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Research Article

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Screening and characterization of bioactive compound from Samalona island sponges, Indonesia

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ABSTRACT

Marine sponges contain unique chemical compounds that are potential to be developed as new drugs. This study aimed to screen and characterize bioactive compounds of sponges obtained from Samalona Island, South Sulawesi. The sponges were extracted with methanol and partitioned with chloroform-water mixture. The bioactivity of sponge extracts was tested against Artemia salina Leach. Extracts that showed high bioactivity/cytotoxicity were fractionated using vacuum liquid chromatography and isolated using preparative thin layer chromatography. The obtained isolates were then tested against A. Salina. The isolated compound with highest bioactivity was characterized using UV light (chemical reactions and spectrophotometer UV and IR. Three out of 15 sponges collected yielded a highly toxic extracts against A. salina and the most active chloroform extract was obtained from Penares sp. sponge. Fractionation and isolation of the Penares sp extract generated an isolate with high toxicity with $LC_{50}$of 0,0971 µg/ml. Characterization of the isolate showed no detectable bandunder UV light 254 and 366 nm and exhibited positive reaction with Dragendorff's reagent. The UV spectra showed the maximum absorbance of the compound was 206 nm. The IR spectra showed that the compound contains amine (-NH), carbonyl (C=O), alkyl (CH_3,-CH_2, -CH-) and alkena (=CH) groups. In conclusion, Penares sp. obtained from Samalona island contains highly cytotoxic isolate that is potential to be developed as anticancer agent.

Key words: bioactive compound, sponges, Samalona Island, *Artemia salina* Leach.

INTRODUCTION

Marine natural products have been explored as sources of bioactive compounds for the development of new drugs. Currently, marine-derived anti-cancer agents predominantly extracted from marine algae [1], and despite of its potency, marine sponges are still considered underexplored [2]. It has been reported that up to 76% of sea sponges contain cytotoxic compounds that are potentially developed as safer and cheaper novel anticancer agents [3].

As an archipelagic country with approximately 80% of water region, Indonesia coastal zone has become one of the hot spots for sponge biodiversity in the world[4]. In 2008, fourteen species of Indonesian sponges have been reported to contain cytotoxic and anticancer constituents [4], including compounds extracted from *Lenden feldia* sp. that possess significant activities against hypoxia-inducible factor-1 (HIF-1) activation of T47D breast tumor cells[5]. Recently, promising cytotoxic metabolites have been discovered from *Dyspediasp* sponges collected in Biak, West Papua, as well as *Theonella swinhoei* from Bunaken, South Sulawesi[6, 7]. Ever since, Indonesia coastal regions have been widely explored, however, many more areas remain to be investigated, including a high diversity of marine sponge area in Samalona Island, South Sulawesi, Indonesia. Therefore, This study aimed to explore the high biodiversity of marine sponges in Samalona Islandin order to isolate bioactive compound with high cytotoxicity.The isolate was subjected to phytochemical group analysis using UV light, chemical reagentsand spectrophotometer UV and IR.

The preliminary assay for cytotoxicity of marine product is frequently conducted using brine shrimp lethality test (BSLT). This assay measures substance toxicity against cultured *Artemia salina* nauplii brine shrimp.The BSLT is considered beneficial for preliminary assessment sincethe assay itself is inexpensive and simple[8], and have a great correlation with cancer cell line toxicity test [9].

EXPERIMENTAL SECTION

Collection and preparation of extracts

All organic and inorganic solvents were purchased from Sigma Aldrich, Indonesia. Sponges were collected by snorkeling and scuba diving from the depth of 4-20 m along the Samalona island coastline (199^o 20' 325" E and 05^o 07' 474" S). The specimens of each species were extracted with methanol. The yielded extracts were then partitioned in chloroform/methanol (1,1) to obtain two types of extracts, the chloroform and methanol extracts.

Brine shrimp lethality test (BSLT)

Sponge chloroform and methanol extracts(50 mg) were dissolved in 10 ml solvent to obtain 5mg/ml stock solution. Each stock solution was pipetted into clear vials to obtain concentration of 1000, 100 and 10 µg/ml. The negative control was made with 1000 µl of corresponding solvent. The solvent was allowed to evaporate at room temperature before 5 ml of seawater was added.

Artemia salina dried cysts (The Great Salt Lake USA, 50 mg) were hatched in 75 ml seawater at 28–30°C with strong aeration and under an incessant light regime for 48 hours. Ten (10) *Artemia nauplii* were placed in the vials contain the extracts or control (vehicle only). Five replications (a total of 50 *nauplii*) were used for each treatment and control. The toxicity was determined after 24 h of exposure. The number of living *Artemia nauplii* was counted and percentage of deaths wascalculated. The concentration that would kill 50% of the *nauplii* (LC_{50}) was calculated probit and regression analysis.

Fractionation of the active extracts

Extracts thatare highly toxic to *A. salina* were pepared for fractionation. Sample (1 g) was dissolved in etil acetate andfractionated using vacuum liquid chromatography (VLC) with combined mobile phases to obtain eluents with graded polarity, etil acetate 100%, etil acetate, methanol (10,1, 5,1, 1,1, 1,5), and methanol 100%. The bioactivity of fractions were then tested using *Brine shrimp lethality test.*

Isolation of the active compound

Fractions with high bioactivity were partitioned using preparative thin layer chromatography. Fraction was dissolved in chloroform,methanol (1,1), then spread as a thin layer over silica gel plate. After the plate was completely dry, the elution process was started using etil acetate,methanol,amonia (5,0.5,0.3). The compound layers were scrapted and dissolved in chloroform,methanol (1,1) and filtered to separate the silica gel from the isolated compounds. The bioactivity of the isolates were then tested using *Brine shrimp lethality test.*

Characterization of the active isolate

The most active isolate against *A. salina* were characterized using UV light 254 and 366 nm and reagent Dragendorff. The phytochemical group analysis of the isolate was elucidated with UV (Hitachi-3100) and IR spectrofotometres(FT)-IR-5300).

Statistic Analysis

The percentage of death data are presented as mean ± SEM and analyzed using one-way ANOVA (SPSS, IBM 20) followed by Tukey's post hoc test for multiple comparisons. LC_{50} was calculated using probit regression analysis. An independent T-test was performed to compare the LC_{50} of active isolates.

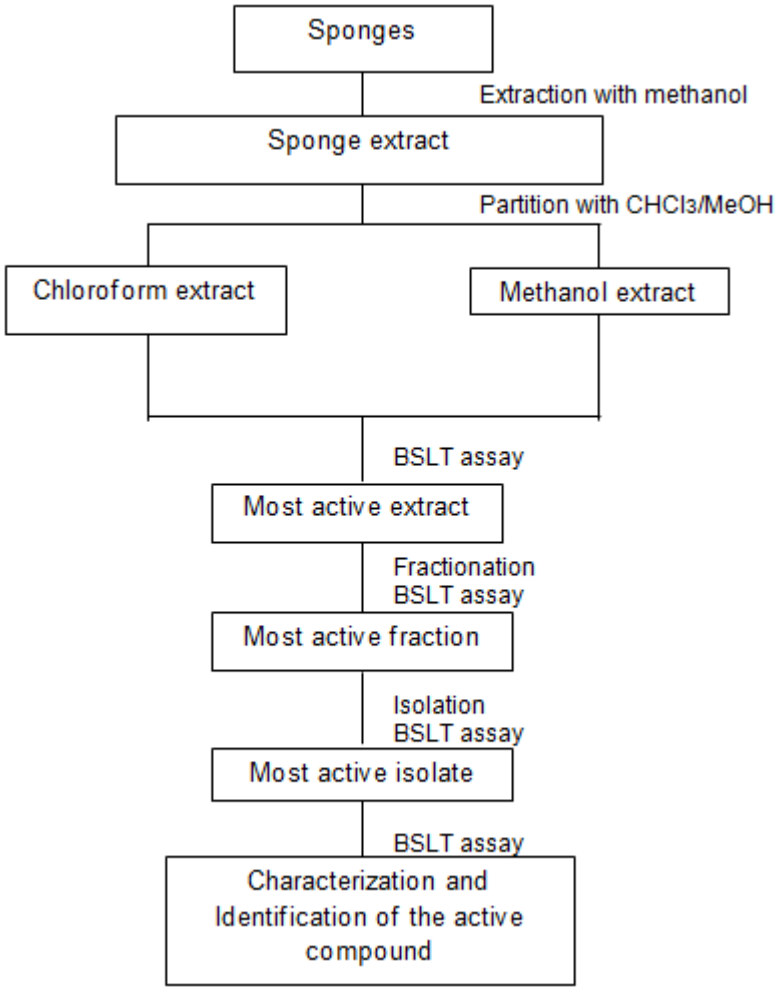

Figure 1. Schematic protocol of study to isolate active (cytotoxic) compound from sponge collected from Samalona Island

RESULTS AND DISCUSSION

Fifteen species of marine sponges were collected and extracted with two types of solvents, methanol and chloroform. Table 1 shows that methanol extracts of the sponges had LC_{50} ranged from 10.98 to 458.15 µg/ml and the chloroform extracts had LC_{50} ranged from 0.001 to 454.91 µg/ml. There were 2 methanol and 5 chloroform extracts with low toxicity (LC_{50}= 100-1000 µg/ml), higher number of extracts showed medium toxicity(LC_{50}= 10-100 µg/ml), and 3 clorofrom extracts exhibited high toxicity (LC_{50}= 1-10 µg/ml). These chloroform extracts were obtained from sponges SP01, SP05, and SP14,which then identifiedas *Pseudoaxinella massa*, *Penares sp*. and *Auletta sp*.respectively (see Table 2).Among them, *Penares sp*chloroform extract had the highest toxicity, which killed 100% of the nauplii at concentration as low as 10µg/ml (Table. 1).

Chloroform extract of *Penares sp.*(SP05) was further fractionated using VLCwith succesive mobile phases, starting with etil acetate 100%, etil acetate,methanol (10, 1, 5, 1, 1, 1, 1, 5), and finally methanol 100%. This fractionationyielded 4 fractions, namely fraction I,II,III, and IV. Among them, only fraction IV killed 100% of the nauplii at the concentration of 10µg/ml (Table 3). The LC_{50} of fraction IV was 0.012 µg/ml,indicating high toxicity of the fraction.Isolation with thin layer chromatography yielded 2 pure isolate, namely isolate IVA and IVB. The BSLT showed that the LC_{50} of isolate IVA was 0,971µg/mland was significantly lower than isolate IVB (138.04 µg/ml) (Table 4).Since only isolate IVA showed high activity against *A. salina*, it was further characterized with UV-VIS and IR spectrophotometers.

Table 1. Percentage of death of *Artemia salina* nauplii exposed to sponge extracts concentration 1000, 100 and 10 µg/ml

Sample	Concentration (µg/ml)	Methanol Extract		Chloroform Extract	
		% Death	LC_{50} (µg/ml)	% Death	LC_{50} (µg/ml)
SP01	1000	100 ± 0.0	11.22	100 ± 0.0	0.05
	100	86 ± 4.0		100 ± 0.0	
	10	54 ± 10.8		94 ± 6.0	
SP02	1000	100 ± 0.0	11.22	100 ± 0.0	28.00
	100	96 ± 2.4		90 ± 6.3	
	10	50 ± 5.5		16 ± 2.4	
SP03	1000	100 ± 0.0	20.21	100 ± 0.0	10.74
	100	74 ± 10.8		92 ± 5.8	
	10	40 ± 8.4		50 ± 7.7	
SP04	1000	100 ± 0.0	38.44	62 ± 4.9	214.07
	100	72 ± 11.6		58 ± 4.9	
	10	14 ± 7.5		42 ± 5.8	
SP05	1000	100 ± 0.0	14.68	100 ± 0.0	0.001
	100	98 ± 2.0		100 ± 0.0	
	10	28 ± 5.5		100 ± 0.0	
SP06	1000	94 ± 2.4	24.58	80 ± 5.1	454.91
	100	60 ± 7.1		24 ± 9.3	
	10	42 ± 9.7		34 ± 7.5	
SP07	1000	80 ± 5.1	102.01	96 ± 2.4	14.54
	100	44 ± 5.1		80 ± 3.2	
	10	24 ± 8.7		62 ± 3.7	
SP08	1000	86 ± 7.5	18.71	94 ± 2.4	17.60
	100	82 ± 6.6		80 ± 2.4	
	10	56 ± 11.2		52 ± 9.3	
SP09	1000	74 ± 6.8	458.15	64 ± 6.8	288.40
	100	24 ± 5.1		34 ± 9.3	
	10	20 ± 3.2		26 ± 8.7	
SP10	1000	100 ± 0.0	18.51	100 ± 0.0	12.14
	100	98 ± 2.0		90 ± 4.5	
	10	20 ± 5.5		48 8.6	
SP11	1000	92 ± 3.7	22.97	80 ± 8.0	123.18
	100	68 ± 5.8		40 ± 8.4	
	10	66 ± 6.8		18 ± 5.8	
SP12	1000	86 ± 5.1	10.98	86 ± 6.8	48.14
	100	80 ± 6.0		58 ± 8.6	
	10	44 ± 9.3		30 ± 9.5	
SP13	1000	98 ± 2.0	18.96	62 ± 5.8	477.61
	100	82 ± 5.8		26 ± 12.1	
	10	36 ± 5.1		22 ± 5.8	
SP14	1000	100 ± 0.0	29.78	100 ± 0.0	0.67
	100	84 ± 7.5		100 ± 0.0	
	10	18 ± 6.6		82 ± 6.6	
SP15	1000	92 ± 5.8	26.60	80 ± 7.5	56.10
	100	68 ± 4.5		40 ± 6.3	
	10	66 ± 9.3		18 ± 4.5	

Table 2. Species identification from Research Centre for Oceanography at Indonesian Institue of Science

No.	Spongecodes	Species
1	SP01	*Pseudoaxinella massa*
2	SP05	*Penaressp*
3	SP14	*Aulettasp*

Table 3. The percentage of death and LC_{50} of fractions derived from chloroform extract of *Penares sp*.

Sample	Fractions	The percentage of death (%)			LC_{50}
		100 µg/ml	10 µg/ml	1 µg/ml	(µg/ml)
Chloroform extract of *Penares sp.*	I	82 ± 6.6	58 ± 8.0	50 ± 5.1	15.71± 1.1
	II	100 ± 0.0	96 ± 4.0	92 ± 3.7	0.35± 0.02
	III	100 ± 0.0	98 ± 2.0	90 ± 6.3	0.45± 0.01
	IV	100 ± 0.0	100 ± 0.0	96 ± 4.0	0.012± 0.00[b]

All data were presented with mean ±SEM. [b]P< 0.05, siginificantly lower than other fractions

Table 4. The percentage of death and LC_{50} of active isolates obtained from fraction IV of *Penares sp.* extract

Isolate	The percentage of death (%)			LC_{50}
	10 µg/ml	1 µg/ml	0.1 µg/ml	(µg/ml)
IVA	100 ± 0.0	96 ± 4.0	48 ± 3.7	0.971 ± 0.02[b]
IVB	22 ± 6.6	10 ± 3.2	2 ± 0.0	138.04± 15.1

All data were presented with Mean ±SEM. [b]P< 0.05, siginificantly lower than other fraction

Despite significant innovation on cancer therapy in the past decades, the search of novel anti-cancer compounds are still in demand due to increased likelihood of prevalence [10]. Studies on marine natural product have discovered at least 150 potential cytotoxic compounds, and around 35 of them have been further developed as anticancer with known mechanisms of activity [11]. A survey conducted in 2013 revealed that about 22marine active compounds are currently involved in clinical trials[10]. These data indicate that marine natural products, including sea sponges, are promising sources of anticancer and antitumor compounds [12]. This present study examined cytotoxicity of chloroform and methanol extracts of 15 species of sponges collected from Samalona Island in South Sulawesi.

The bioactivity of the sponge extracts was tested using brine shrimp lethality test. In addition to toxicity test, the brine shrimp lethality test is also a good indicator for anti-fungal, pesticide and anti-tumor activity [8]. The toxicity of the extract was indicated by its LC_{50} or the concentration that killed 50% of the *Artemia salina* nauplii. From the screening, it is found that 3 three out of 15 sponge species provided extremely toxic chloroform extracts, with LC_{50} less than 1 µg/ml. Indeed, one isolate from most active fraction obtained from sponge *Penares sp* showed significantly high toxicity(LC_{50}0,971µg/ml).

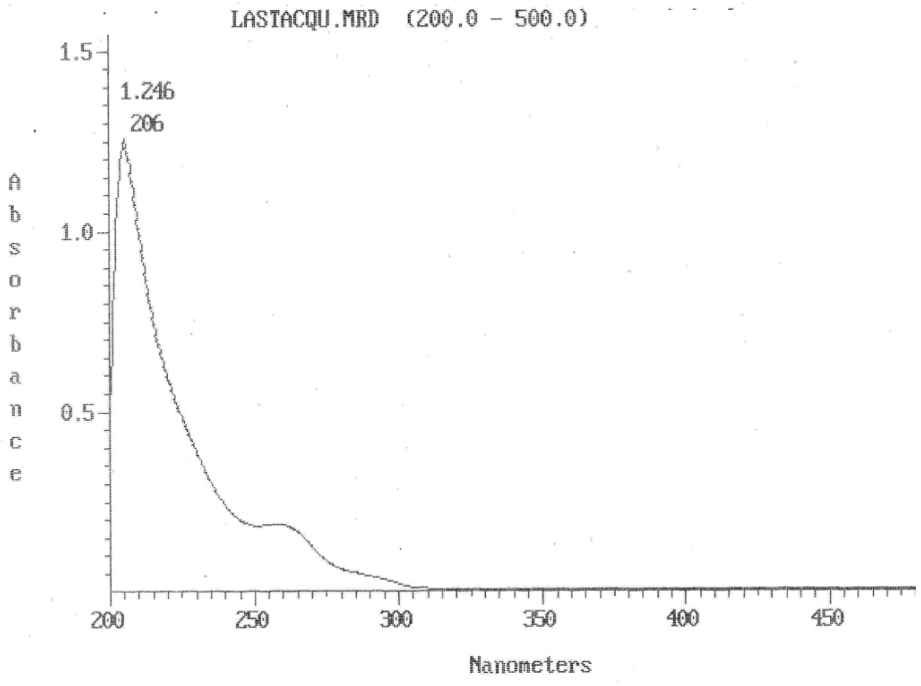

Figure 2. The UV-VIS spectra of isolate IVA derived from *Penares sp.* chloroform extract

The cytotoxicity of marine product is predominantly originated from its secondary metabolites. These include alkaloid [13], steroid glycoside [14], peptides [15] and terpenes[1]. Previously, *Penares sp.* has been shown to contain Ancorinosides B–D, an inhibitor of membrane type I matrix metalloproteinase (MTI-MMP) [16], Penasterol, an antileukemic sterol [17], and cytotoxic constituents, penasin A-E [18]. More recently, bromine-containing alkaloids have been discovered from *Penares sp.*, which have moderate cytotoxicity against HL-60 and HeLa tumor cells, with IC_{50} of 16.1 and 33.2 µM, respectively[19].

Penares-derived isolate IVA appeared as pale green band on thin layer chromatography. Phytochemical group analysis of the isolate showed no detectable band under UV light, indicating the absence of chromophore groups that are able to absorb UV light (254 and 366 nm). Spectra UV of the isolate exhibited a peak at 206 nm (Figure 2). The isolate positively reacted with Dragendorff's reagent, leaving an orange-colored band. The nature of chemical groups of the isolate was shown by IR spectra (Figure 3), depicting the presence of amine in region 3336 cm^{-1} and 1527 cm^{-1}, carbonyl in region 1639.4 $cm^{-1}$and alkil groups in region 1639.4 cm^{-1},while alkyl groups were detected in the region 1384 cm^{-1} (CH_2), 1469 cm^{-1} (CH_3), 2962.5 – 2873 cm^{-1} (CH) and 1735 cm^{-1} (=CH). These data, together with chemical characterization, suggest the presence of alkaloid-type compound in the isolate. Further elucidation of the structure with spectrophotometry ^{1}H NMR and ^{13}C NMR is required o confirm this assumption.

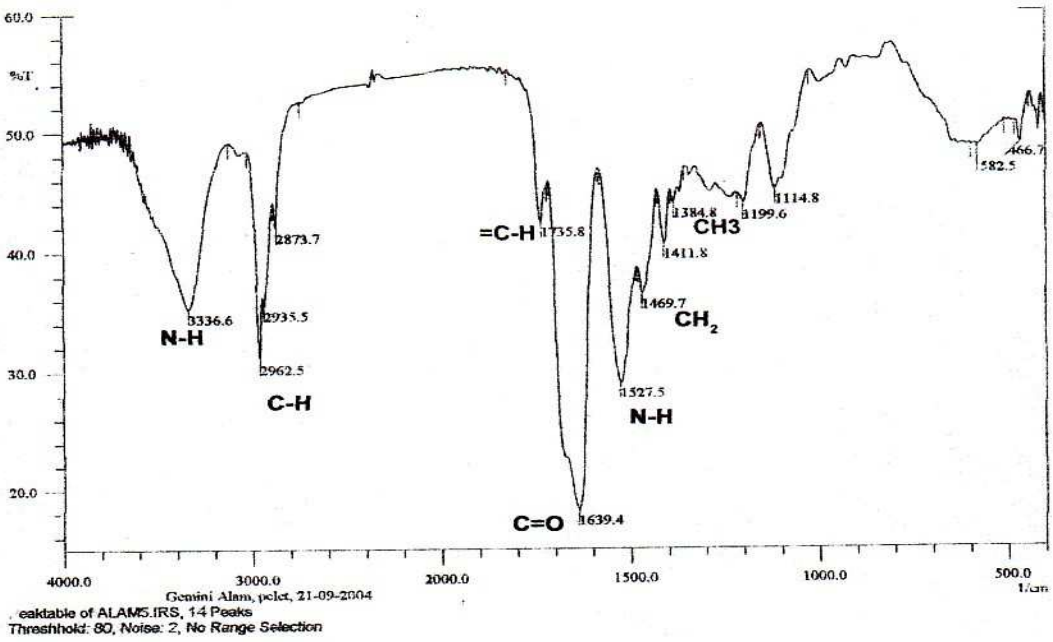

Figure 3. The IR spectra of isolate IVA derived from *Penares sp.* chloroform extract

CONCLUSION

Out of 15 sponge species collected from Samalona Island, chloroform extraction of *Pseudoaxinella massa*, *Penares sp*. and *Auletta sp*. possessa high bioactivity towards brine shrimp larvae. Fractination and isolation of *Penares sp*. chloroform extract yielded a highly toxic isolate against brine shrimp larvae,with $LC_{50}$of 0,971µg/ml. Since BSLT result is highly correlated with anti-cancer effects, this isolate might have promising activities against cancer cells. Further study is required to examine this possibility.

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