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Cytotoxicity and Clastogenic Effect of Metabolite from the Marine Sponge *Halisarca sp.*

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Bioassay-guided fractionation of the cytotoxic butanol-soluble portion of the marine sponge *Halisarca sp.* afforded an aromatic Compound **1**, which exhibited high cytotoxicity towards the nauplii of brine shrimp *Artemia salina* with an estimated acute LD_{50} of 323.59 ± 1.23 ppm. It revealed marginal antibacterial activity against gram-positive bacteria *Staphylococcus aureus* at higher concentrations of 800 and 1000 ppm. It had chromosome-breaking potential as determined using the micronucleus test at dose levels of 15 and 10 mg / kg mouse.

Keywords: marine sponge, *Halisarca sp.*, cytotoxic, clastogenic

INTRODUCTION

The incredible biodiversity of the marine species, particularly the sessile lower invertebrates, which secrete a copeia of allelopathic metabolites utilized by them as chemical warfare, has led to the generation of a voluminous number of promising bioactive bioproducts. Among these bottom-dwelling marine invertebrates, the sponges are the most productive and considered to be a natural reservoir of novel metabolites with unique skeletal systems [1]. Marine sponges belonging to class Demospongiae of the order Haploslerida, Verongida, Dictyoceratida, Dendroceratids, Halichondrida and Haplosclerida are prominent sources of water soluble, highly-polar metabolites like saponins, sugars, hydroxyl and polyhydroxyl containing compounds [2]; of medium polarity compounds such as the peptides and depsipeptides [3]; and of low polarity metabolites such as the hydrocarbons, fatty acids and terpenes [4].

The *Halisarca sp.* is a marine sponge belonging to class *Demospongiae*, order *Halisarcida* and family *Halisarcidae.* The high bioactivity of the marine sponge *Halisarca sp.* towards the nauplii of the brine shrimp *Artemia salina*, the absence of literature concerning its secondary metabolites, as well as instability of its color in life, made this sponge an intriguing candidate for chemical investigation. Bioassay-guided fractionation of the cytotoxic butanol-soluble portion of the marine

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sponge gave an aromatic Compound **1**. This paper reports the isolation and biological activities of this compound.

EXPERIMENTAL

General Experimental Procedures. The UV spectrum was recorded on a Shimadzu UV-160 and IR spectra with a Perkin Elmer Paragon 1000 FT-IR spectrophotometer using the KBr pellet technique. NMR spectra were measured with a JEOL GSX 500 spectrometer (^{1}H; 500 MHz, ^{13}C; 125.65 MHz). Chemical shifts are given on a δ (ppm) scale and were referenced to deuterated chloroform signal (^{1}H 7.26; ^{13}C 77.00). EIMS was recorded on a JEOL SX 102A. Flash column chromatography equipment was manufactured by Biotage, Inc., a Dyex Corp. Company. HPLC was performed on a Knauer with UV detector K-2501. Thin layer analyses were done on pre-coated plates (Si gel 60 F_{254}).

Sampling and Sample Preparation. The marine sponge *Halisarca sp.* was collected by hand scuba in the northern part of Bais Bay, Bais City at a depth of 6-12 m by two Silliman University Marine Laboratory personnel on November 04, 2003. This yellow orange colored soft and mucoid sponge were encrusting in rocks in small colonies, which made it difficult to collect. Upon exposure to the atmosphere, its color turned to burnt dark orange. The fresh sponge sample was placed in thick plastic bags and was immediately stored in an ice cooler. The sponge sample was then transferred to the freezer upon arrival in the laboratory until the work-up.

Extraction and Isolation. Approximately 700 g wet weight of sponge were cut in small pieces and macerated well. The sponge sample was exhaustively extracted with methanol (500mLx3) at room temperature for 48 h. The combined methanol extracts were concentrated *in vacuo* yielding a brownish orange residue (38.0750 g), which was successively partitioned between water and hexane, ethyl acetate and butanol. The butanol soluble portion (7.354 g), which exhibited high cytotoxicity against brine shrimp was subjected to a series of silica column chromatography with MeOH/DCM gradient mixtures as eluent to afford several fractions. Further purification of the most cytotoxic fraction on ODS column employing 0-100% MeOH in H_2O as eluent furnished Compound **1**.

Antimicrobial Assay. The antimicrobial activity of various concentrations of Compound **1**, i.e. 1000, 800, 600, 400, 200, 100 ppm were assessed against gram- positive bacteria *Streptococcus pyogenes* and *Staphylococcus aureus* and gram negative bacilli *E. coli* and *Pseudomonas aeruginosa*, using the paper disk agar diffusion method (disk diameter, 6 mm). Three trials per concentration were done. After incubation of culture for 24 h at 37°C, the zone of inhibition was measured in millimeters.

Cytotoxicity Evaluation. Cytotoxicity was estimated using the brine shrimp lethality test (BSLT). Concentrations of 1000, 800, 600, 400, 200, 100 ppm of Compound **1** were prepared using seawater as solvent. The mortality of the nauplii of the brine shrimp *Artemia salina* was counted after 6 h. Three trials per dose level were done. A parallel of tests with standard potassium dichromate solution as positive control and the blank control were conducted. The quantal data obtained were evaluated using the Muench-Reed method. The LD50 was determined by plotting the number of accumulated deaths and the number of accumulated alive on the same axes versus log concentration. The intersection between the two curves gave an estimated median tolerance or LD50.

Micronucleus Test. Five healthy male mice of approximately same age and weight were injected intraperitoneally with Compound **1**. The treatments were administered twice at 24 h intervals. Three different dose levels were utilized: 15 mg, 10 mg, and 5 mg / kg mouse. Three bone marrow slides were prepared per animal and at least 1000 polychromatic erythrocytes (PCE) were scored for the frequency of micronucleated cells.

RESULTS AND DISCUSSION

Compound **1** was obtained as a light yellow liquid. The IR spectrum showed major absorption bands implying the presence of a hydroxyl group at 3399.91 cm^{-1}, a C-H stretch at 2925.99 cm^{-1}, a C-C stretch for aromatic hydrocarbon at 1502.82, 1457.02, 1430.37 cm^{-1}, and a medium absorption

caused by C-N at 1207.53 cm^{-1}. The UV spectrum showed absorption maxima at 267 nm characteristic of an aromatic ring conjugated with other chromophores (λ_{max} 518.5).

The ^1H NMR spectrum also revealed the presence of an aromatic ring.

After incubation of culture for 24 h at 37°C, all prepared concentrations of Compound **1** showed inactivity against gram-positive bacteria *Streptococcus pyogenes* as well as with the gram negative bacilli *E. coli* and *Pseudomonas aeruginosa.* Small inhibitory zones against gram- positive coccus *Staphylococcus aureus* at higher concentrations of 800 and 1000 ppm were observed but were quite insignificant.

In the cytotoxicity test, the positive toxicant, potassium dichromate solution had an LD_{50} = 589.9 ppm. while Compound **1** had an LD_{50} of 323.59 ± 1.23 ppm.

The ability of Compound **1** to induce numerical chromosomal damage was determined using the micronucleus test Table 1. The dosages of 15 mg and 10 mg Compound **1** per kg mouse revealed positive clastogenic effects, since the number of MPCE were more than twice as those of the negative control. Based on the one-way analysis of variance (ANOVA), Compound **1** showed a significant overall increase of frequency of micronucleated polychromatic erythrocytes (MPCE) across all concentrations since the $F = 110.09 > F_{0.05} = 2.87$; hence, the null hypothesis H_0 was rejected. The pair-wise comparison using the Duncan's Multiplicity Test (Table 2) indicated that each concentrations used were statistically different from the control group in frequency of micronucleated cells since all differences of the MPCE means μ were greater than the Shortest Significant Range SSRp. The Duncan's test indicated too that $\mu_1 \neq \mu_2$, $\mu_1 \neq \mu_3$, $\mu_1 \neq \mu_4$, and $\mu_1 \neq \mu_5$, since all marked differences were at $p < 0.0500$.

Table 1. Effect of various concentrations of yellow sponge extract on chromosome damaging of mice bone marrow

Concentration	No. Micronucleated Polychromatic Erythrocytes per thousand	
	Raw Data	Transformed data ± S.D.
Solvent control	0.00	0.7071± 0.00
Mitomycin-C(4 mg/kg body wt.	7.60	2.845 ± 0.087
15 mg/kg body wt.	3.60	2.021 ± 0.067
10.0 mg/kg body wt	2.40	1.697 ± 0.142
5.0 mg/kg body wt	1.00	1.193 ± 0.281

Table 2. Duncan's test: (Compound 1)

Duncan test; Variable: VAR2 (yellow-data.sta)
Marked differences are significant at $p < .05000$

		{1}	{2}	{3}	{4}	{5}
		M=2.0211	M=1.6970	M=1.1925	M=.70711	M=2.8447
¾ LD_{50}	{1}		0.0081	7.20E-05	5.85E-05	0.000151
½ LD_{50}	{2}	0.00807		0.00031	7.24E-05	7.24E-05
¼ LD_{50}	{3}	7.25E-05	0.0003		0.00041	5.85E-05
Sol. Ctrl	{4}	5.85E-05	7.00E-05	0.00041		3.30E-05
Mito C	{5}	0.00015	7.00E-05	5.80E-05	3.30E-05	

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