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In vitro Antioxidant and Antimicrobial Activity of Soft Corals Collected Off the Coast of Agusan del Norte, Philippines: *Sarcophyton glaucum, Lobophytum pauciflorum, Sinularia flexibilis,* and *Lobophytum crassum*

Harmie N. Luyao[1], Mylene M. Uy[1,2]*
1-Department of Chemistry, College of Science and Mathematics, Mindanao State University – Iligan Institute of Technology, Andres Bonifacio Avenue, 9200 Iligan City, Philippines
2-Premier Research Institute of Science and Mathematics, Mindanao State University – Iligan Institute of Technology, Andres Bonifacio Avenue, 9200 Iligan City, Philippines
*Corresponding author's email: mylene.uy@g.msuiit.edu.ph

ABSTRACT
*The present study aimed to screen for antioxidant and antimicrobial activities in the soft corals Sarcophyton glaucum, Lobophytum pauciflorum, Sinularia flexibilis, and Lobophytum crissum collected off the coast of Carmen, Agusan del Norte, Philippines. Specimens were collected, preserved, extracted and assayed according to standard protocols. Bioactive materials were extracted using 50:50 ethanol-water and 50:50 ethylacetate-methanol to obtain polar (**P**) and nonpolar (**NP**) extracts of the soft corals, respectively. The antioxidant activities were determined using phosphomolybdenum method for total antioxidant capacity (TAC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity while the antimicrobial activity was measured using paper disc diffusion method. All extracts demonstrated variable activities in the different assays. Among the soft coral extracts, the polar extract of S. glaucum (**SgP**) proved to me the most active with total antioxidant capacity (TAC) of 84.65 Ascorbic Acid Equivalence (AAE) and 192.26 ButylatedHydroxyToluene Equivalence (BHTE) respectively at 200-ppm concentration, 8.22% DPPH radical scavenging activity at 500-ppm concentration and most active against Bacillus subtilis with mean zone of inhibition of 17.5 ± 2.9 mm.*
Keywords: *antioxidant activity, antimicrobial activity, bioactive material, soft corals.*

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INTRODUCTION

Next to food, health has always been given utmost attention for survival of mankind. Search for medicines and other bioactive compounds from natural resources has been on top of the list of scientific researches in an effort to find cure for the growing number of infectious diseases. The growing needs for drugs to control new illnesses and to combat very resistant strains of microorganisms encouraged the exploration for new sources of marine bioactive natural products [1]. Marine organisms are an important source of new bioactive molecules; thus the scientific community worldwide has been focusing its efforts on the isolation and characterization of biologically active natural products [2]. Since the early days of marine natural products research in the 1960s, sponges and soft corals have famously yielded the largest number of new metabolites reported per year compared to any other plant or animal phylum known from the marine environment [3].

Octocorallia comprises approximately 3200 species of soft corals (Alcyonacea) found in all marine environments and 94% of new compounds from cnidarians were discovered from soft corals or Alcyonacea [4]. Soft corals belonging to the genus *Lobophytum* (Alcyoniidae) have been shown to be a rich source of macrocyclic cembranoids and their cyclized derivatives are commonly described as defensive substances against predators such as other corals and fishes. Some of these metabolites are of considerable interest and merit continuous attention due to their unique structures and significant biological activities, including anti-tumor, antiviral, and anti-inflammatory properties [5,6,7]. Soft corals

classified in the genus *Sarcophyton* are the predominant species in many coral reefs. They are endowed with diverse secondary metabolites, including cembranoids [8], biscembranoids [9] and steroids [10]. For pharmacological research, some of the metabolites are reported to possess cytotoxic [11], antimicrobial [12], and neuroprotective [13] activities. Ecologically, sarcophytoxide, usually found in *Sarcophyton* species, is an allelopathic chemical used in competition for space with scleractinian corals [14]; while another well-known metabolite, sarcophine, is known to have a toxic effect on fishes [15]. The genus *Sinularia* consists of almost 90 species of which more than 50 have been chemically evaluated, including a hybrid species. Metabolites which have been reported from the genus *Sinularia* display potential bioactivities such as antimicrobial, anti-inflammatory, and cytotoxic activities, closely related to the rich biodiversity of the marine environment. These metabolites include sesquiterpenes, diterpenes, polyhydroxylated steroids, and polyamine compounds. The peculiar structure and potential medicinal value of these metabolites have drawn increasing attention from chemists and pharmacologists [16].
This work focused on the evaluation of the antioxidant and antimicrobial activities of the polar and nonpolar extracts of the four soft corals *Sarcophyton glaucum*, *Lobophytum pauciflorum*, *Sinularia flexibili* and *Lobophytum crassum* collected from the Philippine Sea in Mindanao.

MATERIAL AND METHODS

Collection and identification of soft corals

Four species of marine soft corals (Fig. 1) were collected from Carmen, Agusan del Norte, Philippines (9.086^0N, 125.219^0E) by self-contained underwater breathing apparatus (SCUBA) diving as well as snorkeling last November 2014. T Two colonies of each species were sampled, one for the purpose of extraction and bioactivity testing and the other for identification.
The samples were collected by means of scalpel or a pair of scissors and placed in zip lock plastic bags. The collected soft coral specimens for systematic study were then stored in sterile containers and transported to the Natural Products and Bioorganic Research Laboratory of the Department of Chemistry and the Department of Science and Technology - Philippine Council for Health Research and Development (DOST-PCHRD) Tuklas Lunas Development Center at Mindanao State University- Iligan Institute of Technology (MSU-IIT), Iligan City, Philippines.
The collected soft coral species were kindly identified by E. B. Metillo of the Department of Biological Sciences, MSU-IIT, Iligan City, Philippines. For each species, a method adopted from Yehuda Benayahu and Leendert Pieter van Ofwegen [17] was applied, where small squares of approximately 1 cm were cut from the colony with a scalpel and then mounted on a glass slide with 2 drops of bleach. Once the bubbles have ceased, the sclerites were spread out by stirring, and the specimen was examined under a microscope equipped with a camera and a stage micrometer. When the sclerites were very dark and difficult to distinguish from the tissue remains, clearing was carried out using a mixture of phenol-xylene.

Preparation of the crude extracts

Samples of *S. glaucum* (**Sg**), *L. pauciflorum* (**Lp**), *S. flexibili* (**Sf**) and *L. crissum* (**Lc**) were frozen immediately after collection. The frozen samples of soft corals were left to defrost, broken into small pieces and extracted at room temperature. Polar (**P)** and nonpolar (**NP**) extracts of the soft corals were prepared by sequential extraction of the freeze-dried samples with 50:50 ethanol/water and 50:50 ethylacetate/methanol, respectively. The extracts were filtered through Whatman no. 1 filter paper and dried at 40 ^{0}C using a rotary evaporator (iKA model). The resulting extracts were tested for their antioxidant and antimicrobial properties.

Antioxidant assays

The antioxidant properties of the extracts were tested using phosphomolybdenum method and DPPH (1, 1-Diphenyl-2-picryhydrazyl) free radical scavenging assay. The two standard methods were done in triplicate analysis.

Total Antioxidant Capacity (TAC): Phosphomolybdenum Method

The total antioxidant capacity (TAC) of the marine extracts was measured following the method of Prieto *et al*. [18]. Four concentrations of the sample were prepared (25-, 50-, 100-, 500-ppm) and 0.3 ml of each concentration was added with 3 ml phosphomolybdenum reagent (a mixture of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in screw-capped test tubes. The tubes were then incubated in boiling water bath at 95 ºC for 90 min, cooled to room temperature and the absorbance of the solution was measured at 695 nm. Ascorbic acid and butylated hydroxytoluene (BHT) solutions were also prepared (25-, 50-, 100-ppm) and subjected to the same treatment. Methanol was used as the blank, and ascorbic acid and BHT served as the reference standards since the two are known antioxidants. The antioxidant capacity was then expressed as ascorbic acid and BHT equivalence from the linear equation obtained using the absorbance data of the ascorbic acid and BHT standards.

DPPH Radical Scavenging Activity

The DPPH free-radical scavenging activity of the marine extracts was evaluated following the method of Lee and Shibamoto [19]. For each of the coral extracts, the assay involved four concentrations (500-, 100-, 50-, and 25-ppm). The 500-ppm solution was prepared first and from this solution, the other concentrations were prepared using dilution method with methanol as the solvent. To the 300 µl of the prepared sample solutions in screw-capped test tubes, 3000 µl of methanolic solution of 0.1 mM DPPH was added. The mixtures were shaken thoroughly and permitted to stand at room temperature for one hour in the dark. Then the absorbance for each mixture was measured at 517 nm against methanol as a blank in the spectrophotometer. The percent of DPPH discoloration of the samples were calculated according to the formula:

$$\text{Antiradical Activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

where A_{sample} is the absorbance of the extract and $A_{control}$ is the absorbance of the methanol as the control. The examination of DPPH radical scavenging activity of the samples was compared with the known antioxidant ascorbic acid with the determination of EC_{50} values.

Antimicrobial Screening: Paper Disc Diffusion Method

The paper disc diffusion method of antimicrobial assays as described by Guevara [20] was conducted on all the extracts at 1,000-ppm to evaluate the bioactivity against four test organisms*: Bacillus subtilis*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae*. The solvent used in the preparation of extracts was used as the negative control, while amoxicillin and nystatin were used as the positive control for the antibacterial and antifungal activity, respectively.

Antimicrobial susceptibility testing was done using the paper disc diffusion method to detect the presence of antibacterial and antifungal activities of the sample extracts. A sterile swab was used to evenly distribute bacterial or fungal culture over the petri dish containing agar. The plates were allowed to dry for 15 minutes before use in the test. Sterile Whatman filter paper disc, 6 mm in diameter, was immersed in the soft coral extract for assay. The moistened filter disc was gently laid on the previously prepared petri dish. Full contact of the disc with agar medium was observed. The same extract was used on each plate; with a total of two plates used for each extract including two discs for the positive and negative controls. The negative and positive controls were the same as that used in the tube dilution assay. The plates were incubated in inverted manner at 35 ºC for bacteria for 24 hours and 27 ºC for molds and yeasts for 2 – 3 days after which they were examined for inhibition zones. A caliper was used to measure the inhibition zones. Two replicates were done for each concentration of the different extracts.

RESULTS

Total Antioxidant Capacity

The total antioxidant capacities (TAC) of the marine extracts expressed as Ascorbic Acid Equivalence (AAE) and Butylated Hydroxytoluene Equivalence (BHTE) corresponding to the presence of hydrophilic and lipophilic antioxidants respectively are illustrated in Figures 1 and 2.

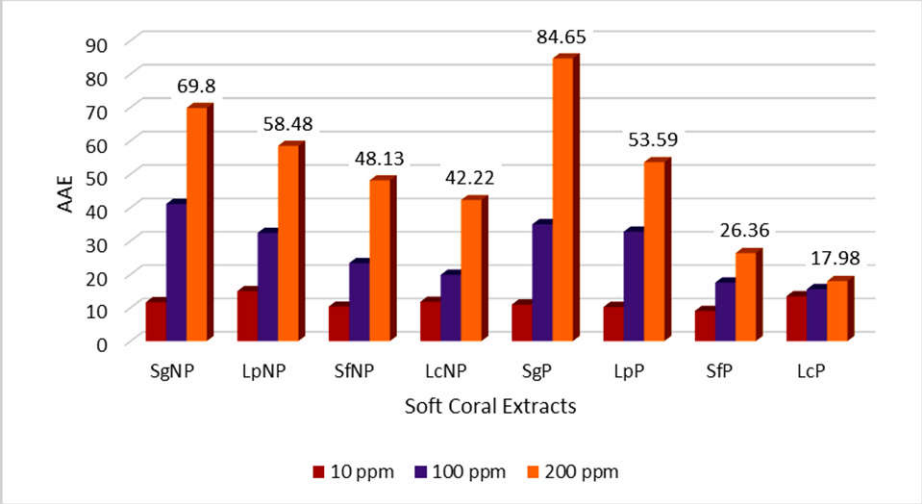

Figure 1. The total antioxidant capacities (TAC) of the various soft coral extracts expressed as Ascorbic Acid Equivalence (AAE).

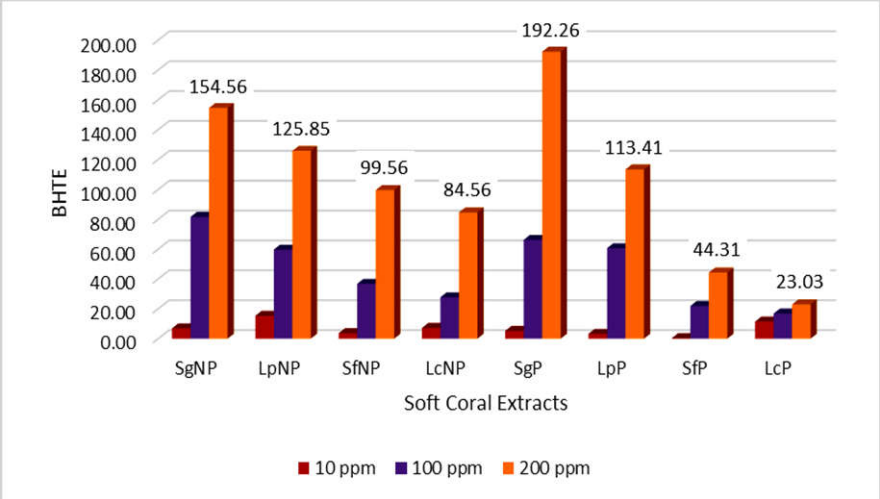

Figure 2. The total antioxidant capacities (TACG) of the various soft coral extracts expressed as butylated hydroxytoluene equivalence (BHTE).

DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of the different soft coral extracts are shown in Table 1.

Table 1: DPPH Radical Scavenging Activity of the Soft Coral Extracts

Soft Coral	Extract***	Antiradical Activity*, %				EC_{50}, ppm
		25-ppm	50-ppm	100-ppm	500-ppm	
Sarcophyton glaucum (**Sg**)	**SgNP**	0.29	0.51	0.54	2.50	>500.00
	SgP	0.00	0.95	1.23	1.58	>500.00
Lobophytum pauciflorum (**Lp**)	**LpNP**	0.38	0.98	1.14	1.49	>500.00
	LpP	0.79	1.27	1.93	2.88	>500.00
Sinularia flexibilis (**Sf**)	**SfNP**	1.14	1.83	2.88	8.22	>500.00
	SfP	0.60	1.08	1.74	2.31	>500.00
Lobophytum crissum (**Lc**)	**LcNP**	1.64	1.83	2.02	2.56	>500.00
	LcP	0.79	1.14	1.20	1.83	>500.00
Ascorbic Acid**		10.72	40.04	92.88	96.96	90.80

*mean of three replicates
**standard
***__NP__-Nonpolar, __P__-Polar

Antimicrobial Assay

For the determination of antimicrobial activity of the soft coral extracts, the diameter of the zone of inhibition was measured in millimeter scale and the results presented as mean zone of inhibition ± SD are shown in Table 2.

Table 2: Zones of Inhibition of the Soft Coral Extracts Against *B. subtilis* and *E.coli*

Test Sample*	Mean Zone of Inhibition**, mm	
	B. subtilis	*E.coli*
SgNP	11.0 ± 1.8	11.0 ± 2.4
SgP	17.5 ± 2.9	10.2 ± 0.4
LpNP	12.0 ± 1.9	11.0 ± 2.5
LpP	10.0 ± 2.4	10.2 ± 1.2
SfNP	12.5 ± 2.7	12.0 ± 3.5
SfP	11.6 ± 1.7	10.0 ± 0.0
LcNP	–	10.0 ± 0.7
LcP	± 2.1	–
Amoxycillin***	45.0 ± 4.7	33.4 ± 3.9

*__Sg__-*S. glaucum*, __Lp__-*L. pauciflorum*, __Sf__-*S. flexibilis*, __Lc__-*L. crissum*, __NP__-Nonpolar, __P__-Polar
**–no observable zone of inhibition; ***Positive control

DISCUSSION

Total Antioxidant Capacity and DPPH Radical Scavenging Activity

The results shown in Figures 2 and 3, convey that the **SgP** extract has the highest total antioxidant capacity with AAE and BHTE values of 84.65 and 192.26 respectively at 200-ppm concentration. The relative increasing trend of total antioxidant capacities among the extracts expressed as AAE accords with the relative increasing trend expressed as BHTE. For the overall results for this assay, the trend follows this order: **SgP** > **SgNP** > **LpNP** > **LpP** > **SfNP** > **LcNP** > **SfP** > **LcP**. An increasing trend of DPPH radical scavenging activity with increasing extract concentration was exhibited by the ten soft coral extracts (Table 1). Among the soft coral extracts at 500-ppm concentration, the highest radical scavenging activity was manifested by **SfNP** extract with 8.22%. However, the scavenging activities of the extracts were not significant compared to those of the standard, ascorbic acid (AA). The results suggest that the soft coral extracts do not possess components which can act as scavengers of free radicals as indicated by the low percent radical scavenging values and high EC_{50} values.

Hydrogen and electron transfer from antioxidant to DPPH and Mo (VI) complex occur in the DPPH and phosphomolybdenum assay methods. The transfers occur at different redox potentials in the two assays and also depend on the structure of the antioxidant. Some literature shows that several flavonoids and polyphenols have been isolated with potent DPPH scavenging activities [21], whereas the phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, a-tocopherol, and carotenoids [18]. Ascorbic acid, glutathione, cysteine, tocopherols, polyphenols, and aromatic amines have the ability to donate hydrogen and electrons and can thus be detected by the two assay models [22]. The marine extracts studied possess amount of antioxidant properties but negligible in free radical scavengers. The activity of each species differs depending on the particular assay methodology, reflecting the complexity of the mechanisms involved. The remarkable example of such diversity is the polar extract of *Sarcophyton glaucum* which in the direct free radical scavenging assay with DPPH had one of the least efficient scavenging ability and highest EC_{50}, whereas in terms of reducing power tested by phosphomolybdenum complex formation was among the most efficient extracts. These results suggest a great complexity of the involved mechanisms that can vary even among the related species. The apparent discrepancy between the antioxidant properties assayed by various techniques have been already mentioned by Mantle *et al*. [23], but the problem still remains unresolved and will require further data accumulation and corresponding analyses of detailed chemical profile of the studied sample.

As for *S. glaucum*, no references could be found despite the thorough literature survey on its antioxidant properties, except toxicity [24] and antibacterial activity [25] on its polar crude extracts. The existing reports on antioxidant properties of *S. flexibilis*, have dealt only with other species such as *S. maxima* [26]. In the previously published papers, there is only little information about antioxidant activity of *L. crassum* [26], however, the method used in the study was different, and so the results are not directly comparable. As for the remaining species, *L. pauciflorum*, Hassan *et al*. [27] found two metabolites that showed significant anti-inflammatory activity which could be linked to antioxidant properties.

Antimicrobial Activity

Zones of inhibition less than 10 mm maybe expressed as inactive against test microorganisms, based on the general standards by Guevarra [20]. Therefore, most of the soft coral extracts exhibited activity against the bacteria tested. The results of the study (Table 3) indicated that *B. subtilis* was most sensitive to the polar extract of *S. glaucum***, SgP**. Meanwhile, the soft coral *S. flexibilis* and *L. pauciflorum* displayed minimal constraining effect towards the growth of Gram-positive and Gram-negative bacteria but are still considered as active. On the other hand, the nonpolar extract of *L. crassum*, **LcNP,** displayed moderate antibacterial activity to *E. coli* and no growth-inhibiting effect to *B. subtilis* while its polar extract, **LcP,** showed moderate antibacterial activity to *B. subtilis* and conversely no growth-inhibiting effect to *E. coli*. In summary, of the eight (8) soft coral extracts, seven (7) exhibited antibacterial activities against *B.subtilis* and *E. coli*. However, all the soft coral extracts exhibited no growth-inhibiting effect on *A. niger* and *S. cerevisiae*. Thus, all the soft coral extracts have no antifungal activity against the tested microorganisms.

The results of the antimicrobial screening indicated *S. glaucum* as the most notable among the marine samples. The finding supports the result of Ibrahim *et al*. [25] wherein *S. glaucum* collected from Egypt exhibited potential antibacterial activity with 18.4 AU (absolute activity unit) against *E.coli.* The results of this study also agrees with that of ElAhwany *et al*. [12] in which *S. glaucum* from the Red sea was found to possess a broad spectrum of antimicrobial activity and could be of great potential in producing novel antimicrobial agents. Various studies about *S. flexibilis* have shown to contain diterpenes that protect it from competitors and predators. Coll *et al*. [28] isolated two diterpenes namely sinulariolide and flexibilide from *S. flexibilis* collected from Orpheus Island. The isolates showed marked of antimicrobial

activity and inhibited the growth of Gram-positive bacteria. This claim is supported by the result obtained in this study. Moderate antimicrobial activity was also found in *L. pauciflorum*, yet Ibrahim *et al*. [25] showed that the polar extract of *L. pauciflorum* was highly potent with 25.0 AU towards the test bacteria *E.coli*. Meanwhile, the suppressing power of *L. crassum* towards the bacterial organism, somehow, were not consistent and if found active, the activity was only mild. The result is opposite to the findings reported by Matthee *et al*. [29] where the polar and nonpolar extracts of *L. crassum* were found to be highly active towards *E.coli* and other fungus. The irregularities of the findings might due to the reasons mentioned by Gallimore [30], that specimens collected in different geographical locations may have different secondary metabolite profiles due to factors such as salinity, temperature, light intensity, pollution levels, as well as predation pressures. Moreover, the nature of bacterial symbionts may play a part in the occurrence and concentrations of specific secondary metabolites.

CONCLUSION

The results of the antioxidant activity assays suggest that the Philippine soft corals *S. glaucum, L. pauciflorum, S. flexibilis,* and *L. crassum* do not have components which can act as scavengers of free-radicals. Nevertheless, the soft corals still possess total antioxidant capacities with the highest values shown by the polar extract of *S. glaucum* indicated by its ability to reduce the Mo (VI) to Mo (V) which could be link to presence of lipophilic and hydrophilic antioxidants. Meanwhile, most of the soft coralextracts exhibited moderate antibacterial activities against *B. subtilis* and *E. coli* except for the polar extract of *S. glaucum*, **SgP**, which displayed a notable activity against *B. subtilis* (ZOI 17.5 ± 2.9 mm), the gram-positive bacteria. No antifungal activity, however, was displayed by any of the soft coral extracts. Among the four investigated Philippine soft corals, *S. glaucum* is quite interesting and promising in terms of finding potential antioxidant and antibacterial secondary metabolites.

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