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In Vitro Antioxidant and Cytotoxic Activities of Some Marine Sponges Collected off Misamis Oriental Coast, Philippines

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Abstract: The phosphomolybdenum method for total antioxidant activity determination showed that the hexane, dichloromethane and ethyl acetate extracts of five marine sponge species collected off misamis oriental coast-*Aaptos suberitoides, Dactylospongia elegans, Stylissa massa, Haliclona* sp. and an unidentified species coded as KL-05, have varying degrees of antioxidant capacity. Expressed as ascorbic acid equivalents in µg/mL of extract, the hexane extract of *Dactylospongia elegans* (DeH) and the ethyl acetate extract of *Aaptos suberitoides* (AsE) showed the highest antioxidant capacity. Although the hexane extract of KL-05 (KL-05H) has considerable antioxidant activity, the ethyl acetate extract (KL-05E) showed no antioxidant activity. The brine shrimp assay for cytotoxicity indicated high bioactivity, with *Haliclona* sp., *Dactylospongia elegans, Aaptos suberitoides* and *Stylissa massa* exhibiting high % mortality and low LC_{50} values. The antioxidant and cytotoxic activities of the marine sponges may be attributed to the zoochemicals present. All sponge species contain alkaloids, saponins, tannins, and flavonoids. Terpenoids are present only in *Haliclona* sp. and the cardiac glycosides, only in *Aaptos suberitoides* and *Haliclona* sp.

Keywords: Antioxidant, Cytotoxic, LC_{50}, Zoochemicals

Introduction

The majority of living organisms on earth thrive in the marine environments. In contrast to those in terrestrial environments, however, many organisms in diverse marine environments have not been investigated chemically[1]. Accordingly, the ocean becomes the new frontier for chemical research. However, despite the vast biomedical potential being offered by the marine resources, less than 1% of the marine species has been studied[2]. The Philippines, specifically Mindanao, with its long coastal lines, has barely drawn on its marine capital, particularly the sponges[3].

Sponges belong to the Phylum Porifera. They are the most primitive of the multicellular animals. They are sessile, sedentary marine invertebrates that usually lack morphological defense structures like spines or protective shell[1]. They produce instead, bioactive compounds that serve as their chemical defense against predators and as chemical offense to combat competitors for space and resources[1,4-6]. These defense compounds have great importance in many possible drugs[7]. Cytotoxic compounds that stop growth of sponge competitors may also inhibit tumor growth in humans and subsequently be channeled into a cure for cancer.

In this study, the *in vitro* total antioxidant activity and the cytotoxic activity of hexane, dichloromethane and ethyl acetate extracts of five species of marine sponges – *Aaptos suberitoides, Dactylospongia elegans, Stylissa massa, Haliclona* sp. and an unidentified species coded as KL-05, collected off Misamis Oriental coast, Mindanao, Philippines, were evaluated.

Experimental

Fresh, cleaned sample of each of the marine sponges - *Aaptos suberitoides*, *Dactylospongia elegans*, *Stylissa massa*, *Haliclona* sp. and KL-05 were separately cut into pieces and divided into two parts, one part was freeze-dried and subjected to zoochemical analysis and the other part was subjected to solvent extraction and partitioning.

Zoochemical analysis

In the absence of an established protocol for the screening of chemical constituents in animal tissues, the qualitative analysis for the possible bioactive components present in the sponge samples were done using established phytochemical screening protocol[8].

Solvent extraction and partitioning

Fresh, cleaned sample of each of the marine sponges were soaked exhaustively with methanol. The resulting crude methanol extracts of the samples were concentrated *in vacuo* and then sequentially partitioned with hexane, dichloromethane and ethyl acetate in a separatory funnel. The solvent partitioning process produced three crude extracts for each sponge species: the hexane extracts – *A. suberitoides* hexane extract (AsH), *D. elegans* hexane extract (DeH), *S. massa* hexane extract (SmH), *Haliclona* sp. hexane extract (HspH), & KL-05 hexane extract (KL-05H); the dichloromethane extracts - *A. suberitoides* dichloromethane extract (AsD), *D. elegans* dichloromethane extract (DeD), *S. massa* dichloromethane extract (SmD), & KL-05 dichloromethane extract (KL-05D) and the ethyl acetate extracts – *A. suberitoides* ethyl acetate extract (AsE), *D. elegans* ethyl acetate extract (DeE), *S. massa* ethyl acetate extract (SmE), & KL-05 ethyl acetate extract (KL-05E). For *Haliclona* sp. only the hexane extract, HspH, was prepared. All crude extracts were subjected to phosphomolybdenum reaction for total antioxidant activity determination and to brine shrimp lethality test for cytotoxicity.

Total antioxidant activity assay

The total antioxidant activity of the extracts was evaluated by the phosphomolybdenum method based on established procedure[9,10]. This assay is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the subsequent formation of a green phosphate/Mo(V) complex at acid pH. A 0.3 mL extract solution (25, 50, 100 and 500 µg/mL) was dispensed into screw-capped test tubes. A 3.0 mL reagent solution (6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate) was added, the tubes were capped and

incubated at 95 °C for 90 minutes. After cooling to room temperature, the absorbance was measured at 695 nm using a spectrophotometer. A blank test was done using the solvent used. The antioxidant activity was expressed as ascorbic acid equivalents which was determined from a linear equation that was established using ascorbic acid as reference standard. The results are reported as means of triplicate analysis.

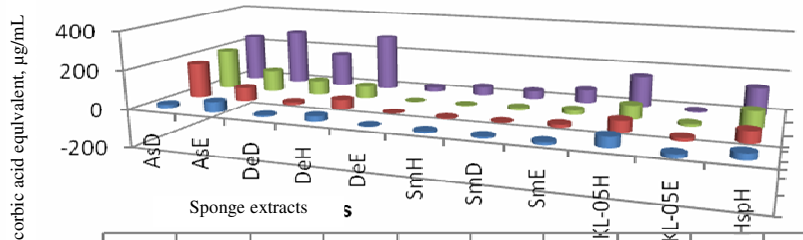
Brine shrimp lethality test

Four doses each of the crude extracts (10, 100, 500 and 1 000 ppm) and the solvent used were prepared with three replicates per dose and were tested for cytotoxicity against the brine shrimp, *Artemia salina* Leach. The number of deaths of nauplii for each dose per replicate was recorded after 6, 12 and 24 hours exposure.

Results and Discussion

Total antioxidant activity

The total antioxidant capacity of the sponges studied, expressed as ascorbic acid equivalents in µg/mL of extract, is concentration dependent as shown in Figure 1. Of the five species, *A. suberitoides* and *D. elegans* have the most antioxidant activity. Among the extracts considered, the hexane extract of *D. elegans* (DeH) and the ethyl acetate extract of *A. suberitoides* (AsE) gave the highest antioxidant capacity of 286 µg ascorbic acid equivalents at 500 µg/mL extract concentration. Although the hexane extract of KL-05 (KL-05H) has considerable antioxidant activity, the ethyl acetate extract (KL-05E) did not exhibit any antioxidant activity indicating that the antioxidative components of KL-05 are nonpolar. The activity values were determined using the following linear equation that was established with ascorbic acid as reference standard: ($A = 0.002C + 0.014$; $R^2 = 0.999$), where A is the measured absorbance and C is the ascorbic acid equivalent**.**



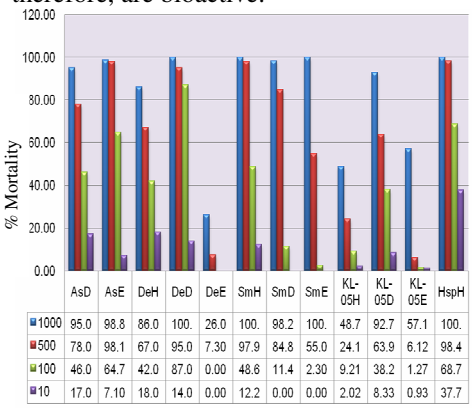
	AsD	AsE	DeD	DeH	DeE	SmH	SmD	SmE	KL-05H	KL-05E	HspH
25ppm	17.5	53	7.5	26	-1.5	-8.5	-8	-13	51	-19	31
50ppm	179.5	70	13.5	48	0.5	-4	2.5	15.5	57	-16.5	58
100ppm	204.5	109.5	73	63.5	0.5	3	11.5	21.5	68	-13	86
500ppm	248.0	285.5	172.5	286	23.5	44	42.5	68	158	-6.5	138

Figure 1. Total antioxidant activity of the extracts of *A. suberitoides, D. elegans, S. massa,* KL-05 and *Haliclona* sp

Brine shrimp lethality test

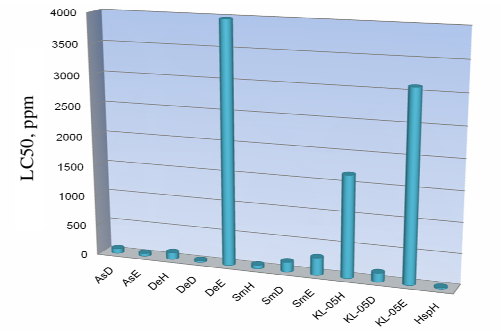
The quantal data obtained from the brine shrimp lethality assay was evaluated by the Reed-Muench method[11] to determine the percent mortality of the brine shrimps that were exposed to different concentrations of the extracts and to estimate the LC_{50} of the extracts. The % mortality results in Figure 2 show that all sponge species studied exhibit cytotoxicity against

A. salina Leach. This is predictable in as much as marine sponges have to produce cytotoxic compounds to stop growth of their competitors for space and resources in their habitat. Figure 2 also shows that the bioactivity of the sponge samples vary with the extracting solvent. The sponge extracts, except DeE, KL-05E and KL-05H, show high % mortality and therefore, are bioactive.



	AsD	AsE	DeH	DeD	DeE	SmH	SmD	SmE	KL-05H	KL-05D	KL-05E	HspH
1000	95.0	98.8	86.0	100.	26.0	100.	98.2	100.	48.7	92.7	57.1	100.
500	78.0	98.1	67.0	95.0	7.30	97.9	84.8	55.0	24.1	63.9	6.12	98.4
100	46.0	64.7	42.0	87.0	0.00	48.6	11.4	2.30	9.21	38.2	1.27	68.7
10	17.0	7.10	18.0	14.0	0.00	12.2	0.00	0.00	2.02	8.33	0.93	37.7

Figure 2. Percent mortality of brine shrimp larvae at 24-hours exposure to different concentrations (ppm) of the extracts of *A. suberitoides*, *D.elegans*, *S. massa*, KL-05 and *Haliclona* sp.



	AsD	AsE	DeH	DeD	DeE	SmH	SmD	SmE	KL-05H	KL-05D	KL-05E	HspH
Series1	79.7	51.17	114.2	30.2	3,975	61.96	166	290.5	1,679	149.5	3,100	23.27

Figure 3. Estimated chronic LC_{50} values of the crude extracts of *A. suberitoides, D. elegans, S. massa,* KL-05 and *Haliclona* sp.

In terms of chronic LC_{50} values of the extracts towards the brine shrimps, Figure 3 shows that most extracts have LC_{50} values less than 1 000 ppm. On the basis that extracts with LC_{50} values less than 1 000 are considered bioactive[12], the sponge extracts, therefore, except DeE, KL-05E and KL-05H, are bioactive, with HspH and DeD as the most bioactive extracts having chronic LC_{50} value of 20 - 30 ppm.

Zoochemical analysis

The antioxidant and cytotoxic activities exhibited by *A. suberitoides*, *D. elegans*, *S. massa*, KL-05 and *Haliclona* sp. may be attributed to the zoochemicals they contain. Adapting the procedure of Edeoga *et al.*[8] for phytochemical analysis, Table 1 shows that the five sponge species contain alkaloids, saponins, tannins and flavonoids. Terpenoids are present only in *Haliclona* sp. and the cardiac glycosides, only in *Aaptos suberitoides* and *Haliclona* sp.

Table 1. Zoochemical analysis results on the marine sponges

Zoochemical	*A. suberitoides*	*D. elegans*	*S. massa*	*Haliclona* sp.	KL-05
Alkaloid	+	+	+	+	+
Tannin	-	-	-	-	-
Saponin	+	+	+	+	+
Flavonoid	+	+	+	+	+
Terpenoid	-	-	-	+	-
Cardiac glycoside	+	-	-	+	-

+(present) - (absent)

Conclusion

Based on the antioxidant activity and cytotoxicity assays conducted, compounds with medicinal potential may be isolated from the marine sponges studied. *A. suberitoides*, *D. elegans*, S. *massa*, KL-05 and *Haliclona* sp. have medicinal potential due to the presence in sponge themselves of chemicals that may have applications in the control of viruses, bacteria, fungi and tumors. Isolation and structure elucidation of compounds with antioxidative and cytotoxic activities from *A. suberitoides*, *D. elegans*, *S. massa*, KL-05 and *Haliclona* sp. is currently in progress.

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