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ANTIBACTERIAL ACTIVITY OF SEAGRASS EXTRACT *Enhalus acoroides* WITH DIFFERENT SOLVENTS AGAINST *Streptococcus mutans* BACTERIA CAUSE DENTAL CARIES

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ABSTRACT

Treatment of *Streptococcus mutans* infection is generally done using antibiotics or chemical mouthwash, but long-term use can cause bacterial resistance and side effects for health. Therefore, it is important to find alternative treatments that are safer and more effective, one of which is through the use of medicinal plants. One plant that has potential as a source of antibacterial compounds is seagrass (*Enhalus acoroides*), which has been recognized in various studies to contain bioactive compounds such as flavonoids, saponins, and tannins, which can function as antibacterial agents. The purpose of this study was to determine the antibacterial activity of *E. acoides* extracts extracted with different solvents against *S. mutans* bacteria. The design used in this study is a completely randomised design (CRD) model. Testing the inhibition zone of *E. acoroides* using piper disc diffusion method with different concentrations of 25%, 50%, 75% and 100% with three replicates. Inhibitory zone diameter data were analyzed used ANOVA significant test and differences between treatments, and test of Real Honest Differences (BNJ) was conducted. Methanol and ethyl acetate extracts of *E. acoroides* can inhibit the growth of *S. mutans* bacteria that cause dental caries, and ethyl acetate has a stronger effect than methanol at higher concentrations.

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INTRODUCTION

Dental caries or cavities are localized dental diseases that damage the hard tissue of the teeth which occur due to the interaction of several factors, namely the host (teeth), bacteria, substrate (diet), and time (Novita, 2016; Makatambah *et al*., 2020). According to Annisa & Mursyid (2020), caries is caused by neglect of oral hygiene resulting in plaque buildup. Plaque is a thin layer that adheres tightly to the surface of the teeth and contains a collection of bacteria (Kolenbrander *et al*., 2006). The beginning of dental caries is a demineralization process that dissolves the tooth surface by acids produced by bacteria that are attached to the tooth surface (Strużycka, 2014; Yadav, K., & Prakash, 2017). *Streptococcus mutans* is a relatively large number of dental caries bacteria, as a stable extracellular polysaccharide former, has the ability to colonize at a relatively low acidity level (pH) of the tooth surface so that it plays a major role in the formation of dental caries (Gao *et al*., 2016; Zhang *et al*., 2022). *S. mutans* is able to produce the enzyme glucosyl transferase which will break down sucrose and glucose in plaque into glucan (Chatterjee *et al*., 2022). Furthermore, *S. mutans* predominantly forms water-soluble dextran chains that have the ability to adhere to the tooth surface so as to form colonies on the tooth surface. Sucrose metabolism by *S. mutans* produces lactic acid which can cause demineralization of hydroxyapatite (Qiu *et al*., 2020).

The use of mouthwash containing chemicals can help maintain dental and oral health (Oktanauli *et al*., 2017). However, continuous use in the long term can change the balance of flora in the mouth, cause stains on teeth, swelling of the parotid glands and other effects and can trigger cancer, so a new strategy is needed to maintain and maintain safer dental and oral health (Sayekti *et al*., 2022). Indonesia as an archipelagic country has great potential from natural resources spread both in the sea and on land. These abundant natural resources have the potential to improve the welfare of its people (Marewa & Parinussa, 2020). According to the Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia (2019), Indonesia has various enormous marine potentials. With an area of waters covering two-thirds of the country's total area, which is around 5.8 million km², Indonesia also has more than 17,000 islands and a coastline of 81,000 km which holds various extraordinary natural resources. Maluku Islands region is dominated by vast and deep oceans, with an administrative area of around 712,480 km^2 consisting of sea waters covering an area of 658,295 km^2 (92%) and land covering an area of 54,185 km^2 (8%) (Purwanto and Mardiani, 2021). This geographical condition gives Maluku the potential for a very diverse and abundant diversity of marine biological resources, thanks to the rich aquatic environment and supportive ecosystem (Kembauw *et al*., 2015; Ukratalo *et al*., 2023). These marine biological resources have an important role as the potential owned by the region to support the needs and sustainability of the ecosystem in the waters (Harahab *et al*., 2021). Among the various marine biological resources available, seagrass plants are one of the important components that can be found in Indonesia (Saputro *et al*., 2018).

Seagrass is a higher plant that grows in shallow sea waters on sandy, muddy, and gravel substrates (Imran *et al*., 2024). In general, seagrass can thrive in coastal and open tidal waters with substrates in the form of sandy mud, gravel, and dead coral debris (Zahra *et al*., 2024). According to Leslida *et al*., (2024), seagrass is very diverse, numbering around 60 species known to the world. In Indonesian waters there are 15 species, but the most frequently encountered are 12 species. One type of seagrass that is easy to find is *Enhalus acoroides*. Seagrass *E. acoroides* is an aquatic plant that is very easy to find on every coastal island (Latar *et al*., 2024). This type of *E. acoroides* has a distinctive morphology and large size compared to other types of seagrass (Irawan and Matuankotta, 2015). The substrate determines the ability of seagrass to grow. Generally, this type of seagrass grows on muddy soil to rocky substrates and differences in substrate characteristics can affect the development and distribution of seagrass (Sahertian and Wakano, 2017). Seagrass contains active compounds so it has the potential to be developed as a drug candidate. These compounds are known as bioactive compounds (Mardiyanti *et al*., 2024). The bioactive compound content of seagrass vegetation varies according to the location where this vegetation grows. Several previous research results have shown that the bioactive compound content of seagrass *E. acoroides* from the waters of Sapeken Island-Madura is tannin, alkaloid compounds (Badriyah *et al*., 2023).

Other compounds contained in seagrass *E. acoroides* from the waters of Sapeken Island-Madura are flavonoids, triterpenoids, saponins (Ningrum *et al*., 2023), while seagrass *E. acoroides* from Sepanjang Beach-Yogyakarta contains tannins, saponins, triterpenoids, flavonoids, and steroids (Permana *et al*., 2020). The bioactive compounds contained in seagrass *E. acoroides* have great potential to be developed as antibacterials, anticancer, and antioxidants. In addition to location factors, the type of solvent used in the extraction of bioactive compounds from seagrass also plays a major role in the resulting chemical content. Each solvent has a different ability to dissolve certain compounds, so choosing the right solvent can increase extraction efficiency and produce bioactive compounds with higher concentrations (Atun, 2014). For example, polar solvents such as water or methanol are more effective in extracting polar compounds, such as flavonoids and saponins (Chaves

et al., 2020), while non-polar solvents such as chloroform or ether are more suitable for extracting lipophilic compounds, such as triterpenoids and steroids (Lefebvre *et al*., 2021).

MATERIALS AND METHOD

Tools and materials

The tools used are shaker, autoclave, petri dish, erlenmeyer, measuring cup, incubator, vernier caliper, spirit lamp, laminar air flow (LAF), refrigerator, Ose needle, oven, tweezers, 10 ml syringe, test tube, analytical balance, rotary evaporator, aluminum foil, micro pipette, micro pipette tip, vial, refractometer, pH meter, DO meter and thermometer. While the materials used are *E. acoroides*, methanol, ethyl acetate, Mg, nutrient agar (NA), chloramphenicol, DMSO, aquades, paper disks, and bacterial isolates *S. mutans*. This research was conducted in an experimental laboratory manner. The measurement result data will be analyzed using one-way Analysis of Variance (ANOVA). If there is a significant effect, it will be continued with the Honestly Significant Difference (HSD) test with a 95% confidence interval. This study used a Completely Randomized Design (CRD) consisting of four treatments and three repetitions. The treatments studied were differences in the composition of *E. acoroides* seagrass leaf extract; P1 25 mg/ml, P2 50 mg/ml, P3 75 mg/ml and P4 100 mg/ml.

Procedures

Sampling of *E. acoroides* on Suli village Maluku of Indoensia. The seagrass samples of *E. acoroides* obtained were then take to the laboratory to be washed and cut into small pieces after which they were air-dried in the laboratory room. After drying, the samples were ground with a blender to obtain powder. Total of 50 g of *E. acoroides* powder was put into a 100 mL Erlenmeyer flask. Then 50 mL of methanol solvent was added. Shaking was carried out with a shaker for 3 hours at a speed of 120 rpm (rotation per minute) and macerated for 24 hours. Then the maceration residue was filtered with a Buchner funnel and re-dissolved using the same solvent until clear. The filtrate obtained was combined in an extract storage container. The above treatment was also repeated for the use of ethyl acetate solvent. The obtained methanol and ethyl acetate extracts were concentrated using a rotary evaporator at a temperature of 30-40 ºC, and N^2 gas was passed through until the solvent evaporated completely.

NA (Nutrient Agar) media is made by taking 2.8 grams dissolved in 100 mL of distilled water in an Erlenmeyer flask then covered with cotton then heated to boiling and put into a test tube aseptically. NA (Nutrient Agar) media is sterilized in an autoclave at a temperature of 121ºC with a pressure of 15 psi for 15 minutes. Bacteria *S. mutans* which comes from pure culture is taken as much as 1 ose then streaked on NA (Nutrient Agar) media aseptically. The tube is brought close to the fire when streaking the bacteria. The tube is then covered with cotton and incubated for 24 hours at 37ºC. DMSO used to dissolve *E. acoroides* extract and was used as a negative control in the inhibition test. Take 3 mL of DMSO into a vial, stir until dissolved and then homogenize. Chloramphenicol is used as a positive control extract of *E. acoroides* in the inhibition test. Weighed as much as 0.375 g of chloramphenicol powder then dissolved in 3 mL of aquades in Erlenmeyer and stirred until dissolved then homogenized, after which the chloramphenicol solution was then put into a vial.

Evaluation of activity antibacterial

Activity antibacterial used agar diffusion method (Agar Disk-Diffusion Assay). The principle of this antibacterial test is the formation of inhibition zone diameter. The concentrations used in *E. acoroides* extract are (25, 50, 75, and 100 mg/mL). The test bacteria used in this antibacterial activity test are *S. mutans*. Paper disks with a diameter of 6 mm were soaked in the resulting *E. acoroides* extract and controls (positive and negative). The paper disks were placed on the surface of the media using sterile tweezers and pressed slightly. Incubated at 37˚C for 24 hours until an inhibition zone appeared. Antibacterial activity test on each solvent was carried out 3 times. The inhibition zone was measured using a caliper to determine bacterial activity.

RESULTS AND DISCUSSION

The method used for antibacterial testing is the diffusion method withpaper diskwhich was repeated three times. This method was chosen because it is effective in providing a clear picture of the ability of antibacterial compounds to inhibit bacterial growth (Aida *et al*., 2021). In the procedure, the treated disc paper is placed on the surface of the bacterial culture medium, allowing the compound to diffuse into the agar. Thus, researchers can observe the inhibition zone formed around the disc, which indicates antibacterial activity. The results of observations of the inhibition zone of methanol and ethyl acetate extracts from *E. acoroides* against bacteria *S. mutans* can be seen in Figure 1.

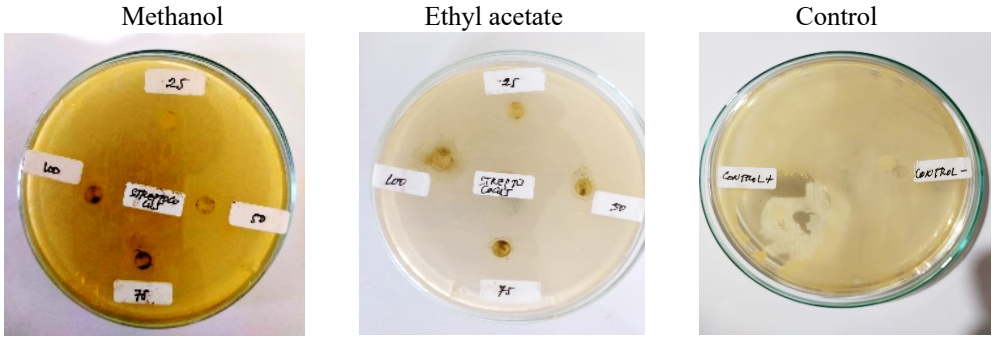

Figure 1. Inhibition Zone Extract *E. acoroides* against bacteria *S. mutans*

The observed inhibition power is indicated by the clear zone around the paper disk that has been given the solvent extract sample. This clear zone is then measured using a caliper. The results of the antibacterial activity test can be seen in Table 1.

Table 1. Antibacterial activity

Test Bacteria	Extract	Concentration mg/mL	Clear Zone Mean (mm)	Criteria
S. mutans	Methanol	25	9.40	Currently
		50	9.53	Currently
		75	10.25	Strong
		100	11.28	Strong
	Ethyl Acetate	25	9.52	Currently
		50	10.00	Currently
		75	10.20	Strong
		100	17.95	Strong

Based on the results of antibacterial activity test measurements in (Table 1) shows that methanol and ethyl acetate extracts have antimicrobial activity against *S. mutans* bacteria at various concentrations. In methanol extract, at concentrations of 25 mg/mL and 50 mg/mL, the clear zones formed were 9.40 mm and 9.53 mm, which are included in the category of moderate antibacterial activity. This shows that although methanol extract provides antibacterial effects, the effect is not too strong at lower concentrations (Table 1). Conversely, at concentrations of 75 mg/mL and 100 mg/mL, the clear zones formed increased to 10.25 mm and 11.28 mm, which are classified as strong, indicating that the higher the concentration of the extract, the stronger the antibacterial activity.

In addition, ethyl acetate extract also showed quite significant antibacterial activity against *S. mutans*. At concentrations of 25 mg/mL and 50 mg/mL, the resulting clear zones were 9.52 mm and 10.00 mm, respectively, which were included in the moderate category (Table 1). Although its activity was lower compared to higher concentrations, ethyl acetate extract still showed potential as an antibacterial agent at these concentrations. The increase in the size of the clear zone indicated that ethyl acetate could also inhibit bacterial growth even at lower concentrations. At higher concentrations, namely 75 mg/mL and 100 mg/mL, ethyl acetate extract showed better results. At a concentration of 75 mg/mL, the clear zone formed reached 10.20 mm, which was still considered strong. However, the most striking thing was that at a concentration of 100 mg/mL, the clear zone formed reached 17.95 mm, indicating that ethyl acetate extract had very strong antibacterial potential at this concentration. This shows that ethyl acetate has a stronger effect than methanol at higher concentrations.

In general, the relationship between inhibitory power and the concentration of extracts used as antibacterials is generally positive, meaning that the higher the concentration of the extract applied, the larger the inhibition zone formed. This is in line with the opinion of Sari and Dahlan (2015), who stated that increasing the concentration of the extract can increase the effectiveness of antibacterials. With increasing concentration, the number of active compounds in the extract also increases, so that the potential to inhibit bacterial growth becomes greater. Increasing the concentration of the extract is directly related to the amount of active substances available to interact with bacterial cells, which results in a larger inhibition zone, indicating that more bacteria are affected by the antibacterial compound (Surjowardojo *et al*., 2015). In addition, studies by Febryanto (2017)

and Prihatini & Dewi (2021) also show that at higher concentrations, changes in environmental conditions such as pH or solubility of compounds can affect the bioactivity of the extract.

The results obtained in this study differ from the findings of Mardiyanti *et al*. (2024), which showed that both dry and wet extracts of *E. acoroides* seagrass were ineffective in inhibiting the growth of *V. parahaemolyticus* bacteria. However, the results of this study are similar to the study of Setyoningrum (2015), which reported the presence of an inhibition zone in *E. acoroides* extract from Paciran Waters, Lamongan Regency against *S. aureus* bacteria. A similar study by Purnama and Brahmana (2018) also found antibacterial activity in *E. acoroides* extract from Karang Tirta Beach Waters, Padang, West Sumatra against test bacteria such as *E. coli*, *S. aureus*, and *B. subtilis*, which showed bactericidal properties, namely the ability to kill bacteria.Purniasih *et al*., (2022) also reported that six isolates of seagrass symbiotic bacteria *E. acoroides* were successfully isolated and showed antibacterial activity with the formation of inhibition zones. Isolates A (En1), B (En2), and C (En4) have antibacterial activity on the test bacteria *E. coli*. Isolate D (En5), has antibacterial activity on the test bacteria *E. coli* and *S. aureus*. Isolates E (Ep1) and F (En3) have antibacterial activity on the test bacteria *E. coli*, *S. aureus*, and *S. mutans*.

Based on the results (Table 3), methanol and ethyl acetate extracts of *E. acoroides* have the potential to inhibit the growth of test bacteria. This shows that the chemical components in the extract are effective in overcoming the growth of pathogenic bacteria, so it can be a good alternative for the development of antibacterial products based on natural ingredients. Methanol extract of seagrass *E. acoroides* is known to contain flavonoids, saponins, phenolics, tannins, steroids, terpenoids, and alkaloids. Meanwhile, ethyl acetate extract contains flavonoids, phenolics, tannins, steroids, and terpenoids (Firman, 2024). Flavonoids are one of the compounds known to have significant antibacterial potential. This compound works by inhibiting important enzymes in bacteria, which play a role in the process of metabolism and survival (Niken & Telaumbanua, 2024). Besides that, Lukman & Aryani (2024) added that flavonoids can also disrupt the integrity of bacterial cell membranes, causing leakage of cell components, and ultimately causing bacterial death. Research by Ibrahim *et al*. (2013) shows that flavonoids have antibacterial activity that can effectively inhibit bacterial growth. Flavonoids can interact with bacterial cell membrane components, changing their structure and function, and disrupting the balance of bacterial cells, leading to irreparable damage and death of the bacterial cells (Donadio *et al*., 2021). This activity makes flavonoids a potential compound to be developed as a natural antibacterial agent in the treatment of infections caused by bacteria (Xiong *et al*., 2023)

In addition to flavonoids, saponins also play an important role in its antibacterial mechanism. Saponins work by damaging the bacterial cell membrane, which is a vital structural component for bacterial survival (Pikhtirova *et al*., 2023). Saponins can reduce the surface tension of bacterial cell membranes, which causes bacterial cells to become more susceptible to damage and eventually experience lysis or rupture (Heryan *et al*., 2018). Regards *et al*. (2015) explained that saponins have a toxic effect on bacterial cell membranes, triggering leakage of internal cell components, such as proteins and nucleic acids, which are important for bacterial metabolic activity. The loss of these important components ultimately causes disruption in bacterial cell function, inhibiting their growth and leading to bacterial death (Fachrial, 2022). The effectiveness of saponins as antibacterials makes them attractive candidates for the development of therapeutic agents, especially in treating infections caused by bacteria that are resistant to conventional antibiotics.

Tannins and alkaloids also have different mechanisms of action but are effective in treating bacterial infections, including *S. mutans*, which is the main cause of dental caries. Tannins work by precipitating proteins found in bacteria and disrupting essential enzymes that play a role in the formation of bacterial biofilms (Smith *et al*., 2005; Schestakow *et al*., 2021). Biofilm formed by *S. mutans* is one of the early stages in the development of dental caries, which leads to a decline in the health of teeth and gums (Jeffrey & Sugiaman, 2022; Zulkaidah *et al*., 2023). Tannins, with their ability to interfere with this process, can reduce the ability of bacteria to form biofilms, thereby preventing more severe dental infections. On the other hand, alkaloids work by inhibiting bacterial protein and DNA synthesis, which are vital processes for the growth and reproduction of bacterial cells (Sholechah *et al*., 2023). Fredela (2021) explains that alkaloids can interfere with the process of bacterial genetic replication and transcription, thereby inhibiting bacterial cell proliferation. In this way, alkaloids reduce the ability of bacteria to reproduce, so that bacteria cannot reproduce effectively and eventually die.

The results of the Analysis of Variance (ANOVA) showed that the treatment of solvent type, extract concentration and interaction between the two treatments had a very significant effect on the inhibition zone of *S. mutans* bacteria. The results of the ANOVA can be seen in Table 2.

Table 2. Anova Inhibition Zone of *S. mutans* Bacteria

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	172.601a	7	24,657	157,412	.000
Intercept	2913.247	1	2913.247	18598.164	.000
Solvent	19,512	1	19,512	124,565	.000
Extract Cone	105,513	3	35,171	224,531	.000
Solvent * Extract Con	47,576	3	15,859	101,241	.000
Error	2,506	16	.157		
Total	3088.354	24			
Corrected Total	175.107	23			

a. R Squared = .986 (Adjusted R Squared = .979)

Tukey's Honestly Significant Difference (HSD) test for the interaction of solvent type and *E. acoroides* extract concentration was not performed because the number of mean values was too many (4 x 2 = 8). According to Torrie and Steel (1960) if the number of mean values to be tested for BNJ is more than 6, then the α BNJ value is no longer 5%, but rather greater than 5% and tends to accept H0 (not significantly different). For such cases, the BNJ test should be carried out on the extract concentration treatment. The results of the BNJ test (Table 5) show that the mean value (average) of the inhibition zone of *S. mutans* bacteria in the *E. acoroides* extract concentration treatment of 25% is not significantly different from the *E. acoroides* extract concentration treatment of 50%, but is significantly different from the *E. acoroides* extract concentration treatments of 75% and 100%. Furthermore, the mean value (average) of the inhibition zone of *S. mutans* bacteria in the *E. acoroides* extract concentration treatment of 50% is not significantly different from the *E. acoroides* extract concentration treatment of 75%, but is significantly different from the *E. acoroides* extract concentration treatment of 100% and the mean value (average) of the inhibition zone of *S. mutans* bacteria in the *E. acoroides* extract concentration treatment of 100%. acoroides 75% was significantly different from the treatment of *E. acoroides* extract concentration of 100%.

Table 3. Treatment Test of E. acoroides Extract Concentration

Kon_Ekstrak	N	Subset		
		1	*2*	*3*
25	6	9.46[a]		
50	6	9.77[a]	9.77[ab]	
75	6		10.22[b]	
100	6			14.62[c]
Sig.		.543	.229	1,000

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square (Error) = .157.
a. Uses Harmonic Mean Sample Size = 6,000.
b. Alpha = 0.05.

In this study, the positive control used was chloramphenicol, while the negative control was dimethyl sulfoxide (DMSO) solvent. DMSO was chosen because it is an effective solvent for dissolving extracts and does not affect antibacterial activity against test bacteria (Ukratalo *et al*., 2022; Kaihena *et al*., 2022). DMSO has non-reactive properties in terms of inhibiting bacterial growth, so its presence will not affect the measured results ((Telaumbanua & Mayasari, 2021). Thus, this negative control ensures that the antibacterial activity observed in this study is truly derived from the extract being tested, not from the solvent used.

Chloramphenicol is used as a positive control because it is an antibiotic with a broad spectrum, effective against various types of bacteria, both aerobic and anaerobic (Wardaniati & Gusmawarni, 2021). Chloramphenicol works by inhibiting the transpeptidation process in bacterial protein synthesis, which is an important step in the formation of bacterial cell walls. According to Putro *et al*. (2022), the mechanism of action of chloramphenicol causes disruption in the protein formation process, which leads to a decrease in the ability of bacteria to reproduce and ultimately results in bacterial death.

CONCLUSION

Methanol and ethyl acetate extracts of *E. acoroides* have been shown to inhibit the growth of *S. mutans* bacteria, which is the main cause of dental caries. Although both extracts showed antibacterial activity, the ethyl acetate extract had a stronger effect compared to the methanol extract, especially at higher concentrations. This can be seen from the more significant increase in the size of the clear zone in the ethyl acetate extract, indicating that this extract is more effective in inhibiting the growth of *S. mutans* than the methanol extract at similar concentrations.

AUTHORS CONTRIBUTION

Firman designed and conducted the study, analyzed and interpreted the data, and wrote a draft of the manuscript. Rieupassa, F designed the research, analyzed and interpreted the data. Mailoa, M.N reviewed the draft manuscript.

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