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Evaluation of the antioxidant and antimicrobial potentials of marine invertebrates collected off Agusan del Norte, Philippines: *Lobophytum baratum, Sarcophyton ehrenbergi, Isis* sp., and *Demospongia* sp.

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Abstract

This study was conducted to investigate the potentials of marine invertebrates as sources of bioactive compounds. Four marine invertebrates were collected off Agusan del Norte, Philippines, and were taxonomically identified as *Lobophytum baratum*, *Sarcophyton ehrenbergi*, *Isis* sp., and *Demospongia* sp. Polar and nonpolar extracts, prepared by extraction of the samples with 50:50 ethanol-water and 50:50 ethylactetate-methanol, respectively, were tested for their antioxidant and antimicrobial activities. None of the extracts showed strong antioxidant activity comparable to the standard ascorbic acid in the antioxidant screening using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging method. However, the polar extract of the soft coral *Sarcophyton ehrenbergi* showed high Ascorbic Acid Equivalents (AAE) as well as high Butylated Hydroxytoluene Equivalents (BHTE) values in the total antioxidant assay conducted using the Phosphomolybdenum method. Among the marine invertebrate extracts subjected to antimicrobial assay, the nonpolar extract of the soft coral *Lobophytum baratum* was shown to be the most active against *Bacillus subtilis* and *Escherichia coli* with zones of inhibition of 21.5 ± 2.1 mm and 12.7 ± 0.9 mm, respectively. However, the extracts failed to show any antifungal activity against the test organisms *Sacaromyce scerevisiae* and *Aspergillus niger*. The results indicated the possibility of obtaining antioxidant and antibacterial compounds from *S. enrenbergi* and *L. baratum*, respectively.

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Introduction

Marine organisms are rich in biologically active compounds, many of which have unique structures that are not found in terrestrial organisms (Jeng *et al.*, 2011). Marine invertebrates comprise approximately 60% of all marine animal diversity. They are delicate animals and are relatively vulnerable to predators (Maia *et al.*, 2014). Marine sponges (phylum *Porifera*) and soft corals (phylum *Cnidarians*) have been unusually productive sources of chemically interesting and biologically significant secondary metabolites. Soft corals rely on their chemical defence system by secondary metabolites accumulating in their bodies or releasing to their surroundings for survival. The chemical defensive functions of these secondary metabolites were found to serve as antipredatory, antimicrobial, allelopathy and antifouling agents (Shahbudin *et al.*, 2011). Soft corals are well-known for their production of sesquiterpenes and diterpenes. Soft corals benefit from the presence of these toxic terpenes. Since they are extremely rich in lipids, they are also extremely vulnerable to predation (Edrada *et al.*, 2000). Marine sponges are a rich source of bioactive compounds and they also show the highest rates of cytotoxic molecules (Ferreira *et al.*, 2007). Compounds isolated in marine sponges include highly unusual macrolides, peptides, steroids, terpenes, and alkaloids which exhibit potent biological activities and many of which are important as drug leads (Nakao and Fusetani, 2000). Overall, there are more than 20,000 marine natural products described to date, and almost half of those produced by invertebrates have been discovered since 1990, with a pronounced increase in recent decades. Because of this, phyla Porifera and Cnidaria have been the two main sources of new marine natural products. These invertebrates have potential towards the development of new products (Leal *et al.*, 2012) and this will continue to be the case in the future (Gordaliza, 2010).

This study investigates the potential of the soft corals *Lobophytm baratum*, *Sarcophyton ehrenbergi* and *Isis sp.* as well the sponge *Demospongia* sp. collected from the Philippine sea in Mindanao, as sources of antioxidant and antimicrobial compounds.

Materials and methods

Collection and preparation of marine invertebrate samples

Fresh samples of the soft corals and sponge were collected off the coasts of Carmen, Agusan del Sur, Philippines (9.086° N and 125.219° E). The samples were immediately stored in sterile containers and transferred to the laboratory. Voucher specimens of each marine invertebrate were separated for taxonomic identification. The remaining samples were then cut and freeze-dried. (Eyela FDU-2200 Freeze-dryer).

Solvent extraction of marine invertebrate samples

Each of the freeze-dried samples was soaked in adequate amount of 1:1 ethyl acetate-methanol mixture for 72 hours. The resulting mixture was then filtered, concentrated *in vacuo* using rotary evaporator and weighed to give the ethyl acetate-methanol (nonpolar) extract. The residue from the first extraction was soaked in 1:1 ethanol-water mixture for 72 hours. The resulting mixture was filtered, concentrated *in vacuo* using rotary evaporator, freeze-dried and weighed to give the ethanol-water (polar) extract.

Free radical scavenging activity

The DPPH free-radical scavenging activity of the polar and nonpolar extracts was assessed following the method of Lee and Shibamoto (2001). Four concentrations (500-, 100-, 50-, and 25 ppm) were prepared from the extracts for the assay. A 500-ppm solution was prepared first from the extracts and from this solution, the other concentrations were prepared by dilution with methanol as the solvent. 300 µL of the prepared solutions was transferred into screw-capped test tubes before adding 3000 µL of methanolic solution of 0.1 m*M* DPPH into the test tubes. The resulting mixtures were shaken thoroughly and allowed to stand at room temperature for one hour in the dark. After that, the absorbance for each solution was measured at 517 nm using a UV-Vis Spectrophotometer (Double-beam Ll-2800) against a

methanol as a blank. The percent of DPPH decoloration of the samples were calculated as % Antiradical Activity.

$$\% \text{ Antiradical Activity} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 10$$

The DPPH free-radical scavenging activity of the samples was also compared with that a known antioxidant, ascorbic acid (AA) with the determination of EC_{50} values.

Total antioxidant capacity by the phosphomomybdenum method

The total antioxidant capacity of the marine extracts was measured following the method of Prieto *et al.* (1999). For each extract, 200- , 100- and 10- ppm solutions were prepared at three replicates. Exactly 0.3 mL of each resulting solution was combined with freshly prepared 3 mL reagent solution (containing 0.6 *M* sulfuric acid, 28 m*M* sodium phosphate, and 4 m*M* ammonium molybdate).

The resulting reaction solutions were then placed in screw-capped test tubes and incubated in a boiling-water bath at 95°C for 90 minutes. After incubation, they were allowed to cool and the absorbance of each solution was measured using a UV-Vis Spectrophotometer (Double-beam Ll-2800) at a wavelength of 695 nm. The antioxidant capacity of each solution was expressed as Ascorbic Acid Equivalents (AAE) and Butylated Hydroxytoluene equivalents (BHTE) using a linear equation (concentrations at 10-, 100-, and 200 ppm versus absorbance) established with ascorbic acid and BHT as the standards and methanol as the control measured at the same wavelength.

Antimicrobial assay

The antimicrobial evaluation of the extracts followed the protocol of Driscoll*et al.* (2012) with slight modifications. Commercially available discs (6 mm in diameter) were pre-impregnated with the marine extracts and the standards. The moistened paper discs were then evenly dispensed and lightly pressed on a previously prepared agar plate swabbed with the test organism. Full contact of the disc with the agar medium was ensured.

The test organisms used were *B. subtilis* (gram-positive), *E.coli* (gram-negative), *A. niger* (fungus), *S. cerevisiae* (yeast).

The zone of inhibition around the discs was then measured after the incubation of the plates in an inverted fashion at 35°C for 24 hours for bacteria 24-48 hours for molds and yeast. Two trials with three replicates per trial were done for this assay.

Results and discussion

DPPH radical scavenging activity

This assay has been the most accepted model for evaluating the free radical scavenging activity of any new drug. A regression line equation was derived for each extract and was then used to calculate the corresponding EC_{50} value (Table 1).

Table 1. The DPPH radical-scavenging activities of the marine extracts at various concentrations.

Type of Extract	% Antiradical activity				EC_{50}, µg/ml
	25 ppm	50 ppm	100 ppm	500 ppm	
L. baratum Nonpolar	0.75	0.43	0.00	1.68	>500
L. baratum Polar	0.68	0.39	1.00	1.43	
*S. ehrenbergi*Nonpolar	0.79	1.04	1.22	4.83	>500
*S. ehrenbergi*Polar	1.64	1.61	1.86	7.62	
Isis sp. Nonpolar	0.79	1.25	1.22	2.04	>500
Isis sp. Polar	2.22	2.11	2.32	3.65	
Demospongia sp. Nonpolar	1.18	1.14	1.07	2.72	>500
*Demospongia*sp.Polar	1.50	1.79	1.61	2.72	
Ascorbic acid (+) control	25.92	50.27	90.81	97.00	21.30

Table 2. Average zones of Inhibition of the marine extracts with antibacterial activity against *B. subtilis*.

Type of extract	Average zone of inhibition, mm
L. baratum Nonpolar	21.5 ± 2.1
*S. ehrenbergi*Nonpolar	15.5 ± 1.2
Isis sp. Nonpolar	14.2 ± 1.9
Demospongia sp. Nonpolar	15.6 ± 2.4
Amoxicillin (+) control	41.4 ± 1.4

The marine extracts did not show strong antioxidant activity as indicated by the low percent radical scavenging values and high EC_{50} values. The results for *L. baratum*in this study is contrary to that obtained by Putra *et al.* (2016) in their investigation of a *Lobophytum* sp. from Indonesia. Such species exhibited a slight DPPH radical scavenging activity with an IC_{50} of 150 □g/mL. Meanwhile, although the octocoral *S. ehrenbergi* collected from other parts of the world has been well-studied, it has not been shown to possess DPPH radical-scavenging activity (Zubair *et al.*, 2016; Elkhatee *et al.*, 2014; Wang *et al.*,2013).

Table 3. Average zone of inhibition of marine invertebrate extracts with antibacterial activity against *E. coli*.

Type of extract	Average zone of inhibition, mm
L. baratum Nonpolar	12.7 ± 0.9
L. baratum Polar	9.8 ± 1.8
*S. ehrenbergi*Nonpolar	9.8 ± 1.7
*S. ehrenbergi*Polar	10.4 ± 1.4
Isis sp. Nonpolar	11.7 ± 2.2
Isis sp. Polar	9.5 ± 1.3
Demospongia sp. Nonpolar	10.0 ± 1.7
*Demospongia*sp.Polar	10.2 ± 1.3
Amoxicillin (+) control	31.4 ± 1.6

The results obtained in this study are in agreement with the results of another study wherein extracts of eleven sponges only showed weak antioxidant activities against DPPH (Bianco *et al.*, 2015). The absence of radical scavengers in these extracts might be due to some factors that hinder the bioactivity of the extracts.

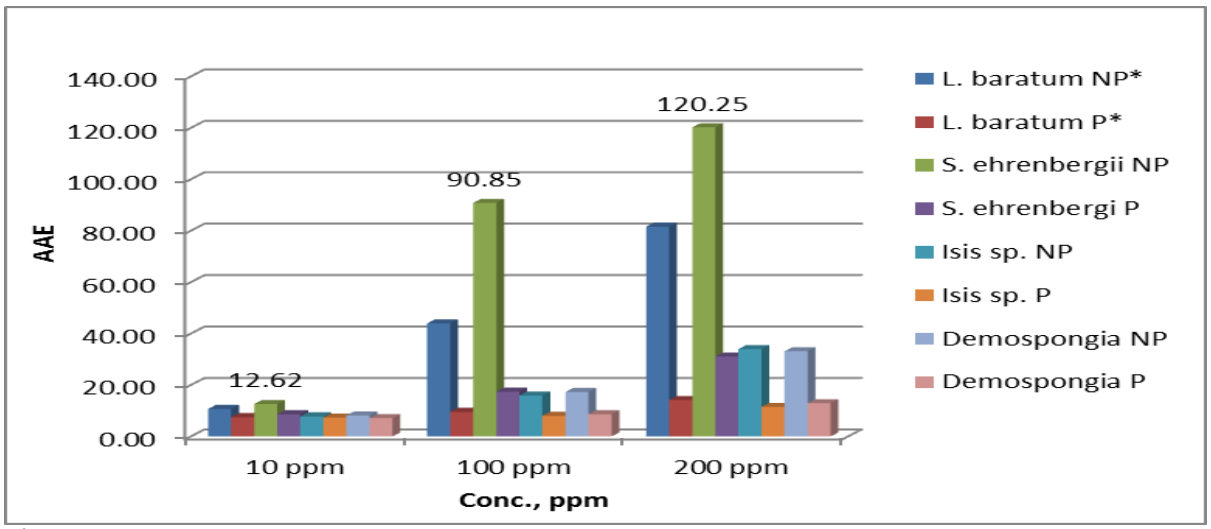

Fig.1. Total antioxidant activities of the marine invertebrate extracts expressed as ascorbic acid equivalents (AAE) at various concentrations.

This discrepancy may be due environmental factors such as seawater conditions, depth, salinity and temperature (Bianco *et al.*, 2015). One possible factor could be that the antioxidant metabolites are present in the extracts in trace amounts only.

Total antioxidant capacity

The total antioxidant activities of the marine extracts expressed as Ascorbic Acid Equivalence (AAE) and Butylated Hydroxytoluene Equivalence (BHTE) correspond to the presence of hydrophilic and lipophilic antioxidants, respectively. As shown in Fig. 1 and 2, almost all of the extracts displayed relatively low AAE and BHTE values. Only the nonpolar extract of the soft coral *S. ehrenbergi* consistently showed high AAE (120.25 at 200 ppm) as well as high BHTE (282.67 at 200 ppm) values. This indicates that this crude extract possesses bioactive metabolites as potential lipophilic antioxidants and hydrophilic antioxidants (Driscoll *et al.*, 2012).

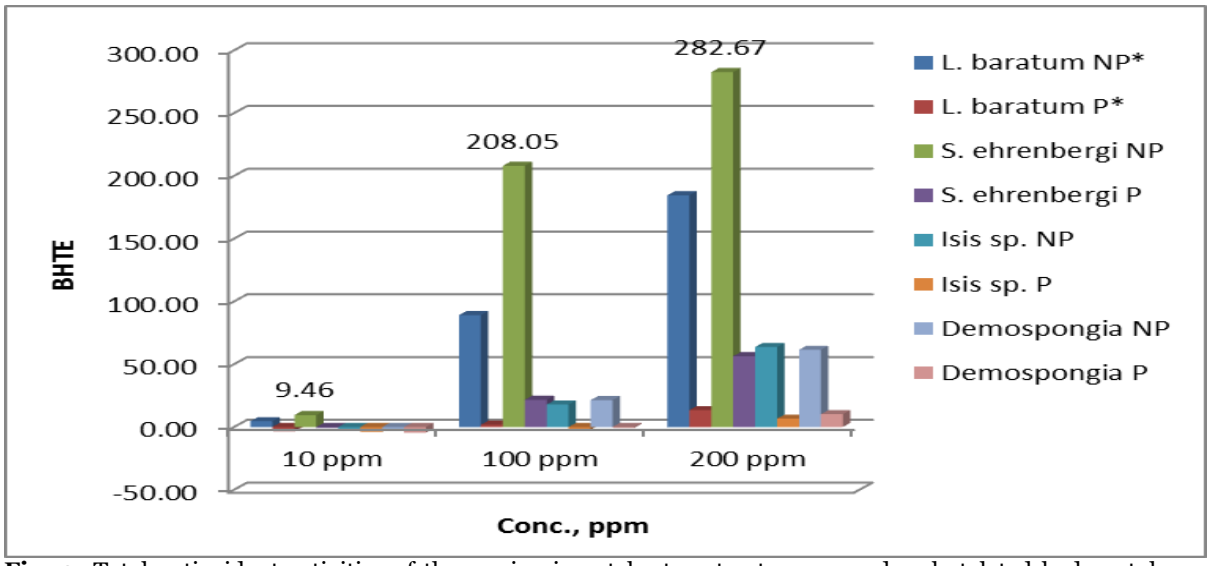

Fig. 2. Total antioxidant activities of the marine invertebrate extracts expressed as butylated hydroxytoluene equivalents (BHTE) at various concentrations.

Antimicrobial assay

Shown in Tables 2 and 3 are the mean value ± standard error of the mean (SEM) of growth inhibition zone diameters obtained from two trials and three replicates per trial. All of the nonpolar extracts exhibited appreciable activity against the test organism with that of *L. baratum* having the greatest zone of inhibition. Interestingly, all the polar and nonpolar extracts displayed moderate activity against *E. coli*. Apparently, this shows that the test organism *E. coli* was susceptible to the 1000- ppm concentration of the extracts. The nonpolar extract of *L. baratum* consistently showed to be the most active among the extracts. These results are consistent with those obtained by Zhao *et al.* (2013) wherein the *Lobophytum* sp. collected from the South China Sea only exhibited moderate antibacterial activities. However, a study of a *Lobophytum* sp. from the Read Sea in Saudi Arabia showed that one of its isolates exhibited a significant antibacterial activity (Al-Footy *et al.*, 2016). .Meanwhile, for the antifungal activity, the test organisms were resistant to all polar and nonpolar extracts at 1000-ppm concentration as no zones of inhibition were manifested. These results are in consistent with the study of Amer and Morsy (2015) wherein extracts of four marine genera from the Tabuk Region of Saudi Arabia did not show any activity against the fungi *A. niger*.

Conclusion

The nonpolar and polar extracts of the Philippine marine invertebrates *L. baratum, S. ehrenbergi, Isis* sp. and *Demospongia*. sp. Were tested with their antioxidant and antimicrobial properties. Effective concentrations from the DPPH assay indicated that all soft coral extracts were weak free radical scavenger

with EC_{50}> 500 µg mL^{-1} compared to Ascorbic acid standard. In the Phosphomolybdenum method, only the polar extract of *S. ehrenbergi* showed high AAE as well as high BHTE values, an indication that it possesses potential lipophilic and hydrophilic antioxidants. Among the marine invertebrate extracts subjected to antimicrobial assay, the nonpolar extract of the soft coral *L. baratum* was shown to be the most active against *B. subtilis* and *E. coli*, while the other extracts showed partial activities. However, the fungi *S. cerevisiae* and *A. niger* were resistant in all extracts, indicating that all extracts possess no antifungal activity. The results encourage further utilization of the assay in the eventual fractionation of active marine invertebrate extracts. Active compounds thus obtained could then be subjected to more elaborate bioassays for specific pharmacologic activities.

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