

Antibacterial and Cytotoxic Activities of Sponges Collected off the Coast of Togean Islands, Indonesia

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ABSTRACT

Context: Marine sponges (Porifera: Demospongia) have astonishing structural diversity and broad biological activities. **Aims:** To evaluate the antibacterial and cytotoxic activities of five sponges collected off the coast of Togean Islands, Indonesia, identified as *Sphēciospongia inconstan*, *Meloplus sarasinorum*, *Oceanapia amboinensis*, *Biemna* sp and *Axinella* sp.

Methods and Material: All dried sponges materials were extracted by maceration method using methanol and then evaporated by the rotary evaporator to obtain viscous extracts. The determination of antibacterial activity was performed by well agar diffusion method against *Staphylococcus aureus* and *Escherichia coli* while the cytotoxic activity was determined by MTT methods on human breast adenocarcinoma (MCF-7) and human colon colorectal carcinoma (HCT-116), followed by determination of the apoptosis mechanism by Annexin V-FTIC assay.

Results: *M. sarasinorum* and *Axinella* sp showed strong inhibition against *S.aureus* and *E.coli* with the diameter of inhibition of 14.21 ± 0.92 mm and 14.36 ± 0.92 mm, and 10.01 ± 2.65 mm and 12.07 ± 1.54 mm, respectively. Moreover, they also exhibited potent cytotoxicity on HCT-116 with IC_{50} values of 0.002 and $8.518 \mu\text{g/mL}$, respectively. Meanwhile, on MCF-7, only *M. sarasinorum* showed moderate inhibition with an IC_{50} value of $87.35 \mu\text{g/mL}$. Annexin V-FTIC assay clearly showed that the cytotoxic mechanism of *M. sarasinorum* and *Axinella* sp on HCT-116 and MCF-7 was via apoptosis induction. **Conclusion:** The sponges of *M. Sarasinorum* and *Axinella* sp are undergoing further analysis to identify the active constituents which could be developed as potential antibacterial and anticancer agents.

Key words: Sponges, Togean Islands, Cytotoxicity, Antibacterial, MTT.

Key Messages: Screening of antibacterial and cytotoxic activities of sponges, collected off Togean Islands, Indonesia, using well agar diffusion and MTT methods, followed by determination of the apoptosis mechanism by Annexin V-FTIC assay have found the high potency of *Meloplus sarasinorum* and *Axinella* sp to be antibacterial and anticancer drugs.

INTRODUCTION

The increasing case of infection diseases caused by bacteria, as well as the growth of antibiotics and anticancer drugs resistance in the worldwide have encourage scientist to search the new source of antibiotic and anticancer drugs, particularly from marine bioactive compounds.¹ The exploration of marine environment have progressively improved in the past 50 year that possessing around thousands of unique chemical structure of new bioactive compounds from marine. Among marine organisms, marine sponges (porifera: Demospongiae) have been considered as a largest source of unusual metabolites and bioactive compounds that attributes to various biomedical pharmaceutical importance such as antibacterial, anticancer, antifungal, antiprotozoal and antiviral activities.²⁻⁵ Some of them are halichondrin B from *Halichondria okadai* which is under preclinical anticancer agent, aurantosides from *Siliquariaspongia japonica* and *Homophynia conferta* and spongistatin 1 from *Hyrtyia serecta* which are commercially available.⁶

Indonesian coast is one of the richest biodiversity for marine organisms in the world. Literature studies and informal database record-based review confirmed the richness of sponges along the Indonesian's shoreline. However, the published knowledge based of Indonesian sponge's organisms is sorely incomplete. Much of the sponge's materials are still needing to be described for species identification and many sponge's locations still need to be explored.⁷

Previously, our study on soft corals of *Sarcophyton trocheliophorum* have found numerous novel metabolites possessing antibacterial and antitumor activities.⁸ In continuing our focus research on marine organism to identify compounds with medicinal prospect, particularly from Indonesian sea, our study now starting on some sponges collected off Togean Islands, Indonesia. Here, we report the screening assay for antibacterial and cytotoxic activities of sponges methanolic extracts on two pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli*,

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two human carcinoma cells MCF-7 and HCT-116 and two human normal cells CCD and NHDF followed by apoptosis assay for the most cytotoxic extracts.

MATERIALS AND METHODS

Study area

Togean islands is in the north, eastward-projecting peninsula of central Sulawesi (Figure 1 and 2). It occupies the central portion of Tomini Bay, stretching over about 90 km. The land area of the Togean Group covers about 755 km² and contains 66 islands of which Batudaka, Talatakoh, Waleakodi, Waleabahi, Una-una and Togean are the largest.⁹

Materials

All samples of sponges were collected in January 2017 on Togean Islands, Tojo Una-una, Indonesia at a depth of 5-10 m and identified by Tri Aryono Hadi (co-author) at Research Center for Oceanography- Indonesian Institute of Science, Jakarta. A voucher sample was deposited at Laboratory of Phytochemistry, Department of Pharmacy, Tadulako University.

Extraction

Material of sponges were minced and repeatedly extracted for 3-5 x 24 h by maceration method with Me-OH as a solvent at room temperature. Then each obtained extract was evaporated by using rotary evaporator to reach a viscous extract. Each of extracts was subjected to antibacterial and cytotoxic activity tests.

Antibacterial Screening

Antibacterial screening was performed against two types of bacteria: *Staphylococcus aureus* and *Escherichia coli* by using agar well diffusion method.¹⁰ Briefly, 0.1 mL of suspended bacterium in sterile medium (1.5 x 10⁸ CFU/mL) was spread on nutrient agar media. Then 50 µL of each sample (1000, 500 and 250 mg/mL) was poured into the wells (6-mm diameter). All plates were left for 1 h at 48°C and then incubated for 24 h at 37°C for bacteria. Inhibition zone diameters formed around the well were measured and the mean diameter of three replicates was calculated. DMSO was used as a negative control and chloramphenicol as a positive control.

Cytotoxic activity

Cytotoxic activity was applied on human breast adenocarcinoma (MCF-7), human colon colorectal carcinoma (HCT-116) and two normal cell lines NHDF and CCD-118 by MTT method as described in our previous study.¹¹ Doxorubicin and fluorouracil were used as a positive standard anticancer drug. The stock samples were diluted with RPMI-1640 medium to desired concentrations of 62.5, 125, 250, 500 and 1000 µg/mL. The final concentration of dimethyl sulfoxide (DMSO) in each sample was 1 % v/v. The cancer cells were batch cultured for 10 d, then seeded in 96 well plates of 1 x 10⁴ cells/well in fresh complete growth medium in 96-well microliter plastic plates at 37°C for 24 h under 5% CO₂ using a water jacketed carbon dioxide incubator (CelCulture, Esco Medical ApS, Denmark). The medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample. After 48 h of incubation, cells were added with 10 µL/well of MTT (5 mg/mL) and incubated for 4 h in incubator at 37°C in 5% CO₂ humidified atmosphere. The reaction was stopped by 100 µL dimethylsulfoxide (DMSO). The plate was then incubated for 15 min. The absorbance of each well was read at 550 nm wavelength in Elisa Reader (Infinite M200 pro Nano Quant, Tecan, Switzerland), using wells without cells as blanks. All experiments were performed in triplicate. The effect of compounds on proliferation of cancer cells was expressed as the % cytoviability, using the following formula:

$$\% \text{ Cytoviability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100\%$$

The IC₅₀ calculation was done statistically by probit analysis using SPSS 17.0 (SPSS, Inc, Chicago IL, USA), in which the series of dose-response data and the percentage of cytoviability were plotted together.

Annexin V-FITC Apoptosis Assay

Annexin V-FITC Apoptosis Assay on cancer cells (MCF-7 and HCT-116) were seeded as described above and then incubated with different treatments for 24 h. Cells were harvested, washed twice with PBS and centrifuged. In brief, 1 x 10⁵ of cells were treated with annexin V-FITC and propidium iodide (PI) using the apoptosis detection kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Annexin V-FITC and PI binding were analysed by flow cytometry on FACScanto II (BD Biosciences, San Jose, CA) without gating restrictions using 10,000 cells. Data were collected using logarithmic amplification of both the FL1 (FITC-A) and the FL2 (PI-A) channels. Quadrant analysis of coordinate dot plots was performed with Cell Quest software. Unstained cells were used to adjust the photomultiplier voltage and for compensation setting adjustment to eliminate spectral overlap between the FL1 and the FL2 signals.

RESULTS

Antibacterial test was performed by well agar diffusion method. *Staphylococcus aureus* and *Escherichia coli* were chosen as tested bacteria as the representative of gram positive and gram-negative bacteria. The inhibition of both bacteria used to be said that the extract has broad spectrum type of inhibition. As it can be seen in Table 1, Only *Melophlus sarasinorum* and *Axinella* sp have inhibition on *Staphylococcus aureus* and *Escherichia coli*, with diameter of inhibition zone of 14.21 ± 0.92 mm and 14.36 ± 0.92 mm, and 10.01 ± 2.65 mm and 12.07 ± 1.54 mm, respectively. Therefore, it can be suggested that the extract of *Melophlus sarasinorum* and *Axinella* sp have a broad spectrum of antibacterial activities. Further cytotoxic activity against human colon carcinoma (HCT-116) of the all extracts of sponges, found that the cytotoxic effects were dose and time dependent. It showed that *Melophlus sarasinorum* and *Axinella* sp methanolic extract have time dependent potent cytotoxicity with the IC₅₀ of 0.002 and 8.518 µg/mL, respectively after 48 h incubation (Table 2). Meanwhile, only *Melophlus sarasinorum* showed cytotoxicity on MCF-7 with the IC₅₀ of 87.35 µg/mL after 48 h incubation. *Axinella* sp showed high selectivity on cell growth inhibition where it is found to be not toxic on both CCD and NHDF normal cells.

Table 1: Antibacterial activity of Togean island sponges.

Sponges	Mean Diameter of Inhibition Zone (mm) at 1000 mg/ml	
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>
<i>Sphaciospongia inconstant</i>	-	-
<i>Melophlus sarasinorum</i>	14.21 ± 0.93	14.36 ± 0.92
<i>Oceanapia amboinensis</i> .	-	-
<i>Biemna</i> sp.	-	-
<i>Axinella</i> sp.	10.01 ± 2.65	12.07 ± 1.54
Chloramphenicol ^a	34.32 ± 9.34	45.53 ± 0.55

^a= Chloramphenicol was tested at 1 mg/mL.

Table 2: Cytotoxic activity of Togeian Islands sponges.

Sponges	Inhibition Concentration, IC ₅₀ (µg/mL)					
	MCF-7		HCT-116		CCD	NHDF
	24H	48H	24H	48H	24H	24H
<i>Sphaciospongia inconstant</i>	281.84	129.25	229.47	229.47	ND	ND
<i>Melophlus sarasironum</i>	51.46	87.35	0.75	0.002	14.20	8.47
<i>Oceanapia amboinensis</i> .	213.34	246.15	58.68	45.33	2.01	132.42
<i>Biemna sp</i>	269.43	213.32	780.40	284.13	ND	ND
<i>Axinella sp</i>	250.75	756.90	27.03	8.52	NT	NT
Doxorubicin	0.024		-		ND	ND
Fluorouracil	-		8.915		ND	ND

ND= Not determined, NT= No toxicity.



Figure 1: Location of study: Togeian Islands, Central Sulawesi, Indonesia.

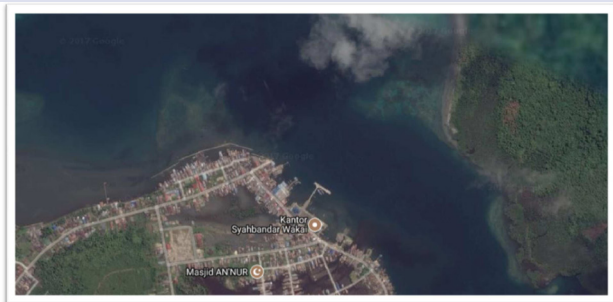


Figure 2: Aerial view of sample collection site.

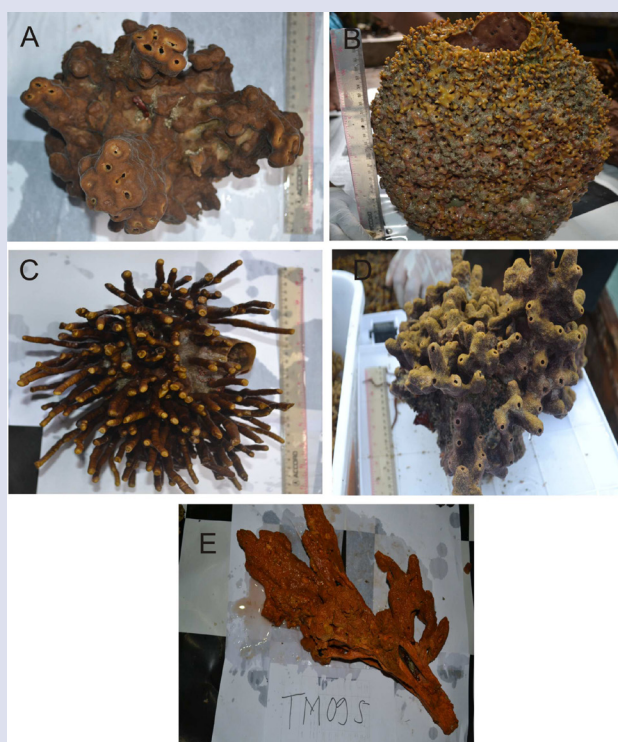


Figure 3: Sponges collected from Togeian Islands. A: *Sphaciospongia inconstan*, B: *Melophlus sarasironum*, C: *Oceanapia amboinensis*, D: *Biemna sp* and E: *Axinella sp*.

DISCUSSION

The aims of this study are to screen for the most potent antibacterial and cytotoxic activity of sponges collected off Togeian Islands, identified as *Sphaciospongia inconstan*, *Melophlus sarasironum*, *Oceanapia amboinensis*, *Biemna sp* and *Axinella sp*. (Figure 3).

Melophlus sarasironum (family Ancorinidae) has been reported to contain nine triterpene glycosides, namely sarasinosides A1, A2, A3, B1, B2, B3, C1, C2, and C3.^{12,13} Interestingly, these sarasinosides compounds exhibited cytotoxic against several cell lines. Schmitz *et al.* (1988) reported the cytotoxicity of sarasinoside A1 against human lymphocytic leukemia cell line with the ED₅₀ of 2.8 µg/mL.^{14,15} Lee *et al.* (2000) proved the cytotoxic activities of sarasinosides A2 and A3 against human leukemia cell line K562 with ED₅₀ of 6.5 and 17.1 µg/mL, respectively.¹⁶ Moreover, four new tetramic acid derivatives, namely Melophlins P, Q, R and S, have potent cytotoxicity against murine leukemic cell lines with the IC₅₀ of

20.0, 10.5, 0.85 and 5.13 µM, respectively.¹⁷ Meanwhile, *Axinella sp* has been reported to contain polyalkylated cyclopentindoles, herbindoles A, B and C, which are cytotoxic to KB cells.¹⁸ These reported data supported our result. However, there is still a lack in the mechanism of cytotoxicity reported. Therefore, we further identify the possible apoptosis mechanism of the potential extracts (*Melophlus sarasironum* and *Axinella sp*) by Annexin V-FITC assay. Apoptosis is the programmed cell death process. It plays an important role in the regulation of tissue development and homeostatis. Therefore, the induction of apoptosis will suppress the tumor progression.¹⁹ The result showed that methanolic extract of *Melophlus sarasironum* and *Axinella sp* have significant percentage of early and late apoptosis on HCT-116 cell lines with the value of 89.70% and 34.00%,

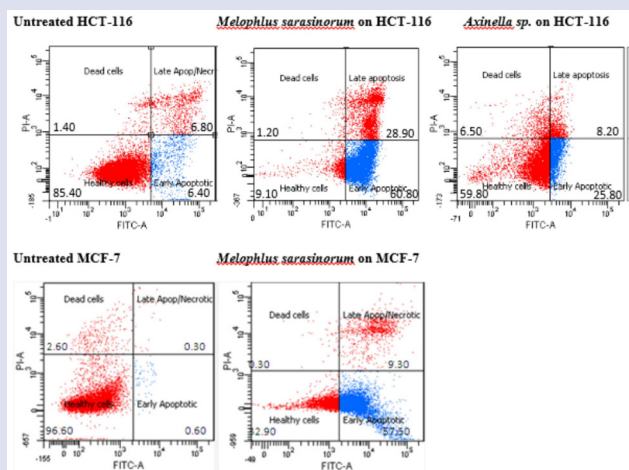


Figure 4: Effect of *Melophlus sarasinorum* and *Axinella sp* methanolic extract on HCT-116 and MCF-7 on annexin V-FITC-positive staining. The four quadrants identified as LL (healthy cells); LR (early apoptotic); UR (late apoptotic) and UL (necrotic).

respectively. Meanwhile, on MCF-7, methanolic extract of *Melophlus sarasinorum* showed significant percentage of early and late apoptosis of 66.80% (Figure 4). Although some studies revealed the potency of bioactive secondary metabolites from these two sponges, the mechanism of anti-cancer via apoptosis induction obtained in this study suggested for further isolation and purification for their metabolites.

CONCLUSION

Melophlus sarasinorum and *Axinella sp* are the most potential extracts from our biological activity screening that have broad spectrum of antibacterial activity. Moreover, it also found to have potent cytotoxicity and apoptosis induction on HCT-116. This study suggested for further isolation and identification of the bioactive compound from these two sponges.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

MCF-7: Michigan Cancer Foundation-7; HCT-116: Homosapiens Colon Colorectal; CCD: Normal Colon Fibroblast; NHDF: Normal Human

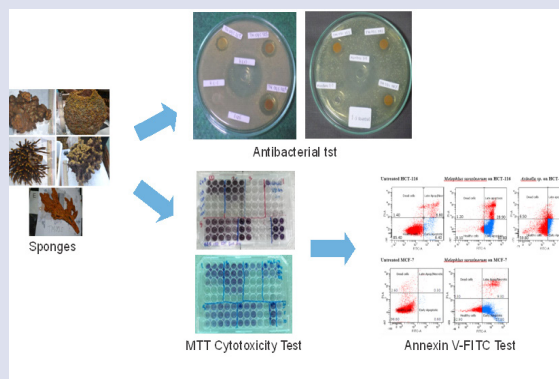
Dermal Fibroblast; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPMI 1640 medium: Roswell Park Memorial Institute; SDS: Sodium Dodecylsulfate; DMSO: dimethyl sulfoxide; CFU: Colony-forming Unit; IC₅₀: Inhibition Concentration; SPSS: Statistical Package for the Social Sciences.

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GRAPHICAL ABSTRACT



SUMMARY

- Only extracts of *M. sarasinorum* and *Axinella sp* exhibited strong inhibition against *S.aureus* and *E.coli*
- *M. sarasinorum* and *Axinella sp* also showed potent cytotoxicity on HCT-116 with the apoptosis induction mechanism
- Only *M. sarasinorum* showed moderate growth inhibition on MCF-7 cell lines.
- The cytotoxic mechanism of *M. sarasinorum* on MCF-7 cell lines was via apoptosis induction.

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