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Biochemical Characterization and Bioactivity of the Tape Seagrass *Enhalus acoroides (L.f.) Royle* from Gosoon, Carmen, Agusan Del Norte, Philippines

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ABSTRACT

Seagrasses have been used for food and traditional medicine for centuries, but only a few studies report their bioactivities and nutritional and medicinal values, particularly in Caraga Region, Philippines. Thus, crude ethanolic extracts of the tape seagrass *Enhalus acoroides* collected from Gosoon, Carmen, Agusan Del Norte, Philippines, were evaluated for biochemical constituents and bioactivity. Proximate analysis of *E. acoroides* yields the following results: moisture: 18.3%, Ash: 16.50%, crude fiber: 11.49%, crude protein: 5.52%, crude fat: 0.91%, and total sugar: 2.06%. Fourier-transform infrared (FT-IR) spectroscopy analysis identified the presence of hydroxyl groups, alkanes, esters, and carbohydrates. *Enhalus acoroides* showed active inhibition against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* (25±0.00 mm and 23.67±0.58 mm, respectively) using the Disk-diffusion method. Furthermore, cytotoxicity against *Artemia salina* nauplii revealed high toxicity of *E. acoroides* after 18 (LC_{50} value of 0.31 ppm) and 24-hour (LC_{50} value of 4.5x10-3 ppm) treatment with complete mortality at 24 hours. The present findings suggest the potential of *E. acoroides* for nutritional and medicinal purposes.

Keywords: *FT-IR, Cytotoxicity, Proximate Composition, Antibacterial Activity, Nutritional, Medicinal*

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1 Introduction

In developing countries, plant materials have been used to treat many infections and diseases due to a lack of access to healthcare (Compean and Ynalvez 2014). Coastal communities rely on marine natural products because they have limited access to terrestrial plants. Interestingly, local communities have little knowledge about the utility of seagrasses (Newmaster et al. 2011). Among marine natural products, seagrasses are not thoroughly studied in relation to drug discoveries (De la Torre-Castro and Rönnbäck 2004; Orno et al. 2020). Despite the efforts exerted on seagrass biodiversity for conservation, the conditions of seagrass still face challenges (Capin et al. 2021).

Enhalus acoroides is a large seagrass found widespread in subtidal zones of the Indo-Pacific region and is commonly known as tape seagrass (Short and Waycott 2010). It is distinctive among seagrass species due to its large size and rhizome, which is densely clothed with numerous stiff black fibrous strands that are remnants of previous leaves (Klangprapun et al. 2018). It serves as foraging and breeding grounds for fish, waterfowl, dugongs, manatees, sea turtles, and many other animals that are of commercial importance (Fajardo et al. 2016) and has also been exploited for feedstuffs (Newmaster et al. 2011).

Several communities in the Philippines and South Sulawesi, Indonesia, have utilized *E. acoroides* seeds for food consumption (Syed et al.

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2019; Gatta et al. 2020). The seeds of *E. acoroides* are highly nutritious as they contain carbohydrates, protein, and fat (Klangprapun et al. 2018). In traditional medicine, the rhizome, roots, fruit, and leaves treat different ailments and, more importantly, diseases such as hypertension (Newmaster et al. 2011). Furthermore, *E. acoroides* displays phytochemical compounds responsible for various bioactivities such as anti-larvicidal, antibacterial, antioxidant, and anti-cancer (Qi et al. 2008; Kannan et al. 2013).

Studies on seagrass species in the Philippines have focused more on its diversity despite its potential biological uses. Studies regarding the nutritional value, biochemical constituents, and bioactivity of *E. acoroides* are scant (Tangon et al. 2019; Tangon et al. 2021), especially within the bounds of the Caraga Region. Additionally, Pradheeba et al. (2011) noted significant seasonal variations of nutritional value in seagrass species. Gosoon, located in the Municipality of Carmen, in the province of Agusan del Norte, is abundant in the seagrass species *E. acoroides.*

Evaluation of the biochemical composition of *E. acoroides* can help educate seagrass in local communities and eventually address it in conservation efforts through a multifaceted and interdisciplinary perspective. Also, as the need for a constant search for potential drugs emerges, seagrasses pose the potential to be a rich source of novel bioactive compounds, allowing for significant bioactivities that will contribute to developing curative agents against emerging diseases. Thus, this study evaluated the biochemical composition of *E. acoroides* from Gosoon, Carmen, Agusan Del Norte and tested its antibacterial activity and cytotoxicity as baseline data.

2 Materials and Methods

Sampling Conditions

Samples of tape seagrass *E. acoroides* were collected between low and high tides in August on the coastal zones of Gosoon, Carmen, Agusan Del Norte, specifically on GPS waypoints of 09°04' 13.04" N, 125°13' 18.8" E (Figure 1). Water parameters such as salinity, temperature, pH, oxidation-reduction potential, and electric conductivity were measured under partly cloudy weather using an Oakton PC 450 Waterproof Portable Meter. Samples were photographed in situ (Figure 2).

Sample Collection and Extract Preparation

Enhalus acoroides samples were identified, and 2 kg samples of the whole plant were placed in a polythene bag and transported to the laboratory

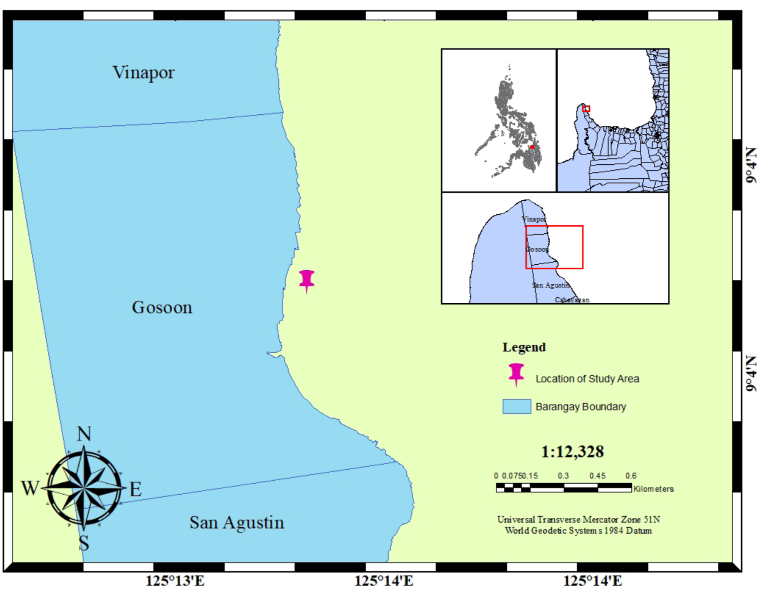

Figure 1. Collection Site for *Enhalus acoroides* at Gosoon, Carmen, Agusan del Norte

Figure 2. Freshly collected samples of *Enhalus acoroides* showing the (A) rhizome and roots and (B) whole plant. Scale Bar= 30cm.

for drying. Fresh samples were thoroughly washed with tap water to remove extraneous contaminants, shade-dried at room temperature (25°C) until brittle, and ground into powder using a mortar and pestle. Extraction was performed by the Department of Science and Technology XIII- Regional Standards and Testing Laboratory (DOST-RSTL). The powder was soaked in ethanol, and plant extraction was conducted as described by Guevarra et al. (2005).

Proximate Analysis

The proximate composition of crude protein, crude fiber, crude fat, ash, moisture, and total sugar (as glucose) was performed at the BIOTECH Central Analytical Service Laboratory at the University of the Philippines and determined following the methods described by the Association of Official Analytical Chemists (AOAC): (1) moisture was determined following AOAC 925.45 B; (2) Ash-AOAC 923.03; (3) Crude fat- AOAC 2003.05; (4) Crude Fiber- AOAC 978.10; (5) Crude Protein-AOAC 081.10; (6) Total Sugars- Phenol Sulfuric Acid Method. The extraction and antibacterial activity were performed by DOST Caraga-RSTL.

Fourier-transform Infrared Spectroscopy

Functional groups were determined via Fourier-transform Infrared (FTIR) spectroscopy at the Chemistry Laboratory of Caraga State University. Peaks were characterized using a PerkinElmer Spectrum Two FT-IR Spectrometer- Attenuated Total Reflectance (ATR).

Antibacterial Assay

Enhalus acoroides samples were subjected to an antibacterial assay following Guevarra et al. (2005). Paper disk diffusion was carried out using a 6mm diameter disc. *Staphylococcus aureus* (BIOTECH 152) and *Escherichia coli* (BIOTECH 1634) sensitivity to samples were tested in three replicates. Antibacterial activity was determined by measuring the clear or bland zones surrounding the disc.

Cytotoxic Activity

The brine shrimp lethality assay described by Guevarra et al. (2005) was adopted to assess the cytotoxicity of *E. acoroides* extract with modifications. Briefly, Artemia salina eggs were hatched in 2 L artificial seawater (38 g rock salt per 1 L distilled water: 38 ppt) for 48 h under constant aeration and illumination (3 watts). Sterile vials containing 4.5 mL artificial seawater and 0.5 mL *E. acoroides* extract with varying concentrations of 100, 10, and 1 ppm were loaded with ten nauplii using a micropipette and kept under illumination for 24 h. Artificial seawater without extract was used as a control. The number of surviving nauplii was determined following a series of 6 h interval observations for 24 h of exposure. The LC_{50} was determined by Probit analysis (Finney, 1952). The criterion for cytotoxic activity (LC50) was according to Clarkson et al. (2004) and Meyer et al. (1982) toxicity index, where; 0-100 ppm (Highly Toxic); 101-500 ppm (Moderately Toxic); 501-1000 ppm (Slightly Toxic); and 1000 ppm and above (Non-toxic).

Statistical Analysis

Descriptive statistics utilized IBM SPSS Statistical Analysis Software to analyze variations in the antibacterial activity, proximate composition, and water conditions. Meanwhile, Microsoft Excel 2016 was used for Brine Shrimp Lethality Test statistical data and probit analysis (Finney 1952).

3 Results and Discussion

Sampling Conditions

Temporal and spatial changes have been recorded to affect seagrass cover, species distribution (Ahmad-Kamil et al. 2013), and biochemical composition (Kolsi et al. 2017; Pradheeba et al. 2011). Several studies have noted variations in secondary metabolites, bioactivity, and biochemical composition of seagrass, including *E. acoroides*, from different sampling locations (Natrah et al. 2015; Pradheeba et al. 2011; Sidi et al. 2018; Gatta et al. 2020; Tangon et al. 2021). In the present study, water salinity, temperature, pH, oxidation-reduction potential, and electrical conductivity were 38.7 ppt, 32.3°C, 7.73 pH, 66.1 mV, and 78.1 mS/cm, respectively. These parameters are prominent in seagrass viability (Arumugam et al. 2013). Therefore, physicochemical properties should be documented to evaluate the factors required for biochemical characterization.

Proximate Composition

The moisture content (Table 1) of *E. acoroides* crude ethanolic extracts was 18.3±0.42%. Determination of seagrass moisture content is necessary since it affects the product's natural stability and encourages microbial contamination and chemical degradation by providing a substrate for various processes (Rohani-Ghadikolaei et al. 2012).

The amount of ash in plants measures their mineral content preserved in food materials; variations in ash content are likely related to ambient environmental conditions, which may affect the mineral transfer process (Gatta et al. 2020). Ash content in this study was found to be relatively low compared to ash values of *E. acoroides* from previous research (Rengasamy et al. 2013; Klangprapun et al. 2018; Tangon et al. 2019) but is similar to the ash content of *Cymodocea nodosa* from the Coast of Chebba (Kolsi et al. 2017).

The fiber content in the present study was comparable with previous findings for the seagrasses *Thalassia testudinum* (Coria-Monter and Durán-Campos 2015) and *C. nodosa* (Rengasamy et al. 2013). The high fiber content in food aids digestion and colon cancer prevention. Seed and seed pods of *E. acoroides* also showed high fiber content, 2.38% and 15.48%, respectively (Ratnawati et al. 2019), which supports the utilization of *E. acoroides* as a remedy to ease indigestion and become a basis for drug formulation (Newmaster et al. 2011).

The fat and lipid content of seagrass is generally in the range relevant to the fat content in the present study. Interestingly, Taba et al. (2019) investigated *E. acoroides* and found 0.27% fat content while identifying 11 fatty acids, with palmitic acid, linolenic acid, and oleic acid having the highest % content.

Every living cell's primary structural component, protein, is crucial for growth and development. It is a complex molecule composed of amino acids, nine of which the body cannot synthesize and must be obtained from food sources. Seagrass typically contains less protein than animals, so it cannot be considered a viable source of protein (Immaculate et al. 2018). In the present study, protein content was relatively low compared to the value reported by Rengasamy et al. (2013) for *E. acoroides*.

Carbohydrate is one of the essential components of metabolism, and it supplies the energy needed for respiration and other necessary processes. The body breaks down carbohydrates into simple forms called monosaccharides, which are used as the primary source of energy for cells, tissues, and organs. Most seagrass species contain soluble products, such as sucrose, as major storage carbohydrates (Pradheeba et al. 2011). In the present study, the total sugars of *E. acoroides* estimated as glucose were 2.06±0.18% which is low compared to the carbohydrate content reported for seagrass species *Zosteria marina* (50.9%) and *Cymodocea serrulata* (19.8%), whose large rhizomes act as reservoirs for food storage (Pradheeba et al. 2011; Tangon et al. 2021).

Accordingly, Tangon et al. (2019) recorded a high carbohydrate content of *E. acoroides* from the coast of Carmen, Agusan del Norte.

Variations in biochemical constituents are most probably due to geographic distribution and ambient environmental conditions (Pradheeba et al. 2011; Cristianawati et al. 2019; Gatta et al. 2020). The findings suggest that nutrient concentrations in sediments and dissolved nutrients in seawater in Gosoon, Carmen, Agusan Del Norte may be deficient due to lower values obtained than in other studies (Table 1).

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy is an essential tool researchers use to discern the chemical constituents present in a sample (Kannan et al. 2011). It is presently used to discover novel chemical and molecular components in unknown compounds (Sumayya and Murugan 2017). The FT-IR spectrum (Figure 3) of the *E. acoroides* ethanolic extract showed the presence of both a broad absorption peak at 3361 cm-1 and a weak signal at 2973 cm-1 assigned as the O-H stretching in the hydroxyl group and CH stretching vibration (Gómez-Ordóñez and Rupérez 2011; Kannan et al. 2011; Kannan 2014), respectively. This spectrum, which depicts the presence of alcohols, may indicate the extraction solvent ethanol (Sumayya and Murugan 2017; Rasheed et al. 2019; Pharmawati and Wrasiati 2020).

The presence of a weak band at 1647 cm-1 is a characteristic of a hydroxyl compound; according to Nandiyanto et al. (2019), the broad absorption band at 3600-3250 indicates a hydrogen bond, which in turn confirms the presence of a hydroxyl group that should usually be followed by the presence of additional spectra at frequencies in the ranges of 1600-1300, 1200-1000, and 800-600. Similarly, Pharmawati and Wrasiati (2020) reported a peak at 1654 cm-1 that is believed to be due to chlorophyll and protein content in *E. acoroides*. Stretching bands between 1500 and 1000 cm-1 are complex regions of ethers and alcohol, making it hard to assign the peaks of these regions. However, this C-O stretching region is a characteristic band

Table 1. Proximate analysis of *Enhalus acoroides*.

Biochemical composition	Amount (%)
Moisture	18.3±0.42
Ash	16.50±0.14
Crude fiber	11.49±0.62
Crude protein	5.52±0.08
Crude fat	0.91±0.03
Total sugar (as glucose)	2.06±0.18

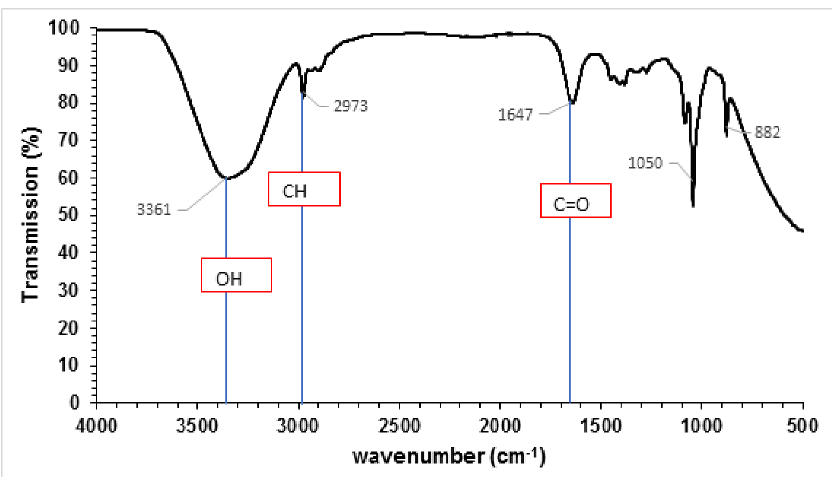

Figure 3. FTIR spectrum of *Enhalus acoroides* ethanolic extract

typical for carbohydrates (Svečnjak et al. 2011). Because seagrass has a high carbohydrate content, the strong peak at 1050 in the fingerprint region might be attributed to the carbohydrates in the samples. Also, A weak-medium peak at 820-920 cm-1 was observed. It could indicate the presence of out-of-plane C-H bending of glucose (Kannan 2014) or C-C skeletal vibration of alkanes, which was confirmed by TLC analysis in a recent study by Pharmawati and Wrasiati (2020) on *E. acoroides* dried leaf powder.

Antibacterial Activity

Seagrasses are rich in secondary metabolites that are effective antibacterial agents (Pradheeba et al. 2011; Tangon et al. 2019; Windyaswari et al. 2019), such as Alkaloids, tannins, and flavonoids, which they produce to survive the harsh marine environment (Qi et al. 2008; Kannan et al. 2013). Plant secondary metabolites target the microbial cell in several ways, including interaction with membrane proteins, disruption of cytoplasmic membrane function and structure, prevention of enzyme synthesis, and interruption of DNA/RNA synthesis and function (Gorlenko et al. 2020). This study's crude ethanolic extract of tape seagrass *E. acoroides* exhibited very active inhibition (Guevarra et al. 2005). Zones of inhibition of extracts showed comparable results between gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* (Table 2). However, *E. coli* exhibited slightly higher clear zones (25±0.00) than *S. aureus* (23.67±0.58). The gram-positive bacteria possess a thick and rigid peptidoglycan in their cell wall but lack an outer membrane.

In contrast, gram-negative bacteria possess a thin peptidoglycan and lipid-rich outer membrane, which consist of a major component called lipopolysaccharide (LPS); this outer membrane is an additional protective layer in gram-negative bacteria. However, this membrane contains channels called porins, which allow the entry of various molecules, such as drugs (Kapoor et al. 2017). Vanitha et al. (2017) determined the phytochemicals in *E. acoroides* using different extracts and found that the ethanol extract of *E. acoroides* yielded quinones and flavonoids. Flavonoids render the membrane more permeable and disrupt it by interacting with membrane proteins found in bacterial cell walls (Gorlenko et al. 2020). Compean and Ynalvez (2014) stated that flavonoids display astounding inhibition against gram-negative bacteria compared to gram-positive bacteria. The susceptibility of gram-negative bacteria against *E. acoroides* showed promising activity (Manikandan and Kolanjinathan 2016), suggesting that *E. acoroides* in the present study exhibits antibacterial activity against gram-negative and gram-positive bacteria.

Cytotoxic Activity

Cytotoxic activity of *E. acoroides* was carried out using the brine shrimp lethality test (BSLT). BSLT provides baseline information for the anti-cancer potential or safety of bioactive compounds in samples (Premarathna et al. 2020). There are alternative methods for testing a sample's cytotoxicity, but BSLT is inexpensive, reliable, and convenient (Vinayak et al. 2011).

Enhalus acoroides ethanol extracts showed cytotoxic activity to *Artemia salina* nauplii following a series of 6 h interval observations for 24 h for all three replicates. Cytotoxicity was expressed as % mortality and LC_{50} values. The 18 hour and 24 hour exposures of *A. salina* nauplii to *E. acoroides* ethanol extract showed a high toxic interpretation. They had complete mortality at 24 hour exposure (4.5x10-3 ppm), while 6 hour (LC_{50} 2951.20 ppm) and 12 hour (LC_{50} 245.8 ppm) exposures were interpreted as non-toxic and moderately toxic, respectively (Table 3).

There are a few studies of the cytotoxic activity of seagrass, e.g., Kannan et al. (2013) recorded lesser cytotoxicity in *E. acoroides* compared to other seagrass species. In another investigation by Orno et al. (2020), different plant parts (e.g., leaf, stem, and rhizome) of *E. acoroides* were found to inhibit the growth of *A. salina* nauplii, categorizing them as non-toxic and moderately toxic. The cytotoxic activity of three novel secondary

Table 2. Antibacterial activity of *Enhalus acoroides* Ethanol Extract.

Bacteria	Zone of Inhibition (mm)	Remarks
Staphylococcus aureus	23.67±0.58	very active
Escherichia coli	25±0.00	very active

Mean ± Standard Deviation.

Table 3. *Enhalus acoroides* brine shrimp lethality assay results.

Conc (ppm)	6H			12 H			18 H			24 H		
	%M	LC_{50}	R	%M	LC_{50}	R	%M	LC_{50}	R	%M	LC_{50}	R
100	19	2951.20 ppm	NT	44	245.89 ppm	MT	99	0.31 ppm	HT	100	4.5x10-3 ppm	HT
10	8			24			88			100		
1	2			14			59			94		

Note: %M- % mortality (mean), Conc- concentration, R- remarks: NT- non-toxic; MT- moderately toxic; HT- highly-toxic.

metabolites, apigen, luteolin 3'-glucuronide, and p-hydroxy-benzaldehyde, isolated from *E. acoroides* against *Spodoptera litura* cells was found to be highly toxic (Qi et al. 2008). The inhibitory effect of the extract is attributed to several cytotoxic compounds such as flavonoids, saponins, and alkaloids that are stated to possess anti-cancer, antiproliferative, and antitumor properties and are reported to be abundant in seagrass (Widiastuti et al. 2021). These compounds inhibit the feeding power of *A. salina* nauplii by disrupting the digestive tract and inhibiting taste receptors, failing to detect food and eventually causing starvation and death (Orno et al. 2020).

The cytotoxic activity exhibited by *E. acoroides* in the present study establishes the presence of potent bioactive compounds. The findings indicate that *E. acoroides* ethanol extracts are dosage dependent since fatality was strongly correlated with the concentration of the ethanol extract. Differing plant organs may contribute to the diversity of cytotoxic activity (Orno et al. 2020). Each organ is an organized group of specialized tissues that perform a specific function. Thus, it may contain its unique bioactive compounds, considering different parts of *E. acoroides* are used for different ailments in local communities (Newmaster et al. 2011).

4 Conclusions and Recommendations

The biochemical characterization of *E. acoroides* reported in this study indicates that seagrass is a good source of nutrients for human consumption and feed formulation. With the rising threat of antimicrobial resistance, new classes of antimicrobial drugs are urgently required. The active inhibition of *E. acoroides* suggests its potential as a center for research and development of new antibacterial drugs. Meanwhile, the high toxicity value of *E. acoroides* found in this study exhibits prospective potential anti-cancer, antitumor, or antiproliferative activity.

In contrast to prior studies, the current research found that the biochemical composition of seagrass species varies according to their geographic distribution. Hence, the findings of this study recommend that we describe and compare the biochemical composition of seagrass found within the bounds of the Caraga Region. Also, the results of the bioactivity of *E. acoroides* warrant further studies to elucidate the phytocompounds contained in them responsible for their bioactivity. Specifically, the researchers recommend that *E. acoroides* be tested for antibacterial activity using different solvents against a broader range of pathogens and tested for their anti-cancer and antitumor action.

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5 Statement of Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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