *Lissoclinum patella* extract showed the highest activity against the Gram-negative bacteria *E. coli* and *V. cholerae*. The LC_{50} values of the crude extracts of *Lissoclinum patella, Didemnum molle, Botryllus schlosseri,* and *Oxycoryna fascicularis* were 74.3, 97.2, 114.7 and 132.9 µg/ml, respectively. In our study, the most promising species for anti-microbial and cytotoxic properties were *Lissoclinum patella* and *Didemnum molle*.

Keywords *: ascidians; antibacterial; antifungal; cytotoxicity*

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INTRODUCTION

The marine environment becomes a new focus of natural product drug discovery because of its relatively unexplored biodiversity compared to terrestrial environment (Malve, 2016). Available resources for pharmacy can be found in the coral reef communities such as algae, soft coral, sponge, ascidian, marine microorganism, etc (Abdillah *et al*., 2015; Deepa *et al*., 2017; Ramos *et al*., 2016). Bio-prospection of marine natural products is one of the main interest of pharmaceutical research such as antibacterial, antiviral, antifungal, cytotoxic activity (Castro-Carvalho *et al*., 2017).

Sessile filter-feeding chordates known as ascidians are an important structural component of coral reef communities. They populate marine ecosystems, whether in tropical, subtropical or temperate waters. Ascidians have been the source of a wide variety of secondary metabolites, some of which have physiological functions, mainly in defense against their natural predators (Joullié *et al*., 2003; Paul *et al*., 1990; Pisut & Pawlik, 2002)northern Sulawesi (Indonesia. While research efforts in bioactive metabolites from marine ascidians are relatively recent, some chemical compounds from marine ascidians are being evaluated as antitumor agents in preclinical and clinical trials. The most prominent example of an ascidian-derived drug is trabectedin or ecteinascidin 743 (ET-743, Yondelis®), an alkaloid isolated from the Caribbean ascidian *Ecteinascidia turbinate.* This is the first marine-derived drug to be prescribed against soft tissue carcinoma (Cuevas & Francesch, 2009; Gordon *et al*., 2016; Menna, 2009).

It has been demonstrated that 7% of the compounds isolated from ascidians are produced by symbiotic bacteria, leaving the exact biosynthetic source of the remaining 93% of described metabolites unknown (Schmidt & Donia, 2010; Tianero *et al*., 2015). Many anti-microbial compounds have been isolated from ascidians or ascidian-associated marine bacteria. Antibacterial activity was observed in brominated rubrolides isolated from an African ascidian Synoicum

globosum (Sikorska, Parker-Nance, *et al*., 2012). Peptidolipins, which are lipopeptide compounds produced by bacteria (Nocardia sp.) from the Florida Keys tunicate Tridemnum orbiculatum, showed activity against methicillin-sensitive Staphylococcus aureus (MSSA) and methicillin-resistant Staphylococcus aureus (MRSA) (Wyche *et al*., 2012).

In the present study, investigations were undertaken on marine invertebrates from coastal areas of the Indonesian archipelago, one of the world's hotspots of marine biodiversity. We examined organic extracts from some of the most abundant marine ascidians in the Buton islands, Southeast Sulawesi, namely *Lissoclinum patella*, *Oxycoryna fascicularis,* and *Didemnum molle.* We also investigated crude extracts of the ascidian *Botryllus schlosseri* collected from Selayar Island, South Sulawesi, for their anti-microbial and cytotoxic effects. Using the disk diffusion assay, the anti-bacterial activity of ascidian extracts was tested against five human pathogenic bacteria, *viz.* Staphylococcus aureus (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Eschericia coli* (ATCC 25922)*, Vibrio cholerae* (ATCC 14035), and *Pseudomonas aeruginosa* (ATCC 10145). Antifungal activity was similarly tested against *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404). We also used the brine shrimp lethality bioassay to test for cytotoxic activity.

MATERIALS AND METHODS

Ascidian Sample Collection and Extraction

Three ascidians, *Lissoclinum patella*, *Oxycoryna fascicularis,* and *Didennum molle*, were collected in July 2016 at a depth of 1-5 m by scuba diving from two sites of the Buton Islands, Southeast Sulawesi, Indonesia. The ascidian *Botryllus schlosseri* was collected in June 2015 at a depth of 5-10 m from Selayar Island, South Sulawesi (Table 1). A voucher record of each specimen (ASC1-BT, ASC2-BT, ASC3-BT, ASC1-SLY) was deposited at the Research Center for Oceanography of Indonesian Institute of Science. Each ascidian sample of 150-200 g wet weight was cut into small pieces, homogenized and extracted three times with MeOH-$CHCl_3$ (3:1) at room temperature overnight. Following this, the crude extracts were concentrated by evaporation under reduced pressure.

Antimicrobial Assays

Disk Diffusion Assay

Antibacterial activity of the ascidian extracts was assessed using the agar disk diffusion test[6]. Five reference strains of human pathogens were used in the assays, *viz.* two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633) and three Gram-negative bacteria (*Eschericia coli* ATCC 25922, *Vibrio cholerae* ATCC 14035*, Pseudomonas aeruginosa* ATCC 10145). The bacteria were grown in liquid marine broth medium for 24 hours at room temperature. The various ascidian extracts were adjusted to a concentration of 100 µg/mL in MeOH and 20 µl of each extract was pipetted on to a sterile paper disk (6 mm diameter) and allowed to dry. A reference control paper disk carried 10 µg ampicillin in 20 µl MeOH. The paper discs were then placed on Mueller Hinton Agar (Himedia) in a petri dish that had been inoculated with the test bacteria (10^7 CFU/mL). The inhibition zone that appeared was recorded after 24 h incubation at 30°C. All the assays were carried out in triplicate.

Candida albicans ATCC 10231 and *Aspergillus niger* ATCC 16404 were used as test fungi and inoculated on to Sabouraud Dextrose Agar (fungus) plates (10^4 CFU/mL). The various ascidian extracts were adjusted to a concentration of 100 µg/mL and 20 µl of each extract was pipetted on to 6 mm sterile paper disks. The discs were allowed to dry and then placed on Sabouraud Dextrose Agar. The inhibition zone was recorded after incubation at room temperature (25 °C) for 48 h. Nystatin (30 mg/disc) was used as the positive control and methanol as the solvent control. All the assays were performed in triplicate.

Microdilution Method

The microdilution method was used to evaluate the

Table 1. Ascidians Species and Place of Collection

Sponge Species	Depth (m)	Place of Collection	Voucher Specimen Number
Lissoclinum patella	1-5 m	Buton Island (Southeast Sulawesi)	ASC1-BT
Oxycoryna fascicularis	1-5 m	Buton Island (Southeast Sulawesi)	ASC2-BT,
Didennum molle	1-5 m	Buton Island (Southeast Sulawesi)	ASC3-BT
Botryllus schlosseri	5-10 m	Selayar Island (South Sulawesi)	ASC1-SLY

minimum inhibitory concentration (MIC) of the ascidian extracts which displayed high activity (growth inhibition halos more than 9 mm.) 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) containing different concentrations (62.5, 125, 250, 500 µg/ml) of *Lissoclinum patella* extract*, Oxycoryna fascicularis* extract, *Didemnum molle* extract, ampicillin or solvent control, as well as the test microorganism (10^7 CFU/mL). After a 24 h incubation period at 37 °C, the turbidity in each well was measured at 600 nm.

MIC for fungal strains was also performed using the 96-well plate. Each well contained potato dextrose broth, different concentrations of *Lissoclinum patella* estract, *Didemnum molle* extract, Nystatin or solvent control, as well as the test yeast strain (104 CFU/mL). Incubation was performed at room temperature (18-20 °C) for 48 h. The yeast growth was measured at 494 nm using the Infinite® 200 PRO microplate reader (Tecan Austria GmbH).

Brine Shrimp Lethality Bioassay

Different concentrations (10, 100, 1000 mg/ml) of the ascidian crude extracts were prepared for cytotoxic activity test using the brine shrimp lethality bioassay. Brine shrimp eggs (*Artemia salina*) were placed in a hatching tank containing 1 L of seawater under aeration at room temperature to hatch under continuous light. After 24 h, ten brine shrimp larvae were placed in a small container filled with sea water and different concentrations of the extracts. Survivors (instar III/IV stage) were calculated after 48 h incubation, and the mortality percentage for each concentration and the control (seawater) was determined. Treatments and controls were prepared in triplicates.

Statistical Analysis

Measurements in all the experimental analyses were expressed as mean ± SD (n = 3). Results of the research were tested for statistical significance with One-way ANOVA. Differences were considered statistically significant at the $P < 0.05$ level. The statistical software package SPSS v.16 (SPSS Inc., Chicago, IL, USA) was used for the analysis.

RESULTS AND DISCUSSION

Antimicrobial Properties

The results of the agar disk diffusion assay on the crude ascidian extracts against five bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 14035, *Pseudomonas aeruginosa* ATCC 10145) and two strains of fungus (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404) are presented in Table 2. All the ascidian extracts showed potential anti-microbial activities against at least one of the test strains. By comparing the inhibition zones, the most active anti-bacterial activity against Gram-positive bacteria (*S. aureus* and *B. subtilis*) was shown for the crude extracts of *Oxycoryna fascicularis* and *Didemnum molle*. Against *S. aureus*, *Oxycoryna fascicularis* and *Didemnum molle* produced inhibition zones of 9.10 and 9.20 mm, respectively. Moreover, against *B. subtilis*, *Oxycoryna fascicularis* and *Didemnum molle* showed inhibition zones of 9.20 and 10.2 mm, respectively.

Lissoclinum patella extract was found to possess the highest activity against Gram-negative bacteria (*E. coli* and *V. cholerae*), with inhibition zones of 9.20 and 9.1 mm, respectively. All ascidian extracts showed moderate activity against the Gram-negative bacterium *P. aeruginosa*. Based on the results from the anti-fungal assays, *Didemnum molle* demonstrated the highest

Table 2. Antimicrobial Activity of Marine Ascidian Extracts Determined by the Disk Diffusion Assay

Species	Inhibition Zone (mm)						
	S. aureus	***B. subtilus***	***E. coli***	***V. cholerae***	***P. aerugi-nosa***	***C. albicans***	***A. niger***
Lissoclinum patella	6.80	7.50	9.20	9.10	3.30	4.8	4.3
Oxycoryna fascicularis	9.10	9.20	8.00	4.60	2.10	5.2	5.8
Didemnum molle	9.2	10.2	8.1	8.5	5.3	9.1	4.2
Botryllus schlosseri	8.60	7.30	0	3.60	4.50	3.7	4.1
Ampicillin[a]	30.60	18.8	29.30	23.9	23.5	-	-
Nystatin[a]	-	-	-			20.3	15.4

Table 3. Minimum Inhibitory Concentrations (MICs in μg/ml) of Marine Ascidian Extracts

Ascidian extracts	Microorganisms						
	S. aureus	***B. subtilus***	***E. coli***	***V. cholerae***	***P. aeruginosa***	***C. albicans***	***A. niger***
Lissoclinum patella	-	-	250	250	-	-	-
Oxycoryna fascicularis	125	250	250	-	-	-	-
Didemnum molle	250	125	-	-	-	250	-

Table 4. Effect of the Extract of Sponges on Brine Shrimp Survival

Acidians	LC_{50} (μg/mL)	Regression Equation	R^2
Lissoclinum patella	74.336	y = 5,3818x - 5,0702	R^2 = 0.9915
Oxycoryna fascicularis	132.91	Y = 1,6445 + 1.5078	R^2 = 0.8028
Didemnun molle	97.145	y = 5,5812x - 6,0923	R^2 = 0.9281
Botryllus schlosseri	114.709	y = 3,9699x - 3,1765	R^2 = 0.9714

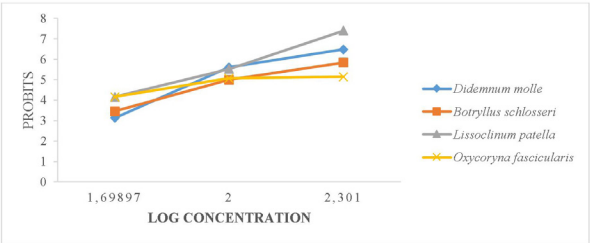

Figure 1. The Plot of Log Concentration of Crude Ascidian Extracts vs. Percent of Shrimp Mortality After 48 H of Exposure (Probits)

inhibitory activity against *C. albicans*, with an inhibition zone of 9.1 mm. MICs were determined for the three ascidian extracts that gave the best results for anti-bacterial and anti-fungal activities. Results from the broth microdilution method are summarized in Table 3.

Cytotoxic Activity

Cytotoxic activity of the ascidian extracts was evaluated using the brine shrimp lethality bioassay. The LC_{50} values of the crude extract of *Lissoclinum patella*, *Oxycoryna fascicularis, Didemnum molle* and *Botryllus schlosseri* were 74.3, 132.91, 97.145 114.71 μg/ml, respectively (Figure 1, Table 4). Based on the results from the cytotoxic assay, extracts of *Lissoclinum patella* and *Didemnum molle* were the most promising from the standpoint of cytotoxic activity.

A large number of natural compounds, especially those with anti-microbial or cytotoxic properties, have been extracted from marine invertebrates. Among such marine invertebrates widely studied are the ascidians, from which a broad range of interesting bioactive compounds, including those showing anti-bacterial, anti-cancer, anti-inflammatory and antimalarial properties, have been extracted. Nonetheless, information on potential pharmacological characteristics of Indonesian ascidians remains rare. With the evolution of multiple resistance among pathogenic microorganisms, the search for new anti-microbials from alternative sources such as the marine environment has taken on a new urgency. We have, accordingly, initiated a program to screen Indonesian marine ascidians for anti-microbial and cytotoxic compounds.

Previous anti-microbial screenings have shown Gram-positive bacteria to be more sensitive than Gram-negative bacteria or fungi to extracts of marine invertebrates such as sponges, and ascidians (Qaralleh *et al*., 2010; Tadesse *et al*., 2008) including the bacteria Staphylococcus aureus ATCC25923, Bacillus cereus ATCC11778, Pseudomonas aeruginosa ATCC27853, Escherichia coli ATCC35218, the yeasts Candida albicans ATCC10231 and Cryptococcus neoformans ATCC90112. All the sponge species in this study showed antibacterial activity against at least one bacterial strain and only one sponge species was significantly active against C. albicans. P. aeruginosa was considered resistant to all tested samples, since no inhibition zone was observed while the Gram-positive B. cereus was shown to be the most sensitive microorganism followed by C. albicans and S. aureus. The highest activity was obtained for the aqueous extract of Neopetrosia exigua against the Gram-positive bacteria B. cereus (inhibition zone 25mm and MIC 0.07mg/mL. Our results that find the tested

extracts showing high activity against Gram-positive bacteria are, therefore, in agreement with these findings. Based on these results, extracts from two ascidians, *viz. Lissoclinum patella* and *Didemnum molle*, were found to be especially promising in their anti-bacterial action towards Gram-positive bacteria, whereas another ascidian *Oxycorina fascicularis* was reactive against Gram-negative bacteria. *Didennum molle* appears to have the greatest potential as an anti-microbial source. This ascidian was reactive against the Gram-positive bacteria (*S. aureus* and *B. subtilis*) and a fungal pathogen *C. albicans*. The anti-microbial activities of the extract from the genus *Didemnum* might be due to the presence of the compounds, Didemnoline A, Didemnoline C or Didemnaketal F, that are reported to be inhibitory to *S. aureus, B. subtilis* and *C. albican* (Palanisamy *et al*., 2017; Schumacher & Davidson, 1999; Shaala *et al*., 2014) *while didemnolines B (2*. The extract from another ascidian, *Lissoclinum patella*, also showed an apparent specificity towards Gram-positive bacteria. This genus is known to contain the anti-bacterial compound Lissoclinotoxins (Litaudon *et al*., 1994).

Gram-positive bacteria tend to be more sensitive to drug action than Gram-negative bacteria (Cos *et al*., 2006; Malanovic & Lohner, 2016; Mcdonnell & Russell, 1999)many of which have been used for hundreds of years, including alcohols, phenols, iodine, and chlorine. Most of these active agents demonstrate broad-spectrum antimicrobial activity; however, little is known about the mode of action of these agents in comparison to antibiotics. This review considers what is known about the mode of action and spectrum of activity of antiseptics and disinfectants. The widespread use of these products has prompted some speculation on the development of microbial resistance, in particular whether antibiotic resistance is induced by antiseptics or disinfectants. Known mechanisms of microbial resistance (both intrinsic and acquired. The former, such as *S. aureus,* are significant pathogens in hospital environments. For this reason, new anti-bacterial agents useful for treating serious Gram-positive infections are in demand.

Several cytotoxic compounds such as cyclic peptides, diterpenes, and alkaloids have been isolated from ascidian *Lissoclinum patella* and *Didemnum molle* (Donia *et al*., 2008; Rashid *et al*., 1995; Sikorska, Hau, *et al*., 2012; Teruya *et al*., 2008). These compounds are cytotoxic against various tumor cell lines, and they are considered potential lead compounds for the development of new anticancer agents.

The brine shrimp bioassay is broadly accepted as a useful indicator of potential cytotoxic activity in terrestrial plants and marine organisms. This assay, which has been shown to correlate well with cytotoxicity in cancer cell lines such as 9KB, P388, L5178Y and L1210 (De Rosa *et al*., 1994; Mclaughlin *et al*., 1998)to establish relevant structure-activity relationships (SAR, has the benefits of simplicity, low cost and reliability in assessing cytotoxicity. In our study that employed the assay, *Lissoclinum patella* and *Didemnum molle* were the most promising in their cytotoxic activity.

CONCLUSION

Indonesian marine ascidians were shown to possess anti-microbial and cytotoxic activities. In our study, the most promising species for anti-microbial and cytotoxic properties were *Lissoclinum patella* and *Didemnum molle*. Extracts from these organisms will be further subjected to bioassay-guided fractionation to isolate the active compounds responsible for these properties of pharmacological relevance.

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