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FLAVONOIDS, PHENOLICS, AND ANTIOXIDANT ACTIVITIES OF ETHYL ACETATE EXTRACTS OF THE SEAGRASSES *ENHALUS ACOROIDES* AND *THALASSIA HEMPRICHII* OF GO-SOON CARMEN, AGUSAN DEL NORTE, PHILIPPINES

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ABSTRACT

The total flavonoids and total phenolics contents are important parameters of the antioxidant that inhibit oxidation or reactions promoted by oxygen, peroxides, or free radicals. The total flavonoids and total phenolics contents and antioxidant activities of the seagrasses *Enhalus acoroides* and *Thalassia hemprichii* collected from Go-soon, Carmen, Agusan Del Norte, Philipines were determined using Aluminum Chloride complex forming assay for the total flavonoid content, Folin-Ciocalteu reagents with analytic grade gallic acid as the standard for the total phenolic contents and the antioxidant activities were determined using DPPH and ABTS. The results showed that ethyl acetate extract of *Thalassia hemprichii* had the highest phenolics and flavonoids contents which values were 0.078 mgGA/g and 0.454 mg Q/g, followed by *Enhalus acoroides* which values were 0.037 mgGA/g and 0.096 mg Q/g. The strongest free radical scavenging activity (DPPH) of the extracts were recorded by *Thalassia hemprichii* followed by *Enhalus acoroides* which values were 0.006 and 0.005 mg trolox/g respectively. The results of the radical cation decolorization power (ABTS) showed *Thalassia hemprichii* 0.252 mg trolox/g and *Enhalus acoroides* 0.005 mg trolox/g. The antioxidant activity determined by DPPH and ABTS demonstrated a strong linear relationship with the phenolics and flavonoids. The results suggested that the seagrasses have strong antioxidant potential and could be a source of natural antioxidant compounds.

KEYWORDS: Seagrass, Flavonoids, Phenolics, Antioxidants, Philippines.

INTRODUCTION

The Philippines is located in a spot in the globe the "Coral Triangle," an area of tremendous importance because it is recognized as the global center for marine biodiversity. It includes portions of the waters of the Philippines, Malaysia, Indonesia, Timor Leste, Papua New Guinea, and the Solomon Islands, which looking at it on a map, very roughly resembles a triangular region. The part is called the Coral Triangle due to the large number of corals in the area. If this region is the center of global marine biodiversity, the middle of this center is no other than the Philippines, and the Philippines' marine resources are made up not just of coral reefs, but "seagrass beds, mangrove and beach forests, fisheries, invertebrates, seaweeds, marine mammals and many others. The Philippines has the second-highest seagrass diversity in the world, second only to Australia (Dorente, 2016).

Coastal communities, knowingly or otherwise, rely on seagrasses for their livelihood, recreation, medicines, and food sources, among other services. However, despite its importance, seagrasses are not receiving similar attention with its adjacent ecosystems, the mangroves, and coral reefs. Because of their role in climate change mitigation, seagrasses and mangroves and salt marshes are gaining attention recently (Quevedo, *et al*. 2020).

Natural products are the primary resource for drug development. A large number of plants, microbes, and marine animals have been examined for bioactive secondary metabolites. Natural products have been an essential resource for the maintenance of life for ages. Several life-saving drugs have been developed from plants. The fact that approximately 25% of prescribed pharmaceuticals are derived from plants which demonstrates the enduring importance of natural products. Medicines derived from unmodified natural products or medicines semi-synthetically obtained from natural sources corresponded to 78% of the new drugs approved by the FDA between 1983 and 1994. With marine species comprising approximately half of the total global biodiversity, large-scale screening continues to play an essential role in the development of new drugs (*Xu et al*., 2004).

Seagrasses are rich in phenolic and flavonoid compounds (Mc Millan, 1979). The total phenolic content and flavonoid content of the seagrass is an important

parameter for their antioxidant properties. The natural antioxidant activities of the seagrasses are influenced mostly by their total phenolic and total flavonoid content. The purpose of this study was to assess the flavonoids, phenolics and antioxidant potential of the seagrasses *Enhalus acoroides* and *Thalassia hemprichii* of Go-soon, Carmen, Agusan Del Norte, Philippines.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents used in the study were ethyl acetate, $NaNO_2$, $AlCl_3$, NaOH, FC reagent, Na_2CO_3, ABTS reagent and DPPH reagent. All the chemicals used in the study were analytical grade and the chemicals were obtained from Elmar Marketing, Iligan City and Merteflor, Cagayan de Oro City, Philippines.

Sample collection

The species of seagrass used in the study was based on the survey of the availability of the various locations where seagrass grows abundantly. The seagrass samples were collected in the coastal waters adjacent to the marine sanctuary of Go-soon, Carmen, Agusan Del Norte, Philippines. The marine sanctuary boasts well-protected coral reefs, and the seafloor is a sight of corals and seaweeds. It is also the home of several exotic tropical fishes. The Municipality of Carmen is in the province of Agusan Del Norte in the Caraga (Region XIII) of the Philippines.

Ethyl acetate extraction

Ethyl acetate Extraction. About 25.00 g of the sample was soaked in 95% methanol or ethyl acetate. A minimum volume of 200 ml was used to soak the sample. The soaking time took about 48 hours. After 48 hours, the sample was filtered using Whatman filter paper. Then another 100 ml of methanol or ethyl acetate was used for the second soaking. Then after an hour, it was filtered again and the filtrate was then placed in the refrigerator for proper storage (Parkash *et al*., 2015).

Total phenolic content

The total phenolic content of all the formulations of seagrasses was determined by using the Folin- Ciocalteu method. 0.5 ml of the seagrass extract was placed in a 25 ml vial, and 4.5 ml of distilled water was added. A 0.5 ml of FC reagent was mixed with the solution, and 10 ml of 7% Na_2CO_3 was added. The FC reagent was prepared by dissolving about 0.0166 g of Gallic Acid monohydrate with absolute methanol, and diluted to 50 ml. 2.5 ml distilled water was added then to make a 12.5 ml solution. The solution was incubated for 90 minutes and then the absorbance was read at 750 nm. The total phenolic content of the seagrass is calculated as Gallic acid equivalents (mgGAE/g). All the experiments were performed in triplicate (Raventos, 2017).

Total flavonoid content

Aluminum chloride complex-forming assay was used to determine the total flavonoid content of the extracts. Quercetin was used as standard, and flavonoid content was determined as quercetin equivalent. 1 ml of the 1,000 ppm of plant extract was placed in a clean vial. 5ml of absolute methanol was added and followed by 300 µL or 0.3 mL of 5% $NaNO_2$. The mixture was allowed to stand for 5 minutes at room temperature. 600 µL or 0.600 mL 10% $AlCl_3$ was added and allowed it to stand again for 6 minutes at room temperature. 2 ml of 1 mM NaOH, and 1.10 ml of absolute methanol were added and the mixture was incubated for 20 minutes at room temperature. The absorbance then was read at 510 nm. Total flavonoid content was calculated as quercetin equivalents (mg QE/g). All the procedures were performed in triplicate (Pekal *et. al*., 2014).

Scanvenging activity (DPPH) assay

The scavenging activity of the seagrass extract was evaluated using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. Intense violet color in methanol is the result of the oxidation of the DPPH due in the antioxidant compound that gives off the electron to the DPPH solution resulting in the reduction and changing its color from intense violet to yellow. 0.2 ml of the 1,000 ppm of plant extract was placed in a clean vial and 5.8 ml of 0.01 mM DPPH reagent was added. The DPPH reagent was prepared by dissolving about 0.0250 g of Trolox with absolute ethanol and diluted to 100 ml in a volumetric flask. The mixture was then incubated for 30 minutes in the dark at room temperature, and the absorbance was read at 517 nm using a UV-VIS spectrophotometer.

ABTS radical cation decolorization

The ABTS radical cation decolorization power was determined according to the method described by Irondi et al. (2012) with slight modification. 0.2 ml of the 1,000 ppm of plant extract was placed in a clean vial, and 5.8 ml of the ABTS reagent was added. The ABTS reagent was prepared by dissolving about 0.0250 g of Trolox with absolute ethanol and diluted to 100ml in a volumetric flask. The mixture was then incubated for 6 minutes at room temperature, and the absorbance was then read at 734 nm using a UV-VIS spectrophotometer.

Statistical analysis

Three replicates of each sample were used for statistical analysis and the values were reported mean $\pm$ SD. Pearson's correlation analysis was carried out using Minitab, version 17 software to study the relationship between the antioxidant activities and the total flavonoid and phenolic content.

RESULTS AND DISCUSSION

Table 1. Showed the results of total phenolics and flavonoids content of the seagrasses *Enhalus acoroides* and *Thalassia hemprichii*. The highest total phenolic content was recorded by the seagrass *Thalassia hemprichii* 0.078 $\pm$ 0.10, followed by *Enhalus acoroides* 0.037$\pm$0.007 mgGA/g. The results showed that the seagrasses in this study are rich in phenolics and were in

conformity with other studies. Choi *et al*., (2009) reported that the total phenolic content of the ethyl acetate fraction of the methanol crude extracts of the seagrass *Zostera marina* was 968.50 mg Gallic acid equivalence / g of extract. Ragupathi et al. (2010d) studied that the ethanolic leaf extract of *Enhalus acoroides* collected from the Chinnapallam in Gulf of Mannar, which showed high levels of phenolic of 0.323±0.028 mg TAE/g and proanthocyanidins of 0.570±0.0003 mg TAE/g when compared to the root and rhizome. The total phenolic content in the test seagrass extracts which were collected from the intertidal region of the Mandapam coast in the Gulf of Mannar was found to be higher in *H. pinifolia* (1.0807±0.0390) followed by *T. hemprichii* of 0.4187±0.007 (Ragupathi *et al*., 2010a).

The highest total flavonoid content was recorded by the seagrass *Thalassia hemprichii* 0.454± 0.036, and followed by *Enhalus acoroides* 0.096± 0.022 mg Q/g. In the study of Libin *et al*., (2017), they reported that *Thalassia hemprichii* recorded 85±0.22 mg.100g-1 total flavonoids content, while *Enhalus acoroides* leaves, rhizomes and roots total flavonoids content values were 34±0.21, 9.3±0.06, and 5.2±0.07 mg.100g-1, respectively. In comparison, higher contents of flavonoids were detected in seagrasses from Palk Bay than those of the Gulf of Mannar, irrespectively of body parts of the seagrass *Thalassia hemprichii* and *Enhalus acoroides*, while the study of Amudha *et al*., (2017) on the total flavonoids content of seagrass *Enhalus acoroides* which was determined using the Aluminum Chloride method with quercetin as standard. Further, the Aqueous extract showed significantly higher content of flavonoids was 57.52 mgQU/g. Earlier reports revealed that plant phenolic compounds, including flavonoids, are potent antioxidants with reported antimutagenic and anticarcinogenic effects. Flavonoids have been used against cancer-causing tumors, and it inhibits the promotion of growth and progression of tumors. The results indicated variations in the flavonoid compounds with reference to the ecosystem and the presence of other plants in the meadows.

Table 1: Total phenolic, total flavonoid, and antioxidant of the seagrasses.

Seagrass	TPC (mgGA/g)	TFC mg Q/g)	DPPH (mg Trolox/g)	ABTS (mg Trolox/g)
E. *acoroides*	0.037±0.007	0.096± 0.022	0.005 ± 0.001	0.005 ± 0.001
T. *hemprichii*	0.078±0.10	0.454± 0.036	0.006 ± 0.000	0.252 ± 0.000

Values are means ± SD for 3 determinations.

The results of the free radical scavenging activities (DPPH). The strongest activity (DPPH) of the seagrass using ethyl acetate as a solvent was *Thalassia hemprichii* with values 0.006 mg trolox/g, and followed by *Enhalus acoroides* 0.005 mg trolox/g. The results of this study suggested that the seagrasses *Thalassia hemprichii* and *Enhalus acoroides* have strong antioxidant potential and could be a source of natural antioxidant compounds. Kannan et al., (2010) study on the Aqueous Methanol extracts of four seagrass species such as *Enhalus acoroides, Thalassia hemprichii, Halodule pinifolia* and *Syringodium isoetifolium* collected from Gulf of Mannar Biosphere Reserve, South India were by DPPH (2, 2-Diphenyl-1-picrylhydrazyl) method and the total phenolic content (Folin-Ciocalteu method). Twenty-five to seventy-five percent of the major seagrasses determined were rich sources of potential antioxidants. The stability of antioxidant activity was shown to be stronger in the biochemical constituents of *Enhalus acoroides, Thalassia hemprichii, Halodule pinifolia.* The total phenolic concentrations of the methanolic extracts ranged from 0.2878 to 1.0807 mg tannic acid equivalent/g. It shows that the seagrasses have strong potential antioxidants. Kannan et al., (2013) analyses on the methanol extracts on the six leaves of the seagrasses *Enhalus acoroides, Thalassia hemprichii, Halodule pinifolia, syringodium isoetifolium, Cymodocea serrulata,* and *Cymodocea rotundata* revealed a high antioxidant activity with 15.75, 8.37, and 6.65 mg ascorbic acid equivalent/g respectively in *Halodule* pinifolia, *Enhalus acoroides* and *Cymodocea. Rotundata, while Cymodocea rotundata* recorded 70.30 percent antioxidant activity and was found to be the most potent DPPH radical scavenger.

The results of antioxidant activity of *Thalassia hemprichii* and *Enhalus acoroides* extracts using the ABTS method showed *Thalassia hemprichii* recorded the highest ABTS radical scavenging activity with value 0.252 ± 0.000, and followed by *Enhalus acoroides* with 0.005 ± 0.001 mg trolox/g using ethyl acetate as solvent. The results suggested that the seagrasses have strong antioxidant potential and could be sources of natural antioxidant compounds. However, the results of this study showed lower ABTS radical cation decolorization power activity compared to the studies of Jeyapragash *et al*. (2016). In their study, the maximum scavenging activity was found in the leaf extracts of seagrass *T. hemprichii* (58.10 ± 0.42), followed by *E. acoroides* (51.01 ± 0.84). *H. ovata* showed the lowest scavenging activity for both leaf (9.12 ± 0.52) and rhizome (4.39 ± 0.65) extracts, respectively. ABTS radical scavenging activity registered a strong linear relationship with the total phenolic content (R2 =0.767) respectively and Wisespongpand *et a*l., (2019), revealed that the six extracts from seagrasses showed significantly different antioxidant activities with four assays ($p<0.05$). These extracts had DPPH and ABTS radical scavenging activities in the range of 22.82±3.35-92.93±0.40 and 30.91±1.01-94.80±0.42% while the reducing power and total antioxidant were in the range of 152.73±16.30-568.97±12.12 and 52.74±2.23-215.41±13.46 mgAAE/g extract, respectively. The extract from *Cymodocea rotundata* had the highest DPPH and ABTS radical scavenging activities with IC_{50} of 27.52 and 62.97

mg/ml. The extract from *Halophila ovalis* had the highest reducing power with a value of 568.97±12.12 mgAAE/g extract, while the extract from *Thalassia hemprichii* had the highest total antioxidant with a value of 215.41±13.46 mgAAE/g extract. The amount of phenol was highest in the extract from *Enhalus acoroides* with a value of 110.60±1.72 mgGAE/g extract.

Table 2: Correlation between total phenolic content, total flavonoid content and antioxidant assay.

Antioxidant assay	**DPPH assay**		**ABTS assay**	
	R^2	P-value	R^2	P-value
TPC	0.99	0.000	0.99	0.000
TFC	0.76	0.048	0.62	0.051

Table 2. Showed the correlation between the total phenolic content and DPPH with R^2 value of 0.99 and P-value of 0.000, while the correlation between the total flavonoid content and DPPH with R^2 value of 0.76 and P-values of 0.048. The correlation between the total phenolic content and ABTS with R^2 value of 0.99 and P-value of 0.000, while the correlation between the total flavonoid content and ABTS with R^2 value of 0.62 and p-value of 0.051. The antioxidant activity determined by DPPH and ABTS all demonstrated significant linear correlations with their flavonoids and phenolics. Yuvaraj *et al.* (2011) reported that in the methanolic extracts of *Enhalus acoroides, Thalassia hemprichii,* and *H. ovalis*, the reducing power and total antioxidant activity level is directly proportional to the concentration of the extract. The greater the concentration of the extract, the greater the reducing power and total antioxidant activity. The antioxidant activity of the seagrass depends on the ability of the solvent to extract the phenolic content of the seagrass.

CONCLUSION

The results of the study showed the antioxidant activities of the seagrasses in this study show potential rich sources of natural antioxidants and influenced mostly by their total phenolic and total flavonoid content. The results suggest that the strong antioxidant properties of the seagrasses could play a significant role in food and pharmaceutical industries. It is recommended that further study should be conducted, particularly in the aspect of determination of total phenolics, total flavonoids, and antioxidant activities of the crude extracts from these seagrass species.

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