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Original Article

THE EFFECT OF EXTRACTION METHODS ON TOTAL FLAVONOID CONTENT AND ANTIOXIDANT ACTIVITY USING THE ABTS METHOD (2,2-AZINOBIS (3-ETHYLBENZOTHIAZOLINE)-*6-SULFONIC ACID)* IN SEAGRASS LEAF EXTRACT *(ENHALUS ACOROIDES (L. F.))*

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ABSTRACT

Objective: This study aimed to determine the effect of different extraction methods on flavonoid levels and antioxidant activity in seagrass leaves (*Enhalus acoroides*), with a particular focus on assessing their potential as natural antioxidants.

Methods: Flavonoids were extracted using infusion and ultrasonic methods with various solvents, including distilled water, ethyl acetate, and n-hexane. Flavonoid levels were measured in mg QE/g, while antioxidant activity was evaluated using the ABTS assay, with IC_{50} values determined using a UV-Vis spectrophotometer. Data were analyzed using several statistical tests, including the Shapiro-Wilk normality test, Levene's homogeneity test, t-test, Kruskal-Wallis test, Mann-Whitney test, ANOVA, and correlation analysis.

Results: The flavonoid content varied depending on the extraction method and solvent used: 4.339 mg QE/g for infusion with distilled water, 8.777 mg QE/g for ultrasonic distilled water, 68.836 mg QE/g for ethyl acetate, and 6.61 mg QE/g for n-hexane. Antioxidant activity results showed IC_{50} values of 13.76 µg/ml for ultrasonic distilled water, 13.66 µg/ml for ethyl acetate, and 15.59 µg/ml for n-hexane. The highest flavonoid levels were observed in the ethyl acetate extract. Statistical analysis confirmed that the extraction method significantly influenced flavonoid content.

Conclusion: Seagrass leaf extract obtained using ethyl acetate solvent showed the highest flavonoid concentration and potent antioxidant activity. The antioxidant activity of this extract was comparable to that of vitamin C, classifying it as a strong natural antioxidant. The findings support the importance of extraction method and solvent selection in optimizing bioactive compound yield and activity.

Keywords: Antioxidants, ABTS, Seagrass leaf

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INTRODUCTION

In Indonesia, the prevalence of degenerative diseases remains significantly high. According to the 2018 Riskesdas data, cancer prevalence increased by 65.7%, hypertension by 63.5%, diabetes mellitus by 5.7%, and coronary heart disease reached 4.5%. Degenerative diseases result from cellular function decline due to oxidative stress. This condition occurs when the body's balance between free radicals and antioxidants is disrupted (with excess free radicals), leading to organ cell damage. Antioxidants are essential to preventing oxidative stress [1].

Antioxidants work by neutralizing free radicals, which negatively impact the body. Antioxidants are categorized into two types: those produced endogenously by the body and exogenously. The endogenous antioxidants alone are insufficient to neutralize free radicals, necessitating the development of external antioxidant sources. Antioxidants can be derived from natural or synthetic materials. However, excessive use of synthetic antioxidants can lead to toxicity and carcinogenic effects in the body, emphasizing the need for safe antioxidants sourced from natural materials [2].

Natural antioxidants are derived from green plants, as they contain high levels of natural antioxidants. One such source is the marine plant *Enhalus acoroides* (L. f). In a study by [3], *Enhalus acoroides* (L. f) demonstrated antioxidant potential with an IC50 value of 38.008 µg/ml, attributed to its flavonoid content of 3.5697. In the study by [4], variations in extraction methods were shown to affect the rutin content, with results differing across maceration, boiling, reflux, UAE (Ultrasound-Assisted Extraction), and MAE (Microwave-Assisted Extraction) methods. Maceration yielded 16 g/kg, boiling 20.38 g/kg, reflux 22 g/kg, UAE 24 g/kg, and MAE 23 g/kg. The highest rutin content was obtained through the UAE method, indicating that the UAE is more effective than the other extraction methods. The infusion method is used. It is simple, cost-effective, non-toxic, and less prone to evaporation, whereas the ultrasonic method is employed because it produces a higher extract yield.

The study by [5] explained that radical scavenging analysis using the ABTS method resulted in 217.83 mg TE/g, the DPPH method 208.8 mg TE/g, and the FRAP method 42.15 mg TE/g. The results from ABTS and DPPH were relatively similar, but it was noted that the ABTS method provided significant absorbance at already visible wavelengths. Therefore, the ABTS method was employed for radical scavenging in this study. Research on the seagrass *Enhalus acoroides* (L. f) has been limited, and this study aims to analyze the effect of extraction methods on flavonoid content and determine the antioxidant activity of the extract with the highest flavonoid content using the ABTS method.

MATERIALS AND METHODS

Determination of seagrass plants

Seagrass leaves were obtained from Rembang District, Rembang Regency, Central Java. Determination of seagrass leaves was carried out at the Pharmacy Laboratory of Sultan Agung Islamic University, Semarang. The results of seagrass leaf determination indicate that seagrass leaves are included in the species *Enhalus acoroides (L. f.)*, no ethical approval was required for the collection of plant materials.

Equipments and materials

The instruments used in this study include an infusion pot, spatula, flannel cloth, stove, 1000 ml beaker (Iwaki), 500 ml beaker (Iwaki), stirring rod, Branson C 3800 ultrasonic extraction device, Whatman No. 1 filter paper, 10 ml volumetric flask (Iwaki), 50 ml volumetric flask (Iwaki), test tubes (Iwaki), dropper, UV-Vis spectrophotometer Agilent Technologies Cary 60, porcelain dish, cuvettes, 1 ml pipette,

1 ml volumetric pipette, measuring glass (Iwaki), Alpha 1-2 lDplus freeze dryer, and Heidolph evaporator. The materials used in this study include seagrass *Enhalus acoroides* (L. f.) simplicia, distilled water, n-hexane solvent, ethyl acetate solvent, quercetin (Merck), ethanol, 10% AlCl3 (Merck), 1M sodium acetate (Merck), potassium persulfate (Merck), ABTS solution (Merck), vitamin C (Merck), sulfuric acid reagent (Merck), Mayer reagent, magnesium powder (Merck), concentrated hydrochloric acid (Merck), and 10% FeCl3 (Merck).

Preparation of seagrass leaf simplicia

Fresh seagrass leaves collected from the sea were cleaned under running water to remove sand and epiphytes adhering to the leaves. After cleaning, the leaves were sorted by separating the damaged ones. The seagrass leaves were cut to reduce size and separated from the stem and roots [6].

Preparation of seagrass leaf extract using the infusion method

One hundred grams of seagrass simplicia was weighed and placed in a blender. 500 ml of water was added, and the mixture was transferred to infusion pot A. In infusion pot B, sufficient water was added, and pot A was placed on top of pot B for boiling. The temperature was monitored using a thermometer until it reached 90 °C. Once the temperature reached 90 °C, the mixture was left for 15 min with occasional stirring. After 15 min, the extract was filtered using flannel cloth, and the filtrate was collected in a glass jar. If the desired volume of filtrate/extract had not been reached, the remaining seagrass residue was added to boiling water to obtain the required volume [7, 8].

Preparation of seagrass leaf extract using the UAE (Ultrasonic-assisted extraction) method

The extraction was carried out with a 1:5 ratio. One hundred grams of simplicia were weighed and placed in a 600 ml beaker. 500 ml of ethyl acetate was added to this beaker, and 500 ml of n-hexane was added to a second beaker. The mixture was then placed in an ultrasonic device set at 40 °C, with a frequency of 40 kHz and duration of 120 min were selected based on preliminary optimization (table 1) and adapted from the study by [9], which demonstrated that these parameters provide an optimal balance between extraction efficiency and preservation of thermolabile compounds such as flavonoids. After 120 min, the extract was filtered using filter paper, and the resulting liquid extract was evaporated using an evaporator [10].

Table 1: Extraction optimization

Sample	Time (min)	Yield (%)
	60	0,05
	120	0,29

Qualitative identification of phytochemical compounds in seagrass leaves

An alkaloid test using a sufficient amount of seagrass extract was placed in a test tube and then dissolved with 2–5 drops of concentrated sulfuric acid. Alkaloid reagents such as Mayer's, Wagner's, and Dragendorff's reagents were added. A sample is considered positive for alkaloids if a white color forms with Mayer's reagent, a brown precipitate forms with Dragendorff's reagent, and a white to orange precipitate forms with Wagner's reagent [11]. Steroid/Triterpenoid test using 3 ml of extract was placed in a test tube, then 10 drops of glacial acetic acid and 3 drops of concentrated sulfuric acid were added and shaken until homogeneous. The sample is considered to contain steroids or triterpenoids if the solution turns red at the beginning of the test and then changes to blue or green at the end of the test [11]. Flavonoid test using a sufficient amount of seagrass extract was placed in a test tube, then 0.1 mg of magnesium powder and 1 ml of concentrated hydrochloric acid were added and shaken. The sample is considered to contain flavonoids if the solution forms a red, yellow, or orange amyl alcohol layer. A saponin test using a sufficient amount of extract was placed in a test tube, then dissolved with an appropriate amount of distilled water. The sample was then heated and shaken until foam appeared. The sample is considered positive for saponins if the foam persists for 10 min. Tannin test using 3 ml of extract was placed in a test tube, and 5 drops of FeCl3 reagent were added, followed by shaking. The sample is considered positive for tannins if the solution turns a greenish-black color [12]. All phytochemical tests were conducted using appropriate positive and negative control.

Quantitative identification of secondary metabolites: flavonoids in seagrass leaves

Preparation of quercetin standard solution, 10 mg of quercetin was placed into a 10 ml volumetric flask and dissolved with ethanol p. a. until it reached the marked volume, yielding a 1000 ppm solution. This 1000 ppm solution was then diluted to 100 ppm by taking 1 ml of the 1000 ppm solution and diluting it in a 10 ml volumetric flask. A series of solutions with concentrations of 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, and 70 ppm were prepared by taking 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, and 7 ml, respectively, and diluting each to 10 ml in a volumetric flask [13]. Determination of operating time**,** 60 ppm quercetin solution was prepared, and 1 ml was taken and placed into a test tube. To this, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M CH_3COONa, 3 ml of ethanol, and 5.6 ml of distilled water were added. The absorbance was measured at the maximum wavelength identified earlier, at 1-minute intervals, until the most stable absorbance value was obtained [14]. Determination of quercetin wavelength**,** a 60 ppm quercetin standard solution was prepared. 1 ml was taken and placed into a test tube, then 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M CH_3COONa, 3 ml of ethanol, and 5.6 ml of distilled water were added. The solution was measured at a wavelength range of 400–500 nm [14]. Preparation of quercetin standard curve, a 1000 ppm quercetin solution was diluted into a series of solutions with concentrations of 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, and 70 ppm in 10 ml volumetric flasks. From each concentration, 1 ml was taken and placed into a test tube. Then, 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M CH_3COONa, 3 ml of ethanol, and 5.6 ml of distilled water were added. The solutions were incubated for 30 min and measured at the corresponding wavelength. Determination of total flavonoid content**,** 500 mg of seagrass extract was weighed and dissolved with ethanol p. a. to 50 ml, yielding a 10,000-ppm solution. From this, 1 ml was taken and combined with 0.2 ml of 10% $AlCl_3$, 0.2 ml of 1M CH_3COONa, 3 ml of ethanol, and 5.6 ml of distilled water. The solution was incubated for the predetermined operating time, and the measurements were replicated three times. The $AlCl_3$ colorimetric method used in this study was validated in a previous study by [14], which evaluated key parameters including linearity (r = 0.993), precision (RSD = 0.00313%), accuracy (106.77%), LOD (249.3 ppm), and LOQ (831 ppm). These results confirm the method's reliability for determining total flavonoid content using UV-Vis spectrophotometry.

Antioxidant activity testing using the ABTS method

Preparation of ABTS stock solution, 7.100 mg (0,00276 M) of ABTS was weighed and placed into a 5 ml volumetric flask, then dissolved with distilled water and incubated for 12–16 h in the dark with a temperature 22-24 °C. Separately, 3.500 mg (0,003 M) of potassium persulfate was weighed, placed into another 5 ml volumetric flask, and dissolved with distilled water under the same incubation conditions. Masurement were performed in triplicate for each concentration. After 12–16 h, the two solutions were mixed in a 25 ml volumetric flask, and ethanol was added to the mark [15]. Preparation of vitamin C standard solution, 10 mg of vitamin C was weighed and placed into a 10 ml volumetric flask. A small amount of ethanol was added and stirred until homogeneous, then ethanol was added to the mark, yielding a final concentration of 1000 ppm. A series of solutions with concentrations of 8 ppm, 10 ppm, 12 ppm, 14 ppm, and 16 ppm were prepared in 10 ml volumetric flasks, with

ethanol added to the mark for each solution [16]. Determination of ABTS wavelength, 1 ml of ABTS stock solution was pipetted into a 5 ml volumetric flask and diluted with ethanol. The absorbance was measured at wavelengths between 700–750 nm [3]. Determination of operating time, 12 ppm series solution was prepared, and 1 ml was placed into a test tube. Then, 1 ml of ABTS solution was added to the test tube and transferred to a 5 ml volumetric flask. The absorbance was measured using the predetermined wavelength over 30 min at 1-minute intervals to determine the time at which absorbance stabilizes [16]. Measurement of vitamin C standard absorbance, each prepared series solution was used by taking 1 ml into a test tube. Then, 1 ml of ABTS solution was added, transferred to a 5 ml volumetric flask, and ethanol was added. The mixture was shaken until homogeneous and incubated for the operating time. Absorbance was measured at a wavelength of 750 nm [16].

Preparation of seagrass extract sample solution, 10 mg of seagrass extract was weighed and placed into a 10 ml volumetric flask. A small amount of ethanol was added, stirred until homogeneous, and ethanol was added to the mark, yielding a final concentration of 1000 ppm. A series of solutions with concentrations of 8 ppm, 10 ppm, 12 ppm, 14 ppm, and 16 ppm were prepared in 10 ml volumetric flasks, with ethanol added to the mark [16]. Measurement of antioxidant activity in the sample, each series solution was prepared by pipetting 1 ml into a test tube. Then, 1 ml of ABTS solution was added, transferred to a 5 ml volumetric flask, and diluted with ethanol. The solution is incubated for the operating time, and the absorbance is measured using a predetermined wavelength [16], to determine the antioxidant activity of the initial step by calculating the percentage of inhibition. The following is the formula for determining % inhibition:

$$\% \text{ Inhibition} = \frac{A0-A1}{A0} \times 100\%$$

With the following information:

A0: absorbance on ABTS without sample/extract

A1: absorbance on ABTS to which extract/sample has been added

If the percentage of inhibition has been obtained, the next step is to calculate the IC_{50} value, because it is used to assess how much antioxidant activity is in counteracting free radicals by 50%. IC_{50} values were calculated based on linear regression of the relationship between concentration and percent inhibition (% inhibition). This approach was used because the data showed a consistent linear relationship over the range of concentrations tested.

To calculate IC_{50} there is an equation used namely the linear regression equation with substitution, namely

Y = bX+a

With the following information:

Y = percentage of inhibition

a = gradient

X = concentration (µg/ml)

b = constant

The equation explains the relationship between log concentration and percentage (%) of antioxidant activity (inhibition). So to calculate the IC50 value, the equation will be:

50 = bX+a

X = 50+ba.

RESULTS

Determination of yield and moisture content of seagrass leaf extract

The study revealed variations in the percentage yield and water content obtained through different extraction methods, as presented in table 2.

Table 2: Yield and water content seagrass leaf extract

Method and solvent	Initial weight (kg)	Extract weight (g)	Yield percentage (%)	Mean water content (%)
Infusion with aqueous solvent	3	14.48	0.48	5.77
UAE with aqueous solvent	4	19.27	0.48	6.34
UAE with ethyl acetate solvent	7	20.56	0.29	5.62
UAE with n-hexane solvent	4	9.55	0.23	5.54

Table 3: Qualitative phytochemical screening test

Type	Results			
	Infusion	UAE aqueous	UAE ethyl acetate	UAE N-hexane
Alkaloids	+	+	+	-
Flavonoids	+	+	+	+
Steroids and triterpenoids	-	-	+	+
Saponins	+	+	-	-
Tannins	+	+	-	-

Phytochemical screening of seagrass leaf extract

Phytochemical screening was performed on the seagrass leaf extract to qualitatively identify the secondary metabolites present, with the results summarized in table 3.

Determination of flavonoid content in seagrass leaf extract

The flavonoid content was analyzed using the colorimetric method with UV-Vis spectrophotometry. This method was selected because flavonoid metabolites possess a conjugated system, enabling detection in ultraviolet and visible light spectra [22]. The flavonoid content analysis of seagrass leaf extract revealed varying results depending on the extraction method, as shown in table 4. The regression equation obtained was Y = 0.006x+0.0436, with an R^2 = 0.9981, as shown in fig. 1.

Table 4: Flavonoid content determination using UV-Vis spectrophotometer

Sample	Total flavonoid content (mgQE/g)
Infusion	4.339±0.28
UAE Aqueous	8.777±1.67
UAE Ethyl Acetate	68.836±10.44
UAE N-Hexane	6.61±2.1

Antioxidant activity testing of sea grass extract

Given that the highest flavonoid content was observed in extracts obtained using the ultrasonic method, this extract was selected for antioxidant activity testing using the ABTS method. The ABTS assay eliminates cations from the ABTS radical, resulting in a color change

that indicates the extent of antioxidant interaction with the cationic radicals. Vitamin C was used as the positive control. The initial step involved determining the maximum wavelength within the 700–750 nm range.

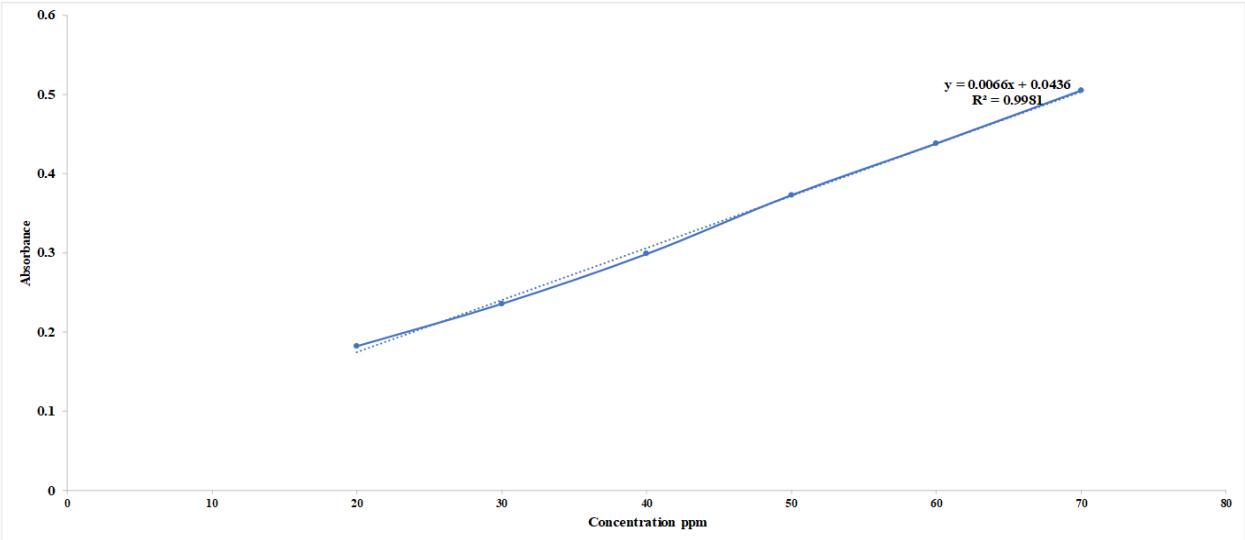

Fig. 1: Linearity graph of quercetin standard curve

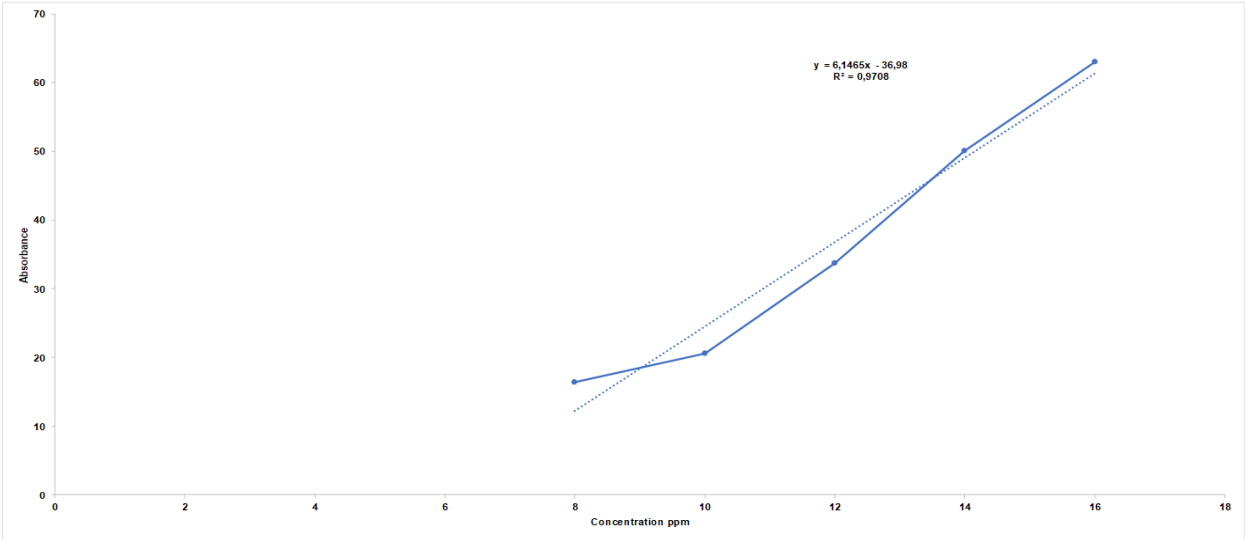

Fig. 2: Linearity graph of vitamin c standard curve

Table 5: Antioxidant test results of vitamin C using UV-vis spectrophotometer

Concentration (ppm)	Mean inhibition (%)	R^2	IC_{50} (ppm)
8	16.35	Y = 6.1465x – 36.98	14.15
10	20.62	R^2 = 0.9708	
12	33.73		
14	50.13		
16	63.06		

Table 6: Antioxidant test results using UAE method with aqueous solvent

Concentration (ppm)	Mean inhibition (%)	R^2	IC_{50} (ppm)
8	14.36	Y = 5.856x – 30.586	13.76
10	29.45	R^2= 0.9831	
12	39.68		
14	54.59		
16	60.35		

Table 7: Antioxidant test results using UAE method with ethyl acetate solvent

Concentration (ppm)	Mean inhibition (%)	R^2	IC_{50} (ppm)
8	4.18	Y = 6.1415x – 45.752	15.59
10	1.64	R^2 = 0.9462	
12	20.04		
14	43.91		
16	52.96		

Table 8: Hasil uji antioksidan metode UAE pelarut N-heksan

Concentration (ppm)	Mean inhibition (%)	R^2	IC_{50} (ppm)
8	10.53	Y = 7,0345x-46,106	13.66
10	21.03	R^2= 0,9863	
12	41.79		
14	53.60		
16	64.59		

DISCUSSION

The infusion method produced an extract yield of 0.48%, while ultrasonic extraction using distilled water as a solvent also yielded 0.48%. In comparison, ultrasonic extraction with ethyl acetate yielded 0.29%, and n-hexane yielded 0.23%. These findings indicate that the highest yield percentage was achieved using polar solvents (table 1). In a related study by [17], methanol as a solvent produced a yield of 12.53%, ethyl acetate 6.7%, and n-hexane 2.8%, with the highest yield observed for the polar solvent methanol. These results align with the findings of this study, where the highest yield for the *Enhalus acoroides* seagrass leaf extract was also obtained using polar solvents. The yield percentage is not influenced by the quantity of simplicia used but rather by the secondary metabolites in the seagrass leaves. The extract yield obtained in this study (0.48%) was indeed lower than the results reported by [17], which was 12.53% using methanol solvent. This difference is likely due to differences in the type of raw materials, harvest time, sample preparation techniques, or differences in the solvent system and extraction process parameters used. The findings further suggest that polar solvents are more effective at extracting polar secondary metabolites [18]. To improve the low extraction yield, future studies should explore sequential extraction with increasing polarity solvents or refine solvent selection and extraction paramaters such as solvent-to-sample ratio, time, and agitation intensity to enhance metabolite recovery. Before utilization, the seagrass leaf extract underwent standardization testing to ensure its stability. One of the standardization tests performed was moisture content analysis. This test aimed to determine the moisture content in the extract, as moisture can promote fungal growth, potentially compromising the stability of the extract. The infusion method yielded a moisture content of 5.77%, ultrasonic extraction with distilled water 6.34%, ultrasonic extraction with ethyl acetate 5.62%, and ultrasonic extraction with n-hexane 5.54% (table 2). According to [19], one indicator of good extract stability is its moisture content, with the requirement for concentrated extracts being ≤10%. The extracts obtained through all methods demonstrated good stability as their moisture content did not exceed 10%. Exceeding this threshold would likely result in rapid fungal growth on the extract, reducing its quality.

The infusion method extract contained alkaloids, flavonoids, saponins, and tannins, while the UAE method with distilled water as a solvent also contained alkaloids, flavonoids, saponins, and tannins. The UAE method with ethyl acetate as a solvent contained alkaloids, flavonoids, and steroids/triterpenoids, while the UAE method with n-hexane as a solvent contained flavonoids and steroids/triterpenoids (table 3). These results are consistent with findings from [20], who reported that seagrass leaf extract using methanol as a solvent contains alkaloids, flavonoids, saponins, and tannins, while n-hexane as a solvent yields flavonoids and steroids/triterpenoids [21] similarly, seagrass leaf extract using ethyl acetate as a solvent contains alkaloids, flavonoids, and steroids/terpenoids. These differences arise due to the varying polarity of each compound, which dissolves according to the solvent's polarity. After the qualitative analysis, a quantitative test was performed to determine flavonoid levels.

The $AlCl_3$ method was employed, which involves forming a complex compound with the keto group at C-4 and the hydroxyl groups at C-3/C-5 of flavones and flavonols. The addition of $AlCl_3$ shifts the wavelength into the visible spectrum, producing a yellow solution. Sodium acetate was used as a shift reagent to detect the presence of 7-OH groups and stabilize the wavelength within the visible spectrum range [22, 23]. Prior to determining the flavonoid content, the maximum wavelength, operating time, and standard curve for quercetin were established. The wavelength determination was conducted in the 400–500 nm range, yielding a maximum wavelength of 440 nm. Operating time determination aimed to identify the optimal incubation time for stable absorbance or reaction completion in the sample. The study found that 25 min was required for the reaction to stabilize. A standard curve was generated using quercetin solution to obtain a linear regression equation. Determination of the curve obtained the results of the linear regression equation Y = 0.006x+0.0436, with a value of R^2 = 0.9981, the correlation coefficient value (R^2) is close to 1, meaning that the data is said to be linear.

The flavonoid content obtained using ultrasonic extraction with aqueous (8.777±1.67 mgQE/g) was higher than that of the infusion method (4.339±0.28 mgQE/g). This discrepancy can be attributed to differences in extraction conditions, such as temperature, extraction time, and the properties of the target compounds—in this case, flavonoids. Flavonoids are thermolabile compounds that can undergo oxidation when exposed to high temperatures (>60 °C). Consequently, the ultrasonic extraction method with distilled water, conducted at lower temperatures, yielded higher flavonoid content than the infusion method, which involved extraction at 90 °C [24]. The extraction method significantly affects the flavonoid content p value 0.041. In a study by [4], a comparison of infusion (24 g/kg) and ultrasonic extraction (20.38 g/kg) methods for rutin compounds revealed that ultrasonic extraction produced higher rutin content. This method enhances compound yields by avoiding degradation due to temperatures exceeding 60 °C. Furthermore, ultrasonic extraction is more efficient and requires less time than conventional methods. The present study also applied ultrasonic extraction with semi-polar and non-polar solvents, such as ethyl acetate and n-hexane. The flavonoid content obtained with ethyl acetate (68.836±10.44 mgQE/g) was higher than that with distilled water (8.777±1.67 mgQE/g) and n-hexane (6.61±2.1 mgQE/g). The relatively high standard deviation observed in the ethyl acetate extract (±10.44 mgQE/g) may be attributed to inconsistent efficiency or heterogeneity in raw material composition. To address this, future studies should consider increasing the number of replications and optimizing extraction paramaters to reduce variability. However, the flavonoid content with n-hexane (6.61±2.1 mgQE/g) was lower than that with distilled water (8.777±1.67 mgQE/g). The correlation coefficient (R^2) close to 1 indicates that the data are linear.

Statistical analysis was performed using the normality test on the UAE method samples of distilled water, ethyl acetate, and n-hexane, obtained p values (0.735, 0.307, 0.996) indicating normal because p>0.05. The homogeneity test obtained a p value of 0.021 indicating non-homogeneous because the p value<0.05. The differences observed in flavonoid content across solvents were due to the characteristics of each different solvent. Significant differences in flavonoid content between solvents (distilled water, ethyl acetate, and n-hexane) with a p value<0.039 (p<0.05). The comparison between distilled water and n-hexane solvents did not show a statistically significant difference (p>0.05), although the flavonoid content with distilled water was higher than with n-hexane with a p value of 0.127. In contrast, significant differences (p<0.05) were observed between ethyl acetate and distilled water, as well as between ethyl acetate and n-hexane. Different solvent polarities affect these variations, as flavonoids can be extracted into polar, semi-polar, and non-polar solvents. The highest flavonoid content was observed with ethyl acetate, outperforming distilled water and n-hexane. Flavonoids such as flavones, flavanones, and flavonols are soluble in semi-polar solvents such as ethyl acetate, while non-polar solvents such as n-hexane dissolve poly methoxylated aglycones or isoflavones, since the sugar or glycoside groups have been removed.

The study identified the maximum wavelength of ABTS as 743.49 nm, which falls within the expected range. Operating time, or incubation duration, was set at 30 min. According to [25], the reaction time for vitamin C with ABTS stabilizes at 30 min. Vitamin C served as the standard for generating a linear regression equation, with the equation obtained being Y = 6.1465x – 36.98 and an R^2 = 0.9708. A regression equation is considered linear if the R^2 value is close to 1, as shown in fig. 2. This high correlation indicates a strong relationship between concentration and absorbance. Antioxidant activity was assessed using the IC50 value, which measures the concentration required to neutralize 50% of free radicals. The IC50 for vitamin C was found to be 14.15 µg/ml (table 5), categorizing its antioxidant activity as very strong. In comparison, reported an IC50 value of 7.81 µg/ml for vitamin C, indicating extreme antioxidant activity. Efficacy of vitamin C as an antioxidant is attributed to its ability to donate electrons to ABTS free radicals, neutralizing them into non-radical forms. Simultaneously, vitamin C is oxidized into semi-dehydroascorbic acid, a stable compound [25].

The ultrasonic extraction method using distilled water as a solvent produced an IC50 value of 13.76 µg/ml, which categorized its antioxidant activity as very strong. The ethyl acetate solvent produced an IC50 value of 13.66 µg/ml, which also showed very strong antioxidant activity. Meanwhile, the n-hexane solvent showed an IC50 value of 15.59 µg/ml (Tables 6, 7, and 8), which also fell into the category of very strong antioxidant activity. These results highlight that the seagrass leaf extract from *Enhalus acoroides* (L. f.) exhibits strong antioxidant activity, which is mainly due to its flavonoid content. According to [26], flavonoids exhibit various pharmacological effects, including acting as antioxidants. The mechanism involves scavenging free radicals or breaking the chain reaction of the oxidative process. Among the three solvents tested, the highest antioxidant activity was observed in the extract obtained using ethyl acetate as a solvent in the ultrasonic method. Post Hoc test showed a significant difference with p value of 0.000 between ethyl acetate and n-hexane extracts when compared to vitamins, indicating that their antioxidant activities are different from vitamin C. However, the antioxidant activity of distilled water extract did not differ significantly from vitamin C values (p 0.229), indicating comparable efficacy.

Despite the variation, all three samples showed extreme antioxidant activity, with ethyl acetate extract showing the highest antioxidant activity among the solvents tested. In the study by [26], antioxidant activity testing was conducted using seagrass leaves with three different solvents, namely methanol, ethyl acetate, and n-hexane. No IC50 value was obtained for the methanol solvent, while the ethyl acetate solvent produced an IC50 value of 25.98 µg/ml and the n-hexane solvent gave a value of 139.50 µg/ml. These results indicate that the ethyl acetate solvent has the highest antioxidant activity. In the study by [3] also conducted an antioxidant test on seagrass *Enhalus acaroides* obtained an IC50 value of 38.008 µg/ml which showed a difference in results. This is because ethyl acetate is semi-polar, and differences in compound polarity can affect antioxidant activity, in addition to being influenced by the location or place of growth because it affects the difference in chemical content so that its bioactivity can also be different. This study tested the flavonoid content and antioxidant activity of seagrass leaf extract using the ultrasonic method with three different solvents. Pearson correlation test produced a correlation value of 0.466 for the aquadest extract indicating a moderate positive relationship between flavonoid content and antioxidant activity. The correlation value of the ethyl acetate extract of 0.941 indicated a very strong positive correlation between flavonoid content and antioxidant activity and the n-hexane extract showed a correlation value of-0.684. The negative correlation observed between flavonoid content and antioxidant activity in n-hexane extract (r =-0.684) is contrary to what was expected. This is likely due to the presence of non-flavonoid compounds such as lipids or other non-polar antioxidants soluble in n-hexane, which may dominate the antioxidant activity in the extract. In addition, possible analytical interference or methodological artifacts in the measurements may also affect the results [27]. In the research that has been conducted, there are suggestions for further research development, namely making seagrass leaf extract *Enhalus acoroides* (L. f.) using dry simplicia and comparing hot and cold extraction methods.

CONCLUSION

The study concluded that the extraction method impacts the flavonoid content in *Enhalus acoroides* (L. f.) leaf extract. The flavonoid content obtained from the infusion method was 4.339 mgQE/g, while the ultrasonic method yielded 8.777 mgQE/g. *Enhalus acoroides* (L. f.) leaf extract also demonstrated robust antioxidant activity across all three solvents. The IC50 values for the solvents were 13.76 µg/ml for distilled water, 13.66 µg/ml for ethyl acetate, and 15.59 µg/ml for n-hexane, all of which fall into the firm antioxidant activity category.

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AUTHORS CONTRIBUTIONS

Naniek Widyaningrum: Research design and supervision. Azmi Rahmadani: Supervision and data collection. Alya Putri Sakura: Phytochemical analysis and statistical analysis. Thendi Abdul Arief: data interpretation and editing.

CONFLICT OF INTERESTS

Declared none

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