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Antioxidant activity of methanol extracts of *Enhalus acoroides* and *Thalassia hemprichii* from the coastal water of Carmen, Agusan Del Norte, Philippines

Efren Tangon[1*], Oliva P. Canencia[1], Romeo M. Del Rosario[1], Elvinia R. Alivio[2]

[1]*Department of Science Education, University of Science and Technology of Southern Philippines, Cagayan de Oro City, Philippines*

[2]*College of Education, Mindanao State University, TawiTawi, Philippines*

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Abstract

Antioxidants play an important role in apoptosis, gene expression, and ion transportation and may reduce the risk of many diseases by protecting the cells against the effect of free radicals. The antioxidant potential of methanolic extracts of the seagrasses *Thalassia hemprichii* and *Enhalus acoroides* collected from the coastal water of Carmen, Agusan Del Norte, Philippines was determined using Aluminum chloride complex forming assay for total flavonoid content, Folin–Ciocalteu reagents with analytical grade gallic acid as the standard for the total phenolic content, DPPH, ABTS and FRAP. The results showed that methanol extract of *Thalassia hemprichii* had the highest content of total phenolics and flavonoids which values were 2.651 and 2.734 mgGA/g respectively. The strongest free radical scavenging activity (DPPH) of the extracts was recorded by seagrass *Enhalus acoroides* which value was 0.301 mgtrolox/g. While the methanol extracts of *Thalassia hemprichii* recorded the maximum radical cation decolorization power (ABTS) and Ferric ion reducing antioxidant power (FRAP) which values were 0.252 and 1.119 mgtrolox/g respectively. The antioxidant activity determined by DPPH, ABTS and FRAP demonstrated a strong linear relationship with the phenolics and flavonoids. The results suggested that the sea grasses *Thalassia hemprichii* and *Enhalus acoroides* have strong antioxidant potential and could be a source of natural antioxidant compounds.

* **Corresponding Author:** Efren Tangon ✉ efrenyutangon@gmail.com

Introduction

Seagrasses are submerged flowering plants found in shallow marine waters, such as bays and lagoons and along the Gulf of Philippines. Nineteen seagrass species were found from more than 529 sites in the Philippines. In relation to seagrass as a resource in need of protection, its status as such is yet largely unknown, becoming a focus of scientific inquiry only in the last 30 years and as an object of conservation, only in the last 15 years (Fortes, 2012). The vast biodiversity and sensitivity to changes in water quality inherent in seagrass communities makes seagrasses an important species to help determine the overall health of coastal ecosystems. Seagrasses grown in the tropical climate like Philippines are expected to bask in strong ultraviolet radiation.

This circumstance can causeincrease levels of reactive radical species. To reduce or protect, they may change their metabolism and stimulate them to produce some active compounds, therefore tropical seagrasses are estimated to possess a large number of active compounds. The secondary compounds like polyphenols and flavonoids are the key factors that are involved in the adaptation to changing biotic and abiotic environments and also mainly for the defense mechanism.

Natural antioxidants and their association with health benefits have gained unprecedented attention in recent years. They have multiple functions in biological systems and mainly defense against oxidation that produce free radicals in food, chemicals and in living systems. During normal cellular activities, various processes produce reactive oxygen species (ROS) inside the cell, which can damage the cellular components such as lipids, proteins, and DNA, when produced at high rates.

The major action of antioxidants in cells is to prevent the damage caused by the action of reactive oxygen species (Kanna *et al*., 2010). The aim of this study was to assess the antioxidant potential of the seagrasses *Enhalus acoroides* and *Thalassia hemprichii* of Butuan Bay, Agusan Del Norte, Philippines.

Materials and methods

Chemicals and reagents

Chemicals and reagents used in this experiment were methanol, $NaNO_2$, $AlCl_3$, NaOH, FC reagent, Na_2CO, FRAP reagent, ABTS reagent and DPPH reagent. All the chemicals were analytical grade and all chemicals were obtained from Elmar Marketing, Iligan city and Merteflor, Cagayan De Oro city, Philippines.

Sample collection

The seagrasses that were used in this study were collectedfrom the coastal water of Carmen, Agusan Del Norte, Philippines. Geographically, Carmen is located at 9°00′N 125°16′E. The collected seagrasses was washed thoroughly with tap water to remove all sand particles and epiphytes then brought to the chemistry laboratory of University of Science and Technology of Southern Philippines (USTP) at Lapasan, Cagayan De Oro city, Misamis Oriental and shade dried at room temperature. The dried seagrass samples were then grounded on the mixer and stored in the refrigerator individually in airtight containers for further use.

Methanolic extraction

About 25.00 g of the sample was soaked in 95% methanol. A minimum volume of 200 ml was used to soak the sample. The soaking took about 48 hours. After 48 hours, the sample was filtered using Whatman filter paper. Then another 100 ml of methanol was used for the second soaking. Then after an hour, it had been filtered again. And for the soaking, another 100 ml of methanol was used, after which, the filtrate was then placed in the refrigerator for proper storage.

Total phenolic content

The total phenolic content of all the formulations of seagrasses was determined by using Folin- Ciocalteu method. 0.5 ml of the plant extract was placed in a 25 ml vial and 4.5 ml of distilled water was added. 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 using UV-VIS Spectrophotometer. The total phenolic content of the sea grass was calculated as gallic acid equivalents (mgGAE/g). All the experiments were performedin triplicate.

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 and then the absorbance was read at 510 nm using UV-VIS Spectrophotometer. Total flavonoid content was calculated as quercetin equivalents (mgQE/g). All the procedures were performed in triplicate.

Scavenging activity (DPPH) assay

The free radical scavenging activities of the extracts was determined by using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. 0.2 ml of the 1,000 ppm of seagrass 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 UV-VIS spectrophotometer.

ABTS radical cation decolorization power

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 volumetric flask. The mixture was then incubated for 6 minutes at room temperature and the absorbance was then read at 734 nm using UV-VIS spectrophotometer.

Ferric reducing antioxidant power (FRAP)

The property of the methanolic extract was determined by assessing the ability of the extracts to reduce FE as described by Irondi *et al.*, (2012) with slight modification. 4.0 ml of the 1,000 ppm of plant extract was placed in a clean vial and 6.0 ml of the FRAP reagent was added.

The FRAP reagent was prepared by dissolving about 0.0139 g of $FeSO_4.7H_2O$ with distilled water and diluted to 100 ml. The mixture was then incubated in a water bath at 37° C and the absorbance was read then at 593 nm using UV-VIS spectrophotometer.

Statistical analysis

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

Results and discussion

Table 1 showed the results of total phenolics and flavonoids content of the seagrasses. The maximum total phenolic content was recorded by the seagrass *Thalassia hemprichii 2.651*$\pm$ 0.001 followed by *Enhalus acoroides 0.201* $\pm$ 0.028 mgGA/g. Seagrasses are a rich source of phenolic substances, including phenolic acids, sulphated phenolic acids, flavones, condensed tannins and lignins, but not hydrolyzable tannins. The phenolic acids that predominate in the seagrasses also occur widely in land plants, but gallic acid was detected in a greater percentage of seagrasses (Zapata *et al.*, 2019).

Phenolic compounds in plants play an important role in pigmentation, growth, reproduction, resistance against pathogens, defense mechanism as well as protecting plants from deleterious effects of ultraviolet radiation and oxidants. The total phenolic content of a plant is an important parameter for their antioxidant properties.

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

Sea Grass	TPC (mg GA/g)	TFC (mg Q/g	DPPG (mg Trolox/g)	ABTS (mg Trolox/g)	FRAP (mg Trolox/g)
E. acoroides	0.201± 0.028	1.805± 0.274	0.301 ±0.003	0.007 ± 0.000	0.063 ± 0.000
T. hemprichii	2.651± 0.001	2.734± 0.047	0.189 ±0.001	0.162 ± 0.001	1.119 ± 0.019

Values are means ± SD for 3 determinations.

The highest total flavonoid content was recorded on the seagrass *Thalassia hemprichii* 2.734± 0.047 and *Enhalus acoroides* recorded 1.805 ± 0.274 mgQ/g. Forty three species of seagrasses were exclusively studied and identified that all contained either flavones and/or phenolic acid sulfates. Among the 12 genera examined, five (*Zostera, Phyllospadix, Enhalus, Thalassia* and *Halophila*) had sulfated flavones (Subhashini *et al.*, 2013). It has been reported that flavonoids are free radical scavengers that prevent oxidative cell damage, and have strong anticancer activities (Pourmorad *et al.*, 2006; Ugwu *et al.*, 2013) and they might induce mechanism that affects cancer cells and inhibit tumor invasion (Rafat *et al.*, 2008). These activities could be attributed to their ability to neutralize and quench radicals (Pourmorad *et al.*, 2006; Omale and Okafor, 2008; Ugwu *et al.*, 2013). It can also be due to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation (Rice-Evans *et al.*, 1995).

In plant systems, flavonoids help in combating oxidative stress and act as growth regulators.

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

Antioxidant assay	DPPH assay		ABTS assay		FRAP assay	
	R^2	P-value	R^2	P-value	R^2	P-value
TPC	0.99	0.000	0.99	0.000	0.99	0.00
TFC	0.66	0.048	0.62	0.064	0.66	0.051

The effect of antioxidants on DPPH radical scavenging is thought to be due to hydrogen donating ability. DPPH is a stable free radical and it accepts an electron or hydrogen radical to become a stable diamagnetic molecule. When a DPPH solution is mixed with a substrate acting as a hydrogen atom donor, a stable non-radical form of DPPH solution is mixed with a substrate acting as a hydrogen donor, a stable non radical from DPPH is obtained with simultaneous change of the violet color to pal Hence, DPPH (1,1-diphenyl-2-picrylhydrazyl) has been used extensively as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging activities of compounds. The maximum free radical scavenging activities (DPPH) of the extracts as shown on Table 1 was recorded by *Enhalus acoroides* 0.301 ±0.003 followed by *Thalassia hemprichii* 0.189 ±0.001 mgtrolox/g. Table 2 showed the results of correlation between total phenolic content, total flavonoid content and antioxidant assays.

There was a strong relationship between total phenol content, total flavonoid content and antioxidant activity determined by DPPH radical scavenging which values R^2=0.99, P value = 0.000 and R^2=0.66, P value=0.048 respectively. Phenol in the form of condensed tannin (proanthocyanidins) was found as the main phenolic compound *E. acoroides* (Kannan *et al.*, 2010).

ABTS or 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) is a chemical compound used to observe the reaction kinetics of specific enzymes. ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and Vitamin C. During this reaction, the blue ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically. This assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay. The reactivity of the various antioxidants tested is compared to that of Trolox, which is a water-soluble analog of vitamin E. The strongest ABTS radical cation decolorization power was recorded by the sea grass *Thalassia hemprichii* with values was 0.162 ± 0.001mgtrolox/g followed by *Enhalus acoroides* 0.007 ± 0.000 mg Trolox/g. There were a strong relationship between total phenol content, total flavonoid content and antioxidant activity determined by ABTS radical cation decolorization power which values R^2=0.99, P value = 0.000 and R^2=0.62, P value=0.064 respectively.

FRAP, also *Ferric ion reducing antioxidant power* is an antioxidant capacity assay that uses Trolox as a standard. The FRAP assay was first performed by Benzie and Strain (1996). The method is based on the formation of O-Phenanthroline-Fe^{2+} complex and its disruption in the presence of chelating agents. This assay is often used to measure the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols. It measures the ability of antioxidants in plasma or in foods to reduce the ferric component (Fe^{3+}) of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex (which is contained in the FRAP reagent) to the ferrous form (Fe^{2+}). During this reaction which takes place at a low pH, the reduction of ferric iron (Fe^{3+}) to the ferrous form (Fe^{2+}) is accompanied by the formation of a blue color which can be measured at an absorption maximum of 593nm using a spectrophotometer. The strongest Ferric ion reducing antioxidant power was recorded by the seagrass *Thalassia hemprichii* with value of 1.119 mg Trolox/g and followed by *Enhalus acoroides* 0.063 mg Trolox/g. There were a strong relationship between total phenol content, total flavonoid content and antioxidant activity determined by (FRAP) Ferric ion reducing antioxidant power which values R^2= 0.99, p value = 0.000 and R2= 0.66, P value=0.051 respectively.

The results of this study are in line with the previous researches conducted by Kannan *et al.*, (2010a), Kannan *et al.*, (2010b), Gavin and Durako, (2011), Sanatoso *et al.*,(2012), Kannan *et al.*, (2013), Athiperumalsami *et al.*, (2008) and Baby *et al.*, (2017) all showed that the antioxidant activity determined by DPPH, ABTS and FRAP demonstrated a significant positive linear correlations with their phenolics and flavonoids.

Conclusion

This study showed that the seagrasses were rich sources of natural antioxidants. The total content of polyphenols and flavonoids in the methanol extracts of the seagrass species positively correlated with their antioxidant properties. The results obtained suggest that the strong antioxidant properties of the seagrass species could play an important role in the food and pharmaceutical industries and realizing the true potential of seagrass meadows therefore requires an international cooperation to conserve and protect seagrass.

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