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## Phytochemical profile of sea grass extract (*Enhalus acoroides*): A new marine source from Ekas Bay, East Lombok

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# Phytochemical profile of sea grass extract (*Enhalus acoroides*): A new marine source from Ekas Bay, East Lombok

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**Abstract.** The coast of Southeast Indian and the tropical part of the Western Pacific is a habitat for the tropical sea grass *Enhalus acoroides*. In Ekas Bay, East Lombok, *E. acoroides* grows well in seashores, which is important to tropical marine ecosystem. Previous research reported the pharmacological activity of *E. acoroides* such as antioxidant and antibacterial. *E. acoroides* was tested to identify their secondary metabolites using phytochemical screening. The secondary metabolites were further characterized using thin layer chromatography with specific spray reagent. The phytochemical screening identified primary metabolites such as carbohydrates and proteins. On the other hand, the phytochemical screening also detected secondary metabolites such as alkaloid, phenolic, tannin, saponins, flavonoid, monoterpenes, and sesquiterpenes. The TLC profile with specific spray reagent confirmed the bioactive components such as phenolic, flavonoid, and terpenes in methanol extract of *E. acoroides*. These results had a significant impact on profiling the marine plant as a new drug candidate. The information of secondary metabolites from *E. acoroides* will contribute to further research in determination of antioxidant, and antibacterial activity of *E. acoroides*, as a promising marine drug candidate from Indonesia.

**Keywords:** antioxidant, *Enhalus acoroides*, phytochemical screening, sea grass, TLC

## 1. Introduction

The high productivity of estuarine and near shore marine regions are affected by major functioning element, such as sea grasses in the complex cyclical processes [1]. Previously, to cure various human diseases, medicinal plants play an important role in healing. In the other side, phytochemical characterizations are divided into categories: primary and secondary constituents. The chlorophyll, proteins, sugars, and amino acids are primary constituents. Meanwhile, the terpenes, flavonoids, phenolic, saponins and alkaloids are secondary compounds. The pharmacological activity from seagrasses such as antifungal, antibacterial and anti-inflammation have been reported [2]. Primary metabolites involve directly in metabolism process. All metabolic reactions such as catabolic and anabolic are influenced by primary and secondary metabolites [3]. According to biosynthesis pathway, many chemical constituents from plants exerting pharmacology activities come from plant metabolites. The primary metabolites such as carbohydrates, proteins and lipid are food material. On the other side, the triterpenoids, alkaloids, glycosides, tannins, flavonoids, essential oils and other similar secondary



metabolites are proven natural medicines. The investigation of plant metabolites as a new candidate medicine need a complete study. The evaluation and standardization of crude extracts must include plant metabolites and chemical study of the plant. To establish a profile of chemical composition of an extract f, the qualitative chemical tests need to be performed [4]. Alkaloids, tannins and flavonoids are the important secondary metabolites from natural product [5].

The coast of Southeast Indian and the tropical part of the Western Pacific is a habitat for the monotypic marine genus *E.* in the family Hydrocharitaceae. One of the sea grass species is *E. acoroides* (L.f.) Royle [6]. As one of food materials in Ekas Bay, Lombok, Indonesia, *E. acoroides* has been utilized as traditional medicines. The seagrass was used as a remedy against stings of different kind of rays, and stone lion and scorpion fish (Scorpaenidae), as well as rabbit fish (Siganidae). The empirical uses from *E. acoroides* including analgesic, antipyretic ('mafusho'), antiseptic, and anti diarrhea. The patient inhales the vapours from a mixture of plants and herbs ('mafusho') in order to decrease body temperature [7-9]. The preliminary screening of antioxidant activities of *E. acoroides* collected from Gulf of Mannar Biosphere Reserve, India [10]. The major sterol and fatty acid component of fresh leaves of *E. acoroides* [11]. The chemical constituents and antifeedant, antibacterial and the antilarval activities of methanol extracts of *E. acoroides* from South China Sea and recorded eleven pure compounds including four flavonoids and five sterols [12]. More recently, reports have revealed that sea grasses are rich sources of antioxidant compounds [10, 13-15]. Antioxidants in biological systems have multiple functions, which include protection from oxidative damage and in the major signalling pathways of cells. The major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (ROS), such as, superoxide radicals ( $O_2^-$ ), hydroxyl ( $OH^\cdot$ ), peroxide ( $ROO^\cdot$ ) and nitric acid radicals. These reactive oxygen species are generated in living organisms during excessive metabolism and they cause extensive oxidative damage to cells leading to age related degenerative diseases, cancer and a wide range of other human diseases [10]. The aim of the study was to provide evidence that the bioactive substances from methanol extract of *E. acoroides* will become a promising drug candidate from marine tropical sea.

## 2. Materials and Methods

### 2.1. Materials

**2.1.1. Sample collection.** Sea grass *E. acoroides* were collected from Ekas Bay, East Lombok, Indonesia. The identity of the collected plant was confirmed in the Department of Aquatic Resources Management, Bogor Agricultural University. Fresh plants and crude extracts of sample were used for phytochemical analysis.

**2.1.2. Preparation of plant samples.** To prepare standardized plant samples, the fresh plants of *E. acoroides* were thoroughly washed with tap water and dried at a temperature of 40°C for three days. Subsequently, the dried samples were ground into powder using a grinder. The powdered samples were then stored in a refrigerator for future use.

### 2.2. Methods

**2.2.1. Extraction.** Maceration technique with methanol solvents (1:7) was adopted for extraction. One kilogram of sample was extracted in room temperature for 18 hours. All techniques were repeated for three times. A rotary evaporator is an instrument to evaporate the liquid methanol extract into the thick methanol extract at the boiling point of methanol (70°C).

**2.2.2. Phytochemical analysis: Primary metabolites.** The screening included carbohydrate and amino acid tests. Carbohydrate test was conducted by adding the methanol extract (1 mL) with two drops of Molisch's reagent. Two mL of concentrated  $H_2SO_4$  was then added to the solution. The presences of carbohydrates was indicated by the formation of a violet ring at the junction. Amino acid test was conducted by adding the methanol extract (3 mL) with the mixture of Biuret's reagent and mixed with 1 mL of 4% w/v sodium hydroxide and 1 mL of 1% w/v copper sulphate. The presence of proteins was confirmed by a violet or pink colour. The methanol extract (3 mL) was added to the mixture of

Ninhydrin's reagent and mixed with 3 drops of 5% v/w lead acetate solution, and boiled in a water bath for 10 minutes. The resulted purple or blue color indicates the presence of protein.

**2.2.3. Phytochemical analysis: Secondary metabolites.** The screening included alkaloid, flavonoid, phenols, tannin, quinnon, saponin, sterol, and terpenoid test. Alkaloid test was conducted by adding 0.2 mg of crude extract with 3 mL of hexane was mixed in it, shaken well and filtered. Then 5 mL of 2% HCl was acquired and poured into a test tube [2, 3].

- a. Wagner's test: Into 2 or 3 mL of methanol extract, a few drops of Wagner's reagent were added. The presences of alkaloid by a reddish brown precipitate.
- b. Dragendorff's test: Into 2 to 3 mL of methanol extract, a few drops of Dragendorff's reagent were added. The presences of alkaloid, by an orange brown precipitate.

For the purpose of flavonoid analysis, distilled water was extracted with 2 mL of test solution and 0.2 mg of crude extract. The filtrate was used for analysis. Add 2-3 mL of filtrate was acquired and a few fragments of magnesium metal into a test tube, followed by a drop wise addition of concentrated HCl. The presences of flavonoid were indicating by a formation of magenta color [3].

For the purpose of phenol analysis, distilled water was extracted with 0.2 mg of crude extract. The filtrate was used for analysis. An amount of 2 mL of test solution was mixture with 0.5 mL  $\text{FeCl}_3$  (w/v) solution. The presences of phenols were indicating by spots and yellow colour [5].

For the purpose of tannin analysis, distilled water was extracted with 0.2 mg of crude extract Add gelatine solution into the extract. The presences of tannin were indicating by the formation of white precipitate [5].

For the purpose of quinone analysis, distilled water was extracted with 0.5 mg of crude extract. Add 1 mL of concentrated  $\text{H}_2\text{SO}_4$  to 1 mL of extract. The presences of quinone were indicating by the formation of a red color [5].

For the purpose of saponin analysis, 20 mL of distilled water was diluted with 0.5 mg of the crude extract, shaken in a graduated cylinder for 15 minutes. The presences of saponin were indicating by layer of foam (1 cm) [5].

For the purpose of sterol analysis, the test tube were added with 0.2 mg of crude extract and 3 mL of hexane was mixed in it, shaken well and filtered, before the chloroform was mixed into 2 mL extract. After that, 1-2 mL acetic anhydride and 2 drops of concentrated  $\text{H}_2\text{SO}_4$  were dropped into the test tube. The presences of sterols were shown in the formation of a red, then blue and finally green colour [3].

For the purpose of terpenoids analysis, 0.5 mg of crude extract was extracted in distilled water. One mL of concentrated  $\text{H}_2\text{SO}_4$  was added to 1 mL of extract. The presences of terpenoid were shown by the formation of a red color [3].

**2.2.4. Qualitatively antioxidant screening.** For the confirmation of antioxidant screening, the 1% extract was diluted into 5 mL of methanol. Thus, the extract was used for dynamolysis using Whatman's filter paper. The radical stock solution of 0.2% DPPH in methanol was used for identification of the antioxidant activity.

**2.2.5. Thin layer chromatography profile [6].** Generally, TLC technique requires a plate with Silica gel 60 F254 TLC (Merck, Germany) as stationary phase, and different solvent system as mobile phase. For TLC analysis, 7x6 cm was cut with scissors and soft pencil were use for plate markings. To spot the sample, applied sample volume 1  $\mu\text{L}$  of sample by using capillary at distance of 1 cm at 5 track. For TLC analysis of ethanol extract *E. arocoides*, the mixture of Chloroform: Ethyl acetate: Methanol (4:4:1) as solvent mobile system were used. The incubation time after pre saturation applied was about 20 minutes. To detection for the bands, the freshly prepared iodine spray reagents were used. The

movement of the spot was expressed by its retention factors (Rf). The Rf values were calculated for different samples using formula (1).

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent front TLC plates}} \quad (1)$$

### 3. Results and Discussion

#### 3.1. Percentage of crude extract

According to specification, the figure and the characterization of *E. acoroides* crude extract is shown in figure 1 and table 1.



**Figure 1.** Sample of *E. acoroides*.

Generally, the specification of crude extracts to extend the storage is dependant on the percentage of water content, which is less than 10%. Meanwhile, the water content of *E. acoroides* crude extract is 7.80%. Thus, the water content of *E. acoroides* crude extract is according to requirement [16].

**Table 1.** Characterization of *E. acoroides*.

Characterization	Result (%)
Water content	7.80±0.20
Ash content:	
a. Total ash	14.78±0.14
b. Total ash (water soluble)	13.23±0.04
c. Total ash (acid insoluble)	0.86±0.09
Extractive values:	
a. Water extractive	12.16±0.04
b. Ethanol extractive	10.09±0.12

The presence of contamination from plant or environment (air, soil and water) is obtained from the value of total ash content from *E. acoroides* crude extract which is 14.78%. On the other side, the percentage of ash soluble in water (13.23%) is evidence of the alkaline metals presence, meanwhile the percentage of insoluble acid ash (0.86%) is evidence of the silica metals presence. Determinations of bioactive compounds in water soluble (12.16%), and ethanol soluble (10.09%) are the valid proof of the influence of solvent polarity to extract the bioactive compound in *E. acoroides* crude extract [17].

#### 3.2. Phytochemical analysis

**3.2.1. Primary metabolites.** The result of phytochemical analysis provided pharmacological activities as well as information as pathological discovery of novel drugs [7]. The information data are shown in table 2 and table 3.

**Table 2.** Phytochemical result of primary metabolites from *E. acoroides*

Primary Metabolites	Result	
	CE	ME
Carbohydrates	+	+
Proteins	+	+

(+) : detected      CE : Crude extract  
 (-) : undetected      ME : Methanol extract

### 3.2.2. Secondary metabolites

Secondary metabolites play importance role in therapeutics agent. These phytochemicals have the ability to produce physiological changes on the human body. The phytochemicals have various complex substances. Qualitative phytochemical analysis of these materials confirms the presence of various phytochemicals. The present preliminary phytochemical analysis may form the basis for drug development in the future using the whole plant or parts of *E. acoroides*. The phytochemical screening results of *E. acoroides* is presented in table 3.

**Table 3.** Phytochemical result of secondary metabolites from *E. acoroides*

Secondary Metabolites		Result	
		CE	ME
Alkaloids	Mayer	-	-
	Dragendorff	+	+
Flavonoids		-	-
Phenols		+	+
Tannins		+	+
Quinones		-	-
Saponins		+	-
Sterols		-	-
Terpenes		+	+

(+) : detected      CE : Crude extract  
 (-) : undetected      ME : Methanol extract

Based on result, the indications of secondary metabolites were present in qualitative analysis from crude extracts and methanol extract of *E. acoroides*. In crude extracts and methanol extracts, the alkaloids, phenols, tannins, saponins, terpenes were found to be present. Meanwhile, the flavonoid, quinones, sterols compounds were present in very low amounts or absent in the crude extracts and methanol extract, as seen in table 3.

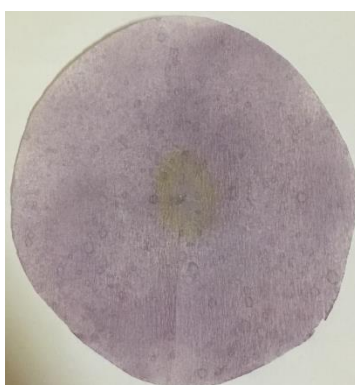
Due to the positive result of alkaloid identification, the white aggregates formed in the sample because of the complex reaction between Mayer reagent and sample. One pair of free electrons from nitrogen in the alkaloid group will bind to heavy metals ion and turn to aggregate. The confirmation test using ethanol is needed to exhibit false result in alkaloid detection. The confirmation test using KOH filter paper under UV<sub>365nm</sub> light is highly recommend to exhibit false detection between coumarine and alkaloid detection.

The positive result of tannin detection using gelatine is white aggregate, whether the Steasny reagent is given a negative result. The recent study proves that the hydrolysis of tannin content in crude extracts and extract of *E. acoroides*. The saponin group was only detected in crude extracts, while the extract of *E. acoroides* was undetected. According to terpenes detection, a positive result is given by a reddish to purple colour with Vanillin Sulphate reagent. The mechanism of reagent is damaging chromophor a functional group in visual color.

The specific reagent used for identified sterols using Liebermann Burchard, a mixture of anhydride acetic acid and sulphate acid, as oxidation agent [22]. Detection of crude extracts and methanol extracts of *E. acoroides* using Liebermann Burchard gave negative result.

### 3.3. Qualitatively antioxidant screening

Dynamolysis profile of extract reflects the presence of antioxidant activity in *E. acoroides*. The qualitatively antioxidant screening provides guidance to further research to quantify the activity. The specific spray reagent for identified the antioxidant activity is the mixture of 0.02 % DPPH in methanol [8]. The yellow area in the purple background is an evidence of the activity of antioxidant, as shown in figure 2.



**Figure 2.** Dynamolysis Profile of *E. acoroides* extract.

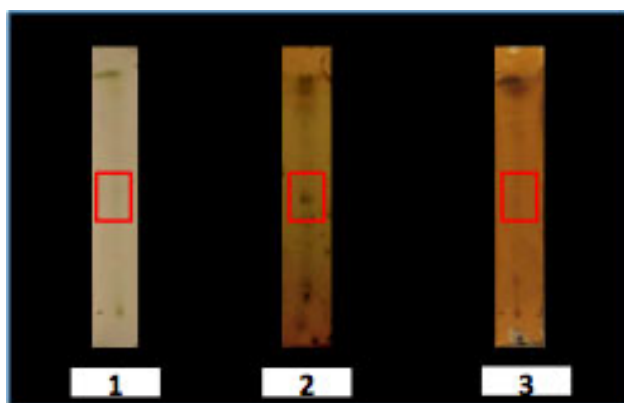
Considering the relationship between antioxidant activity and secondary metabolites: phenols, flavonoids and terpenes are present in the phytochemical analysis of *E. acoroides* extract. The guidance to analyse the secondary metabolites were TLC techniques using specific reagents for each secondary metabolites, such as  $\text{H}_2\text{SO}_4$ ,  $\text{FeCl}_3$ ,  $\text{AlCl}_3$ , citroborate, Liebermann-Bouchard, vanillin sulphate.

### 3.4. Thin layer chromatography profile [6,7]

Based on phytochemical screening and qualitatively analysis of antioxidant, TLC profile was a technique to findings secondary metabolites in *E. acoroides* extract. The result of chromatogram based on spots, fluorescence and  $R_f$  value.

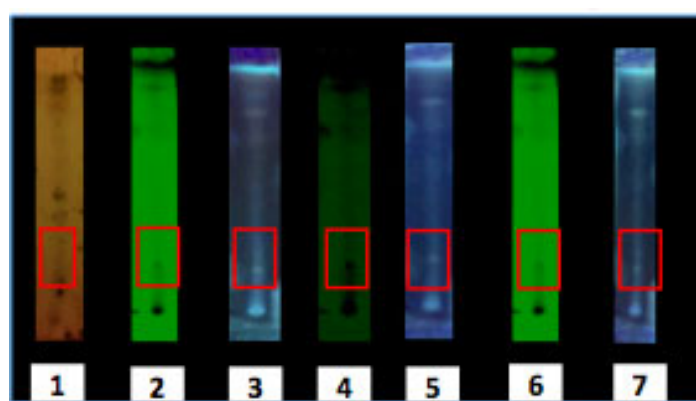
**3.4.1. TLC profile of phenols.** TLC of *E. acoroides* extract used the mixture of solvent phase system to separate many spots. The solvent system contain Ethyl acetate: Methanol: Water (5.8: 8: 0.2). One spot is obtained with  $R_f$  value = 0.5 that gives a positive result when reacting with specific agent spray  $\text{FeCl}_3$  5%. The spot changed the color into blue black without heating. That is the valid proof of phenols contained in *E. acoroides* extract, shown in figure 3.

The presences of phenols were identified after spraying with  $\text{H}_2\text{SO}_4$  reagent. Acid sulphate is an organic reagent; initiate the oxidation reaction and degrade the double bond in organic compound and gave brown colour in TLC plate [16]. However, the visual observation of dark blue spot in TLC plate after spraying with  $\text{FeCl}_3$  spray reagent, clearly proves that the hydrolysis reaction was completed between Lewis acid ( $\text{FeCl}_3$  reagent) and phenols from *E. acoroides* extract [17].



**Figure 3.** TLC Profile of *E. acoroides* extract with specific spray reagent of phenols, (1) visual ( $R_f=0.50$ ), (2) after spraying with  $H_2SO_4$  reagent ( $R_f=0.50$ ), (3) after spraying with  $FeCl_3$  reagent ( $R_f=0.50$ ).

**3.4.2. TLC profile of flavonoid.** Flavonoid was not identified in the phytochemical analysis, but TLC of *E. acoroides* extracts with the mixture solvent system as a mobile phase had separate many spots. The solvent system contains Ethyl acetate: Methanol: Water (5.8:8:0.2) was the best system for the detection the TLC profile. One spot is obtained having  $R_f$  value = 0.25 that gave a positive result when reacting with specific agent such an acid sulphate, Citroborate, and Aluminum chloride. The fluorescent of spot from visual,  $UV_{254}$  and  $UV_{356}$  are different, but have correlation. The valid proof of flavonoid contain in *E. acoroides* extracts, were shown in figure 4.

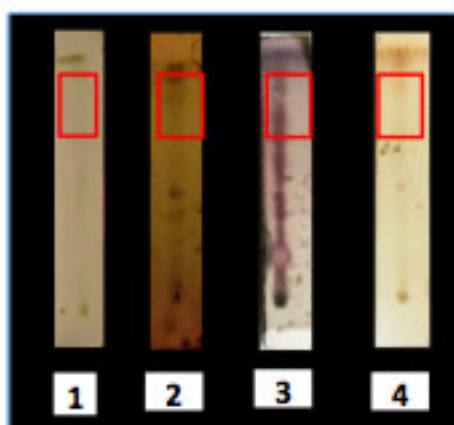


**Figure 4.** TLC Profile of *E. acoroides* extract with specific spray reagent of flavonoids, (1) visual ( $R_f=0.25$ ), (2) after spraying with citroborate reagent,  $UV_{254nm}$  ( $R_f=0.25$ ), (3) after spraying with citroborate reagent,  $UV_{365nm}$  ( $R_f=0.25$ ), (4) after spraying with  $AlCl_3$  reagent,  $UV_{254nm}$  ( $R_f=0.25$ ), (5) after spraying with  $AlCl_3$  reagent,  $UV_{365nm}$  visual, (6)  $NH_3$  ( $R_f=0.50$ ),  $UV_{254nm}$ ,  $NH_3$  ( $R_f=0.25$ ), (7)  $NH_3$  ( $R_f=0.50$ ),  $UV_{254 nm}$

The blue fluorescent of yellow spots after spraying with  $AlCl_3$  ( $R_f$  0.25) in  $UV_{365nm}$  had identified flavonoid content in the extract of *E. arocooides*. The specific spray reagent  $AlCl_3$  builds a complex between hydroxyl and cheton from extract.

**3.4.3. TLC profile of terpenes.** TLC of *E. acoroides* extract use the mixture of solvent phase system to separate many spots. The solvent system contains Ethyl acetate: Methanol: Water (5.8:8:0.2). One spot is obtained having a  $R_f$  value = 0.80 that gives a positive result when reacting with a specific agent such as sulphate acid, vanillin sulphate, and Liebermann Bourchard. The visual color of spots from specific spray reagent is different, but obtained positive results. The valid proof of terpenes contains in *E. acoroides* extract, shown in figure 5.





**Figure 5.** TLC Profile of *E. acoroides* extract with specific spray reagent of terpenes, (1) visual ( $R_f=0.25$ ), (2) after spraying with  $H_2SO_4$  reagent, ( $R_f=0.80$ ), (3) after spraying with vanillin sulphate reagent, ( $R_f=0.80$ ), (4) after spraying with Liebermann Bourchard reagent, ( $R_f=0.80$ ).

#### 4. Conclusion

The extract of *E. acoroides* containing a rich source of primary and secondary metabolites. The phytochemical screening has identified primary metabolites such as carbohydrates and proteins. The phytochemical screening also detected secondary metabolites such as alkaloids, phenolics, tannins, saponins, flavonoids, monoterpenes, and sesquiterpenes. The TLC profile with specific spray reagent has confirmed the bioactive component such as phenolics, flavonoids, and terpenes in the methanol extract of *E. acoroides*, that is responsible for antioxidant activity. Thus, *E. acoroides* could be a potential source of natural antioxidant from marine ecosystem.

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