

Bioactivity of extracts from ascidians collected in North Sulawesi as seeds of marine-derived drugs

by Deiske Jansen 7

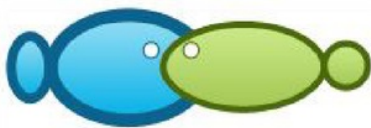
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Bioactivity of extracts from ascidians collected in North Sulawesi as seeds of marine-derived drugs

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Abstract. The ethanol extracts from 95 ascidians collected at North Sulawesi in 2010 were evaluated for their inhibitory activity against the colony formation of Chinese hamster V79 cells, proliferation of two human cancer cell lines (colon HCT-15 and T-cell lymphoma Jurkat), and the growth of *Staphylococcus aureus* (Gram-positive bacterium), *Escherichia coli* (Gram-negative bacterium), *Candida albicans* (yeast), and *Mucor hiemalis* (filamentous fungus). Thirty-eight extracts showed the inhibitory effects (> 50% at 50 µg mL⁻¹) on V79 cells, and 13 and 15 extracts were active (> 50%) against HCT-15 and Jurkat cells, respectively. Among 13 extracts, five extracts were selectively active against HCT-15 cells. Five extracts showed both cytotoxic and antimicrobial activities. The growth of *S. aureus*, *E. coli*, *C. albicans*, and *M. hiemalis* was inhibited by seven, one, five, and nine extracts, respectively. Among nine extracts, seven extracts were active only on *M. hiemalis*. Two compounds, shermilamine B (**1**) and kuanoniamine D (**2**), were isolated from *Cystodytes* sp. as cytotoxic components. Compound **1** was selectively active against HCT-15 (IC₅₀ = 6.7 µM) with no apparent effect on V79 and Jurkat. Compound **2** inhibited HCT-15 (IC₅₀ = 4.1 µM) and Jurkat (19.0 µM) cells, and the EC₅₀ value against V79 cells was 6.2 µM.

Key Words: cytotoxicity, HCT-15, Jurkat, antimicrobial activity.

Introduction. Ascidians (tunicates) are a rich source of biologically active natural products with unique structures as marine sponges, soft corals, and so on (Faulkner 2002; Blunt et al 2018). These marine natural products obtained from marine invertebrates and also from algae and microorganisms are thought to be used for ecological purposes such as prevention from predation, parasitism, biofouling, infection, and so on (Puglisi et al 2014). Some of the marine natural products and their chemical derivatives have been examined in the clinical trials (Butler et al 2014; Mayer et al 2017).

Under the bio-prospective research program, we have been conducting the chemical studies on the organisms in North Sulawesi marine environments and reported the isolation of bioactive compounds and the results from screening bioassays of the extracts from ascidians collected in 2009 (Wewengkang et al, submitted, and citations in this report). The evaluation on bioactivities of ascidians in North Sulawesi has also been performed in 2010. The EtOH extracts from 95 ascidians collected in nine sampling sites at Manado and Lembeh in North Sulawesi were examined for the inhibitory activities on the colony formation of Chinese hamster V79 cells, proliferation of human colon cancer HCT-15 and T-cell lymphoma Jurkat cell lines, and the growth of *Staphylococcus aureus* (Gram-positive bacterium), *Escherichia coli* (Gram-negative bacterium), *Candida albicans* (yeast), and *Mucor hiemalis* (filamentous fungus).

Bioassay-guided separation from the EtOH extract of *Cystodytes* sp. led to the isolation of two cytotoxic compounds, shermilamine B (**1**) (Carroll et al 1989; Bontemps et al 2010) and kuanoniamine D (**2**) (Carroll & Scheuer 1990; Bontemps et al 2010). We describe herein the collection and extraction of ascidians, results from screening bioassays of the extracts, and isolation and identification of compounds **1** and **2**.

Material and Method

General experimental procedures. EI mass spectra were obtained by a JEOL JMS-MS 700 mass spectrometer (Tokyo, Japan). ^1H NMR spectra were recorded on a JNM-AL-400 NMR spectrometer (400 MHz for ^1H) in $\text{DMSO}-d_6$ (δ_{H} 2.46). Preparative HPLC was carried out with a Hitachi L-6200 system.

Fetal bovine serum (FBS) was obtained from GIBCO and other culture materials were purchased from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and organic solvents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Collection of ascidians. Ascidians were collected by scuba diving in the coral reefs in North Sulawesi, Indonesia, in September 2010: at Bunaken Island (South edge) (code No. 10M01–10) on September 11th, Mantehage Island (South edge) (10M11–21) and Bunaken Island (North edge) (10M22–33) on September 12th, Lembeh Strait (10M34–38 and 10M35–43), on September 13th, Manado Tua Island (Bualo, South edge) (10M44–55) and Manado Tua Island (Negeri, East edge) (10M56–71), on September 15th, and Kolongan Beach, Malalayang (10M72–87) and Mutiara, Malalayang, (10M88–95) on September 16th (Figure 1).

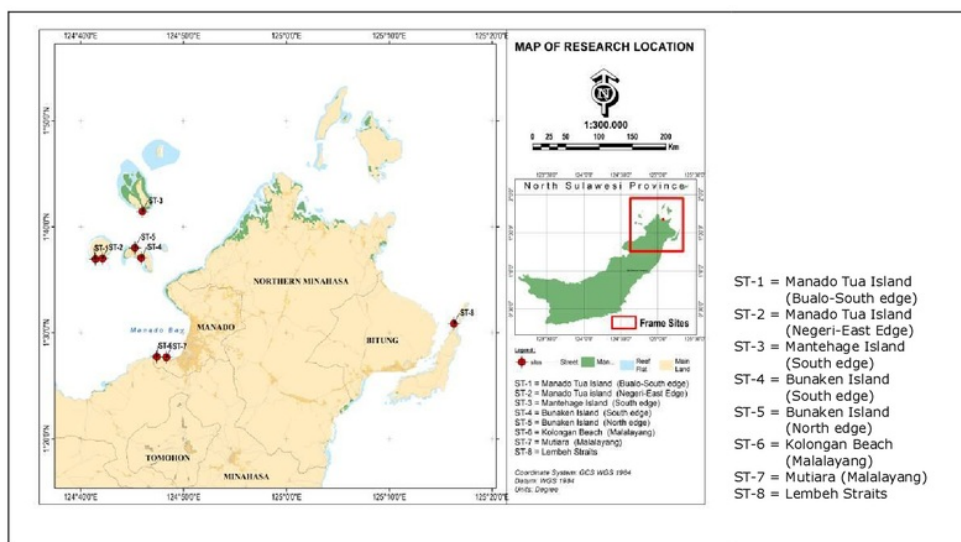


Figure 1. Sampling sites of Ascidian.

Extraction and isolation of compounds. Each ascidian was cut into small pieces and packed in EtOH on a boat immediately after collection. The weights of ascidians were calculated by subtracting the weight of bottle and EtOH from the total weights. The extraction of each ascidian was performed three times with EtOH, and the extracts were evaporated to remove EtOH for screening bioassays.

The EtOH extract (300 mg) from *Cystodytes* sp. 10M34 (79 g, wet weight) was separated by preparative HPLC [column, PEGASIL ODS (10 mm x 250 mm); solvent, 55% MeOH in water containing 0.1% trifluoroacetic acid (TFA); flow rate, 2.0 mL min⁻¹; detection, UV at 254 nm] and yielded 6.3 mg of shermilamine B (**1**) (eluted at 15.9 min) and 8.5 mg of kuanoniamine D (**2**) (eluted at 22.2 min) from 20.0 mg of the extract.

Relative plating efficiency. Chinese hamster (*Cricetulus* sp.) V79 cells were grown as a monolayer culture in Eagle's MEM (Nissui Seiyaku Co., Ltd., Tokyo, Japan) with 10% heat-inactivated FBS. The relative plating efficiencies against V79 cells were determined as

the ratio of the number of colonies in various concentrations of samples to that in the sample-free control, as described in previous papers (Sakakibara et al 1991; Sato et al 1992; Sumilat et al 2017). Two hundred cells were seeded onto a 60/15-mm plastic plate with 17 mL culture medium and incubated overnight at 37°C. After each sample in DMSO (4 µL) was added to the culture medium, cells were further cultured for four days. The numbers of colonies in the sample plates were counted and compared with those in the control cultures.

Cytotoxicity assay. HCT-15 and Jurkat cells were obtained from the Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Miyagi, Japan). Two cell lines were cultured in RPMI-1640 medium. The medium was supplemented with 10% FBS, 100 units mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin. Exponentially growing cells cultured in a humidified chamber at 37 °C containing 5.0% CO₂ were used for the experiments.

Cytotoxicity was evaluated using the colorimetric MTT assay (Mosmann 1983). HCT-15 cells (1 × 10⁴ cells in 100 µL) or Jurkat cells (2.0 × 10⁴ cells in 100 µL) were added to each well of a 96-well plastic plate (Corning Inc., Corning, NY, USA). A sample (1.0 µL in EtOH) was added to each well to make the final concentration from 0 to 27 µM and the cells were incubated for 48 hours at 37°C. MTT (10 µL of 5.5 mg mL⁻¹ stock solution) and a cell lysate solution (90 µL, 40% N,N-dimethylformamide, 20% sodium dodecyl sulfate, 2.0% CH₃COOH and 0.030% HCl) were added to each well, and the plate was shaken thoroughly by agitation at room temperature for overnight. The optical density of each well was measured at 570 nm using an MTP-500 microplate reader (Corona Electric Co., LTD., Ibaraki, Japan).

Antimicrobial assay. The growth inhibitory activity was examined by the paper disc method (disc diameter, 6 mm) against *M. hiemalis* IAM 6088 (fungus), *C. albicans* IFM 4954 (yeast), *S. aureus* IAM 12544T (Gram-positive bacterium), and *E. coli* IAM 12119T (Gram-negative bacterium) as test microorganisms by 250 µg disc⁻¹.

Results and Discussion

Collection and extraction of ascidians. Solitary and colonial ascidians were collected in the coral reefs at eight sites in North Sulawesi as mentioned in the Materials and Methods section. The ascidians were respectively extracted three times with EtOH, and the extracts were evaporated to remove EtOH and used for the screening bioassays.

Screening bioassays. The effect on the colony formation of Chinese hamster V79 cell was evaluated to detect the direct action of the samples on the cells. The inhibitory activity on the proliferation of human colon cancer HCT-15 and T-cell lymphoma Jurkat cell lines and on the growth of four microorganisms, Gram-positive bacterium *S. aureus*, Gram-negative bacterium *E. coli*, *C. albicans* (yeast), and *M. hiemalis* (filamentous fungus), was also examined as screening bioassays for 95 ascidian extracts.

The colony formation of V79 cells was inhibited (> 50% at 50 µg mL⁻¹) by 38 extracts (40.0%), and the growth of HCT-15 and Jurkat cells were affected (> 50% at 50 µg mL⁻¹) by 13 (13.7%) and 15 (15.8%) extracts, respectively (Table 1). It is very interesting that five extracts (10M05, 06, 17, and 90) exhibit the selective cytotoxicity against the solid tumor cells (HCT-15). These samples had no antimicrobial activity. Five extracts (10M03, 35, 48, 59, and 89) showed both antimicrobial and cytotoxic activities. The antimicrobial activities against *S. aureus*, *E. coli*, *C. albicans*, and *M. hiemalis* were detected in seven (7.4%), one (1.1%), five (5.3%), and nine (9.5%) extracts (Table 1; Figure 2). Seven extracts out of nine were selectively active against only the filamentous fungus *M. hiemalis*. The number of extracts possessing the antifungal activity against *C. albicans* and *M. hiemalis* of the 2010 samples (12 extracts) was much higher than that of the 2009 samples (only three extracts against *C. albicans*) (Wewengkang et al, submitted).

Table 1
Cytotoxicity and antimicrobial activity of extracts from ascidians collected at North Sulawesi in 2010

Code No.	Remarks (identification)	Antimicrobial activity (mm)				Cytotoxicity		
		S. a.	E. c.	C. a.	M. h.	V79	HCT-15	Jurkat
<i>Bunaken (South)</i>								
10M03	Colonial	13	12	26	-	100	99.7	99.3
10M05	Solitary	-	-	-	-	93.1	76.3	25.5
10M06	Colonial	-	-	-	-	60.1	51.0	3.8
10M08	Solitary	-	-	-	-	100	7.5	70.3
10M10	Solitary	-	-	-	-	97.7	14.6	41.2
<i>Mantehage (South)</i>								
10M14	Colonial	-	-	-	16	99.1	29.1	21.9
10M15	Colonial	-	-	-	-	100	0.0	0.0
10M17	<i>Eudistoma reginum</i>	-	-	-	-	100	81.2	40.0
10M19	Colonial	-	-	-	-	53.2	50.5	15.0
10M21	Colonial	-	-	-	-	98.2	0.3	24.6
<i>Bunaken (North)</i>								
10M22	Colonial	-	-	-	-	100	99.8	99.3
10M26	Colonial	-	-	-	-	58.7	0.0	1.5
10M29	Colonial	-	-	-	-	56.9	7.4	47.9
10M33	Colonial	-	-	-	-	66.1	1.0	42.9
<i>Lembeh (Putus Island)</i>								
10M34	<i>Cystodytes</i> sp.	-	-	-	-	84.9	44.4	9.9
10M35	Colonial	9	-	18	-	100	99.5	99.5
<i>Manado Tua (Bualo)</i>								
10M47	Colonial	-	-	-	-	100	45.2	22.7
10M48	Colonial	8	-	22	18	100	86.2	100
10M49	Colonial	-	-	-	15	30.3	38.0	25.1
10M50	Colonial	-	-	-	-	57.3	5.1	29.2
10M51	Colonial	-	-	-	18	67.0	7.4	31.6
10M52	Colonial	-	-	-	-	69.3	33.8	15.6
<i>Manado Tua (Negeri)</i>								
10M56	Colonial	-	-	-	-	50.9	0	26.1
10M57	Colonial	-	-	-	22	56.0	7.4	10.9
10M58	Colonial	-	-	-	-	78.4	43.2	62.2
10M59	Colonial	9	-	22	26	100	96.9	100
10M60	Colonial	-	-	-	-	60.6	21.7	22.3
10M61	Colonial	-	-	-	22	32.6	22.8	86.5
10M62	Colonial	-	-	-	32	32.6	5.7	66.1
10M64	Colonial	-	-	-	-	40.8	0.0	57.3
10M69	Colonial	-	-	-	-	55.0	12.5	10.5
<i>Malalayang (Kolongan Beach)</i>								
10M76	Colonial	-	-	-	-	53.7	0.0	10.6
10M78	Colonial	-	-	-	-	71.1	10.9	23.8
10M81	Colonial	-	-	-	-	50.9	0.0	42.8
10M84	Colonial	9	-	-	-	100	95.9	98.7
10M85	Colonial	-	-	-	-	61.0	34.6	0.0
10M87	Colonial	-	-	-	-	100	31.5	53.1
<i>Malalayang (Mutiar)</i>								
10M88	Colonial	-	-	-	-	99.5	0.0	89.0
10M89	Colonial	15	-	15	-	10.0	93.3	99.3
10M90	Colonial	-	-	-	-	99.5	75.9	25.2
10M91	Colonial	-	-	-	15	63.3	26.8	5.4
10M93	Colonial	19	-	-	-	100	67.5	87.5
10M94	Colonial	-	-	-	-	50.5	1.3	47.5

Antimicrobial activity: inhibition zone (mm) at 250 µg disc⁻¹ (disc diameter, 6 mm): S. a.: *Staphylococcus aureus* (Gram-positive bacterium), E. c.: *Escherichia coli* (Gram-negative bacterium), C. a.: *Candida albicans* (yeast), M. h.: *Mucor hiemalis* (filamentous fungus); V79: inhibitory activity (% at µg mL⁻¹) on the colony formation of Chinese hamster V79 cells; Antiproliferative activity (% at µg mL⁻¹): MTT method; HCT-15: human colon cancer cell line; Jurkat: T-cell lymphoma cell line.

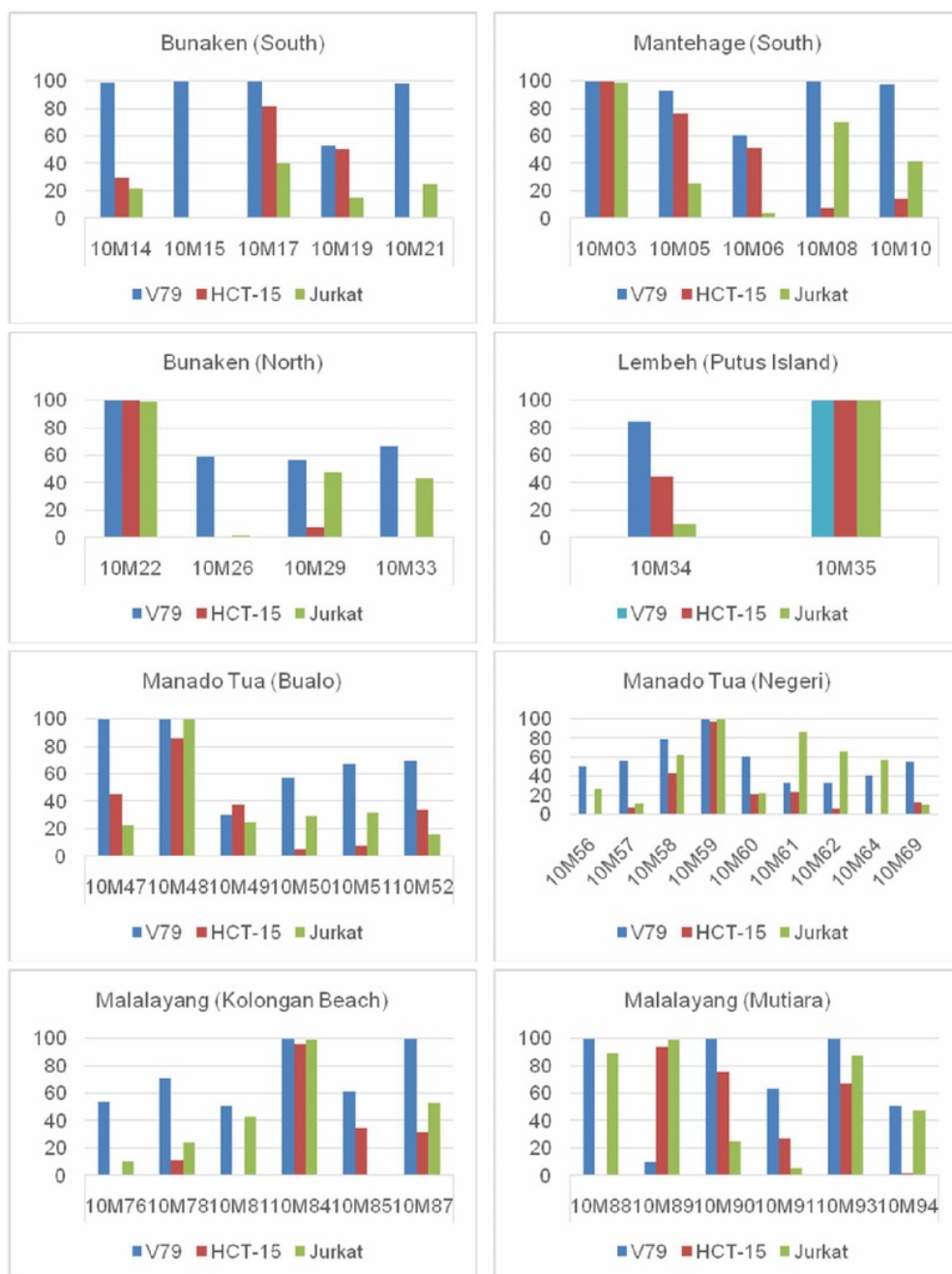


Figure 2. Cytotoxicity and antimicrobial activity of extracts from ascidians collected at North Sulawesi in 2010.

Isolation, structures, and bioactivity of compounds 1 and 2. The HPLC analysis of the EtOH extracts revealed two major peaks in the extract from *Cystodytes* sp. (code No. 10M34), and compounds **1** and **2** were isolated from the EtOH extract by preparative HPLC. The EI mass spectra of **1** and **2** gave the molecular ion peaks at m/z 390 and 360, respectively. The ^1H NMR spectra of **1** and **2** measured in $\text{DMSO}-d_6$ showed the characteristic signals ascribable to fused heteroaromatic rings at δ 7.0-11.0 ppm. From

these spectroscopic data, the literature study in the SciFinder provided shermilamine B and kuanoniamine D as candidates for the structures of **1** and **2**, respectively. The comparison of spectroscopic data for **1** and **2** with those of the reported values for shermilamine B (Carroll et al 1989; Bontemps et al 2010) and kuanoniamine D (**2**) (Carroll & Scheuer 1990; Bontemps et al 2010) identified the structures of **1** and **2** as shown in Figure 3.

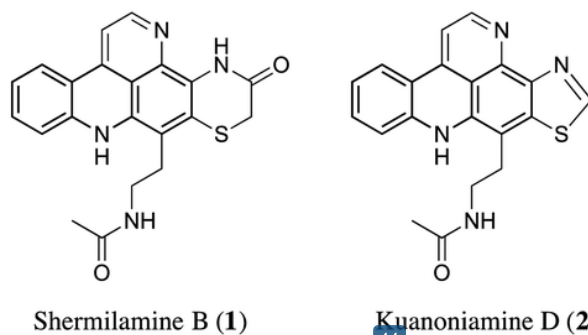


Figure 3. Structures of compounds **1** and **2** isolated from *Cystodytes* sp. collected in North Sulawesi.

The effect of compounds **1** and **2** isolated in this study was examined on the colony formation of V79 cells and two human cancer cell lines (HCT-15 and Jurkat), and the results are listed in Table 2. Compound **1** showed the selective cytotoxicity against the solid tumor cell line (HCT-15) and no apparent activity against Jurkat (lymphoma cell line) and V79 cells.

Table 2
Effect of compounds **1** and **2** against V79, HCT-15 and Jurkat cells

Compound	V79	HCT-15	Jurkat
	EC_{50} (μM)	IC_{50} (μM)	
1	> 20.0	6.7	> 26.0
2	6.2	4.1	19.0

Shermilamine B (**1**) was first isolated from a colonial ascidian *Trididemnum* sp. collected at Guam (Carroll et al 1989) and kuanoniamine D (**2**) from the Micronesian ascidian and its predatory mollusk *Chelynotus semperi* as a cytotoxic compound against KB cells (Carroll & Scheuer 1990). Compounds **1** and **2** were also obtained from the Mediterranean ascidian *Cystodytes dellechiaiei* and tested against *E. coli* and *Micrococcus luteus* (Bontemps et al 2010). These compounds are classified in the pyridoacridine alkaloids (Wang & Namikoshi 2007; Blunt et al 2017), which have been known to exhibit various bioactivities such as cytotoxicity, inhibition of topoisomerase II, anti-HIV activity, Ca^{2+} releasing activity, and intercalation with DNA (Skyler & Heathcock 2002), Ca^{2+} releasing activity, and intercalation with DNA (Marshall & Barrows 2004).

Shermilamine B (1). Obtained as a dark purple gum; 1H NMR spectrum (DMSO- d_6 , Figure 4) δ 1.93 (3H), 2.99 (2H), 3.12 (2H), 3.13 (2H), 7.06 (1H), 7.24 (1H), 7.45 (1H), 7.53 (1H), 8.07 (1H), 8.54 (1H), 8.58 (NH), 9.33 (1H), 10.43 (1H); EI-MS m/z 390 [M^+] (Figure 6).

Kuanoniamine D (2). Obtained as a dark purple gum; 1H NMR (DMSO- d_6 , Figure 5) δ 1.85-1.89 (3H, s), 3.09 (2H, t), 3.16 (2H, dt), 7.00 (1H, dt), 7.49 (1H, d), 7.49 (1H, d), 7.87 (1H, d), 7.99 (1H, d), 8.39 (1H), 8.57 (1H, d), 9.29 (1H, s), 10.94 (1H, s); EI-MS m/z 360 [M^+] (Figure 7).

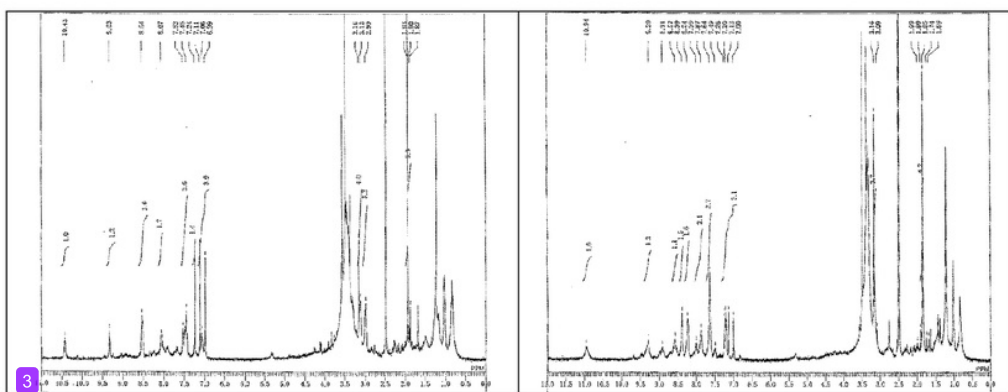


Figure 4. ^1H NMR spectrum of compound 1. Figure 5. ^1H NMR spectrum of compound 2.

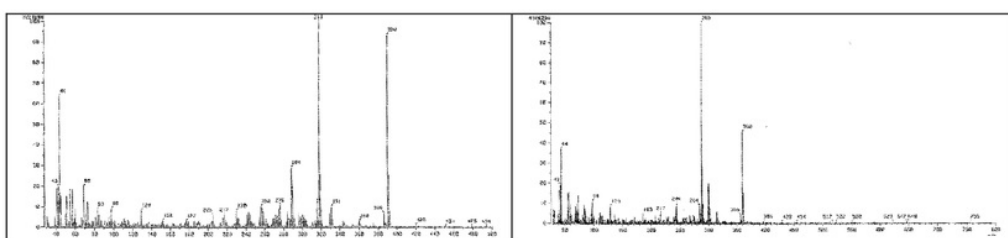


Figure 6. EI mass spectrum of compound 1. Figure 7. EI mass spectrum of compound 2.

Conclusions. The extracts of 95 ascidians collected in North Sulawesi were tested for their inhibitory activity on the colony formation of V79 cells and for the growth of two human cancer cell lines and four microorganisms. The colony formation of V79 cells was inhibited (> 50% at $50\ \mu\text{g mL}^{-1}$) by 38 extracts, and 13 and 15 extracts were active (> 50%) against HCT-15 and Jurkat cells, respectively. Five extracts out of 13 were selectively active against HCT-15 cells. Five extracts showed both cytotoxic and antimicrobial activities. Seven, one, five, and nine extracts inhibited the growth of *S. aureus*, *E. coli*, *C. albicans*, and *M. hiemalis*, respectively. Among nine extracts, seven extracts were selectively active on the filamentous fungus *M. hiemalis*.

Two cytotoxic components were isolated from the EtOH extract of *Cystodytes* sp. by HPLC and identified the structures as shermilamine B (**1**) and kuanoniamine D (**2**). Compound **1** showed selective cytotoxicity ($\text{IC}_{50} = 6.7\ \mu\text{M}$) against the solid tumor cell line (HCT-15).

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