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# Analysis of Flavonoid Levels of *Enhalus acoroides* in Different Coastal Waters in Ambon Island, Indonesia

**Prelly M.J. Tuapattinaya [&] Dominggus Rumahlatu**

Study Program of Biology Education, Faculty of Teacher Training and Education, Pattimura University, Ambon, Indonesia

## Abstract

Seagrass contains bioactive compounds or secondary metabolites which can be used as potential drugs. This research aims at analyzing the levels of flavonoids of the leaves of seagrass *Enhalus acoroides* in three different coastal waters. The sampling of seagrass leaves was carried out in three locations in the coastal waters of Ambon Island, namely the coastal waters of Galala, Rutong, and Waai. The levels of flavonoid were identified using microscopic-microchemical methods. The test results of the flavonoid levels were analyzed descriptively. The results of this research showed that samples of seagrass leaves from the coastal waters of Galala, Rutong, and Waai, after added with NaOH, $AlCl_3$, and $NH_3$ reagents showed a color change. It means that the samples from the three coastal waters were positive to contain flavonoids. Therefore, the analysis was continued to determine the average levels of flavonoids of *E. acoroides*. The average levels of flavonoid in the three coastal waters of Galala, Rutong, and Waai were 0.0192%, 0.1475%, and 3.5697% respectively. The environmental conditions and substrate of seagrass *E. acoroides* in the coastal waters of Rutong and Waai caused higher levels of flavonoids than the levels of flavonoids in the coastal waters of Galala.

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## Introduction

Seagrass is classified into angiosperms plants which grow in marine environment (Borum and Greve, 2004; Subhashini et al., 2013; Immaculate et al., 2018 ) The diversity of seagrass species in the world reaches 70 species, 12 of which are found in Indonesian waters, including *Cymodocea serrulata*, *C. rotundata*, *Enhalus acoroides*, *Halodule uninervis*, *H. pinifolia*, *Halophilia minor*, *H. ovalis*, *H. decipiens*, *H. spinulosa*, *Thalassia hemprichii*, *Syringodium isoetifolium* and *Thalassodendron ciliatum* spreading in Java, Sumatra, Bali, Kalimantan, Sulawesi, Maluku, East Nusa Tenggara and Papua (Tuahatu et al., 2016; Subhashini et al., 2013; Tuhumury, 2008; Nontji, 2005 ) On the waters of Kumbang Island, Karimunjawa Islands are found 6 species of seagrass, namely *T. hemprichii, C. rotundata, C. serrulata, H pinifolia, H. uninervis and H. ovali* (Hartati et al., 2012). In Maluku waters, 10 seagrass species were found, *namely C. rotundata, C. serrulata, H. pinifolia, H. uninervis, S.*

*isoetifolium, T. ciliatum, E. acoroides, H. minor, H. ovalis, and T. hemprichii* and spread on the Ambon Island Seram Island, Aru Islands, Buru Island and Saparua Island (Irawan, 2017).

Ecologically, seagrass is different from terrestrial plants including the compounds they contain. Different environments cause biochemical adaptations by seagrasses to produce certain compounds called bioactive compounds or secondary metabolites (Owolabi et al., 2018; Subhashini et al., 2013; Mansson et al., 2011). The research conducted by Santoso et al. (2012) found secondary metabolites of saponins, triterpene and sterols in 80% ethanol extract in 7 seagrass species. Mani et al. (2012), reported that secondary metabolites was found in methanol extract of seagrass *Syringodium isoetifolium,* such as saponins, phenols and alkaloids.

Many secondary metabolites from seagrass have been known to be biologically active and important biomedicine and can be used as potential drugs. The roots of *E. acoroides* are used as medicine for stings of various types of stingrays and scorpions. *Halophila sp*. is a powerful drug against malaria, skin diseases, and it is found to be very effective in the early stages of leprosy (Mani et al., 2012). Riniatsih and Setyati (2009) utilized simplicia extracts and powder of *E. acoroides* and *T. hemprichii* as agents to control vibrio bacteria. In addition, Kannan et al. (2010a) reported that there were antimicrobial activities from the extracts of seagrass *H. stipulacea, C. serrulata,* and *H. pinifolia.* One of the secondary metabolites found in all parts of seagrass plants is flavonoids (Baby et al., 2017; Zidorn, 2016; Subhashini et al., 2013). Baby et al. (2017) and Gustavina et al. (2018) confirmed that in addition to flavonoids, *Enhalus acoroides* contains the compounds of triterpenoid, steroids, saponins, tannins. Qi et al. (2008) explains that *Enhalus acoroides* contains 11 pure compounds classified as flavonoids and steroids. *Enhalus acoroides* is also reported to contain phenolic bioactive compounds which tend to be potential antioxidants (Kannan et al., 2010b).

Based on the survey observed on the coastal waters of Ambon islands, namely on the Rutong, Waai, and Galala beaches, it was found that *Enhalus acoroides* was used as vegetables and herbal medicines by the community. Moreover, it is also known that seagrass *Enhalus acoroides* on the Rutong and Waai coastal waters can grow well. This is because the coastal waters in Rutong and Waai are still natural and have not been polluted with harmful substances or metals. On the other hand, the number of seagrass *Enhalus acoroides* in the coastal waters of Galala is relatively small, because the waters have been polluted by factory waste and household waste from the community (Rijal et al., 2014).

## Materials and Methods

### The collection of *Enhalus acoroides* samples and measurement of waters condition factors

The sample collection of the leaf of seagrass *E. acoroides* and the measurement of water condition factors (temperature, salinity, and substrate) were simultaneously carried out in three locations in the coastal waters of Ambon island, namely Galala, Rutong, and Waai coastal waters (Figure 1). The identification and analysis of the flavonoids levels of the leaves of seagrass *E. acoroides* were carried out at the Laboratorium Kimia Dasar (Basic Chemistry Laboratory), Faculty of Mathematics and Natural Sciences (FMIPA), Pattimura University. The seagrass leave samples were intact leaves (not damaged nor defective).

### The extraction process of the leaves of *Enhalus acoroides*

The leaf samples of *E. acoroides* were cleaned from sand and salt impurities attached to the leaves using distilled water. The seagrass leaves were then dried and mashed. 70 g powder of leaf samples of *E. acoroides* was extracted using 400 mL of methanol 90%. The extraction process (maceration) lasted for 24 hours, which was then evaporated using vacuum rotary evaporator at a temperature of 55°C to produce a *viscous* extract, then followed by drying process in the desiccator until the extract in the form of paste was obtained.

### The Identification of Flavonoid Levels

The identification of flavonoid levels was qualitatively carried out using microscopic-microchemical methods (Mulyani and Laksana, 2011). Methanol extract of *E. acoroides* was inserted into the test tube and then identified with the addition of reagents and without reagents. The identification without reagent was carried out by adding 5 drops of aquedes to the test tube, while the identification process with reagents was carried out by adding several reagents, namely $NH_3$ 25%, NaOH 40%, and $AlCl_3$ 5% (5 drops each), into the tube. The use of reagents in this study with concentration $NH_3$ 25%, NaOH 40%, and $AlCl_3$ 5% based on the reason that these reagents can provide color when seagrass leaf extract contains flavonoids. According to Mabry et al. (1970) in Chang et al. (2002) that the principle of the calorimetry method is that reactants will form stable complexes with hydroxyl groups from flavones and flavonols.

Color changes in extracts were observed in each treatment (with and without reagents). The color before and after the reagents were given was compared, and the color changes obtained were observed. The results of the flavonoid level test were analyzed descriptively. The descriptive analysis was used to compare the results of flavonoid levels based on the three coastal waters, namely the coastal waters of Galala, Rutong, and Waai.

The quantification of flavonoid levels of the sample *E. acoroides* was carried out by using spectrophotometric method with quercetin as standard. The standard curve was obtained by measuring the absorbance of the standard quercetin solution at concentrations of 5, 10, 15, 20, 25 ppm. The maximum wave length determined for absorbance was 438 nm.

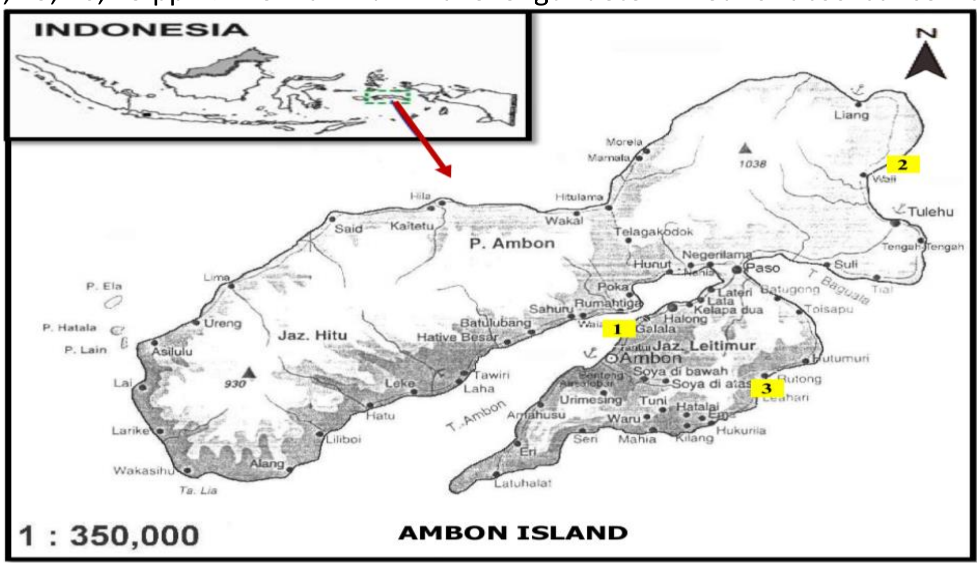


**Figure 1. Location for collecting samples of *Enhalus acoroides***

## Results and Discussion

### The Results of Flavonoids Identification of the leaves of Seagrass *Enhalus acoroides*

The results of the identification of flavonoid content in the leaves of seagrass *E. acoroides* in the coastal waters of Galala, Rutong, and Waai (Table 1) showed that the extract test with aquades reagents on the three coastal water locations did not show any color changes. While the extract test with the three other types of reagents namely NaOH, $AlCl_3$, and $NH_3$ indicated color changes.

**Table 1. Results of identification of flavonoids in the leaves of seagrass *Enhalus Enhalus***

| Sample | Reagent | | | |
|---|---|---|---|---|
| | Aquades | NaOH 40% | $AlCl_3$ 5% | $NH_3$ 25% |
| Leaves of *E. acoroides* of Galela coastal waters | - | + | + | + |
| Leaves of *E. acoroides* of Rutong coastal waters | - | + | + | + |
| Leaves of *E. acoroides* of Waai coastal waters | - | + | + | + |

Description:
(-) = no change in color after given reagent, does not contain flavonoids
(+) = there is a change in color after given reagent, containing flavonoids

The reaction on extract of the leaves of seagrass *E. acoroides* added with NaOH, $AlCl_3$ and $NH_3$ reagents produced green changes to yellow on each test tube. Thus, it could be concluded that they positively contained flavonoids. The color change in the flavonoids color test results with various reagents was caused by the effect of reactant concentration (the level of polarity of reagents / solvents) and surface area, where the concentration and surface area are related to the frequency of collisions. The greater the concentration, the greater the possibility of particles colliding with each other, so that the reaction increases faster. Similarly, the wider the surface area (the size of fine pieces), the more the collision, and the faster the reaction rate.

Color changes that occured in the reagent treatment indicated that the samples positively contained flavonoids. The indication of the color change that occurred in this test was caused by the reaction between reagent ($AlCl_3$) and the carbonyl and hydroxyl groups on flavones and flavonols which produced yellow to brownish color, while ammonia and NaOH with flavonoids would form a red quinoid compound (Mulyani and Laksana, 2011; Popova et al., 2004; Robinson, 1995). The presence of flavonoids in the test material can also be visualized by adding concentrated Mg and HCl powder into the alcohol extract. It will become orange to red when it contains flavones, red to dark red (flavanols), dark red to magenta (flavonone) (Fannsworth, 1966).

Flavonoids detected from leaf samples of *E. acoroides* are very important as antioxidant compounds. This is in accordance with the statement by Redha (2010); Santoso et al. (2012); Subhashini et al. (2013), that flavonoids as a group of phenolic compounds are mostly found in plant tissues both roots, stems, and leaves, and they can act as antioxidants. In addition, the extract of *E. acoroides* contains bioactive compounds from the triterpenoids, steroids, saponins, tannins types (Dewi et al., 2012; Baby et al., 2017; Gustavina et al., 2018).

## Flavonoid Levels of the seagrass leaf *Enhalus Acoroides*

The flavonoid levels of the seagrass leaf *Enhalus acoroides* (quantity) were determined by using a spectrophotometric method with quercetin as standard. The data on the standard solutions (quercetin) and the absorbance are presented in Table 2.

**Table 2. Absorbance of standard quercetin solutions**

| No | Concentration (ppm) | Absorbance (nm) |
|---|---|---|
| 1 | 5 | 0.207 |
| 2 | 10 | 0.396 |
| 3 | 15 | 0.613 |
| 4 | 20 | 0.811 |
| 5 | 25 | 1.091 |

After the value of the absorbance and the standard solution was obtained, the data were visualized in the form of a graph of the quercetin standard solution concentration and the absorbance (Figure 2).

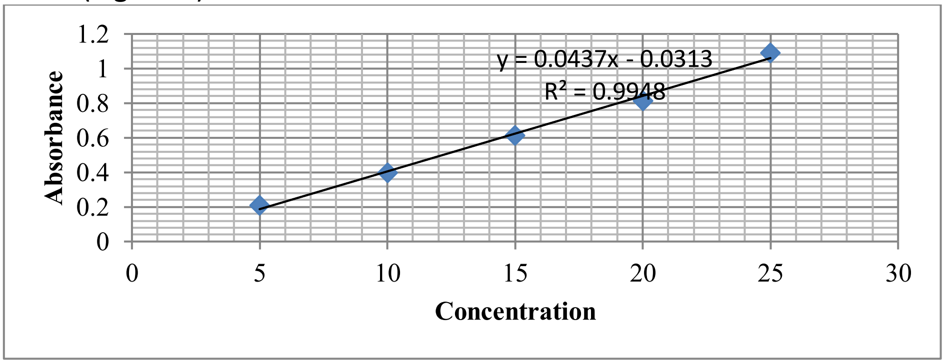


**Figure 2. Quercetin standard curve; standard concentration on standard absorbance**

The graph of the quercetin standard curve (Figure 2) shows a linear correlation between absorbance and concentration. This shows that the greater the concentration, the greater the absorbance value. After that, the results of the flavonoid level analysis of the seagrass leaf *Enhalus Enhalus* in coastal waters of Galala, Rutong and Waai were calculated based on the linearity correlation on the standard curve and are presented in Table 3.

**Table 3. The results of the flavonoid level analysis of the seagrass leaf *Enhalus enhalus***

| Sample Code | Repetition | Sampel Weight (mg) | Flavonoid Weight (mg) | Flavonoid Levels (%) | Average Levels of Flavonoid (%) |
|---|---|---|---|---|---|
| | 1 | 70.0312 | 36.25 | 0.0192 | |
| Galala | 2 | 70.0617 | 38.75 | 0.0193 | 0.0192 |
| | 3 | 70.0615 | 35.75 | 0.0191 | |
| | 1 | 70.3821 | 40.00 | 0.0198 | |
| Rutong | 2 | 70.5984 | 47.50 | 0.2116 | 0.1475 |
| | 3 | 70.5985 | 41.50 | 0.2110 | |
| | 1 | 10.0516 | 352.5 | 3.5069 | |
| Waai | 2 | 10.0417 | 360.0 | 3.5850 | 3.5697 |
| | 3 | 10.0516 | 352.5 | 3.5069 | |

The results of the highest average levels of flavonoids of seagrass leaf *E. acoroides* were at the coastal waters of Waai, which was 3.5697, while the lowest average levels of flavonoid were at the coastal waters of Galala, which was 0.0192%. This occured due to the differences in the substrate that became the growing place of seagrass on the three beaches. The substrate in the Waai and Rutong beaches was in the form of sandy mud, while the substrate in the Galala beach was coarse sand and household waste from the community that affected the growth rate of seagrass *E. acoroides*. This is in accordance with the research results by Rahman et al. (2016) that the water conditions which has the most significant effect on the growth rarte of seagrass leaf were sandy mud substrates. Leaves with a good growth rate will affect the compounds contained in the leaves.

Flavonoids have varying forms and are in free form (aglycone). They are also bound as glycosides and generally have bonds with sugar groups, so that flavonoids are easily soluble in polar solvents or water (Corradini et al., 2011). The presence of flavonoid chemical compounds in the extract of seagrass leaf *E. acoroides* in this research shows that seagrass *E. acoroides* has the potential to be a natural chemical antifouling, antibacterial, antifungal, and other pharmaceutical raw materials (Dewi et al., 2018).

**Table 4. Results of the measurements of water condition factors**

| Sampel Code | Repetition | Water Condition Factors | | |
|---|---|---|---|---|
| | | Temperature ($^0$C) | Salinity (‰) | Substrate |
| Galala | 1 | 28.29 | 28 | Coarse Sand |
| | 2 | 28.31 | 28 | Coarse Sand |
| | 3 | 28.30 | 28 | Coarse Sand |
| Average | | 28.30 | 28 | |
| Rutong | 1 | 29.67 | 30 | Muddy Sand |
| | 2 | 29.67 | 30 | Muddy Sand |
| | 3 | 29.66 | 30 | Muddy Sand |
| Average | | 29.67 | 30 | |
| Waai | 1 | 29.66 | 29 | Muddy sand |
| | 2 | 29.67 | 29 | Muddy sand |
| | 3 | 29.67 | 29 | Muddy sand |
| Average | | 29.67 | 29 | |

The results of the measurements of the physical condition of the coastal waters (Table 4) of Galala, Rutong and Waai covering temperature and salinity show an average range of 28.30$^o$C-29.67$^o$C and 28-30‰. It is a condition that seagrass *E. acoroides* can tolerate for life. According to Erftemeijer and Middelburg (1993), *E. acoroides* lives at a temperature of 26.5$^o$C-32$^0$C in shallow waters and can even tolerate temperatures up to 38$^0$C during low tide during the day. Hutomo (1999) revealed that the optimum range of tolerance to sea water salinity was 35‰.

The results of this research (Table 3) show that the highest levels of flavonoids of the seagrass leaf *E. acoroides* were in Waai coastal waters, which was 3.5697%, while the lowest levels of flavonoids of the seagrass leaf were at Galala coastal waters of 0.0193%. Differences in flavonoid levels in plants can be caused by differences in environmental conditions where these plants grow, temperature, ultraviolet light, nutrient availability, water and $CO_2$ levels in the atmosphere (Rohaeti et al., 2011).

The results of measurements of water condition factors (Table 4) show that the environmental conditions where seagrass *E. acoroides* grows in Rutong and Waai villages are almost the same. The environmental conditions are muddy sand substrate, and the water has not been polluted by human activities. However, the environmental conditions in the Galala village are different, coarse sand substrate with a mixture of waste and the water conditions with high human activities, namely the activities of boats and factories operating around the waters, waste from ships, factories and communities.

Rumahlatu et al. (2018) reported that the levels of heavy metal Cd in the coastal waters of galala village ranged from 0.35-1.15 ppm for sediments and 0.10-0.35 ppm for water, while the levels of heavy metal Pb in the coastal waters of Galala village ranged from 1.05-1.27 ppm for sediment and 0.01 ppm for water. In addition, Rizal (2010) reported that the highest levels of bioaccumulation of lead (Pb) and cadmium (Cd) were found in seagrass leaves. Alkaline Pb and Cd have an effect on the flavonoid levels. This is due to the chemical nature of flavonoids which are acidic, so they can dissolve in bases (Singh *et al*., 2003). The high content of Pb and Cd found in the leaves of seagrass *Enhalus acoroides* causes the flavonoids content to be dissolved in bases thereby reducing flavonoid levels in the leaves of seagrass *Enhalus acoroides* which grow in the coastal waters of Galala village.

The environmental conditions and substrate of seagrass *Enhalus acoroides* in the coastal waters of Rutong and Waai caused flavonoid levels to be higher than those in the coastal waters of Galala. Muddy sand substrate in both waters is a suitable substrate for the growth of seagrass *Enhalus acoroides.* Erftemeijer and Middelburg (1993) explain that the rates of the leaf growth and the production of seagrass *Enhalus acoroides* are higher on muddy sand substrates (*terigenous sediments*) than on other types of substrate, because muddy sand substrates generally have higher N and P nutrient availability.

Even though the conditions of the water and the growing substrate of seagrass *Enhalus acoroides* in the coastal waters of Rutong and Waai were almost the same, the results of flavonoid level teasting on the leaves of seagrass *Enhalus acoroides* from the coastal waters of Waai were higher than that in the coastal waters of Rutong. This is influenced by the availability of nutrition in the two waters. The research conducted by Soedradjad & Syamsunihar (2014) reported that the phenolic and flavonoids content of plants increase along with the increasing doses of organic fertilizer in the growing media. High fertilizer dosages will support the availability and absorption of more nitrogen elements, so that the phenolic and flavonoids content of plants can increase.

The important elements needed by seagrass are nitrogen (N), phosphate (P) and organic C. Nitrate ($NO_3$) is the main form of nitrogen in natural waters and is the main nutrient in seagrass ecosystems (Kilminster et al., 2006; Effendi, 2003). Amri et al. (2011) affirm that the nutrient composition of N, P, and S of a waters is strongly influenced by the anthropogenic activity. According to Philips and Menez in Badria (2007), seagrass growth comes from recycling nitrogen in sediments and water columns. Dead Rizoma and seagrass roots add nitrate in the sendimen. The differences in the flavonoid levels in the coastal waters of Waai and Rutong were due to the differences in nitrogen levels.

## Conclusions

*Enhalus acoroides* in the coastal waters of Galala, Rutong, and Waai contain flavonoids. The average flavonoid levels in the three coastal waters were 0.0192%, 0.1475%, and 3.5697% respectively. In addition, the levels of flavonoids in the *Enhalus acoroides* in the research location were influenced by the differences in environmental conditions and water substrate. The average levels of condition factors of temperature and salinity were in the range of 28.30$^o$C-29.67$^o$C dan 28-30‰ with the coarse sand substrate conditions in the coastal waters of Galala, while muddy sand substrate condition in the coastal waters of Rutong and Waai. The differences in environmental conditions and substrate conditions for the life of *Enhalus acoroides* in the coastal waters of Rutong and Waai cause the flavonoid levels to be higher than the flavonoid levels in the coastal waters of Galala.

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