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In Vitro Phytochemical, Antioxidant, and Antibacterial Evaluations of Various Extracts of *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch

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ABSTRACT

Eleocharis dulcis Burm.f. Trin. ex Hensch, has been empirically used in Chinese folk medicine to treat cough, laryngitis, hepatitis, enteritis, hypertension, and pharyngitis. The study is aimed to compare the phytochemical components, antioxidant and antibacterial activity of hexane (H), ethyl acetat (EA), and methanol extracts (MeOH) extracts obtained by Ultrasonic Assisted Extraction (UAE) method. Total Phenolic Content (TPC) was determined by the Folin-Ciocalteu method and Total Flavonoid Content (TFC) by quercetin method. Antioxidants activity using the DPPH, ABTS, and FRAP method. The inhibition zone of antibacterial activity using the disc diffusion method and Minimum Inhibitory Concentration with microdilution method. Results of the extracts' total phenolic and flavonoid contents showed that EA has a higher content of these phytochemicals. The EA also exhibited a higher zone of inhibition for each concentration against *Aeromonas hydrophila* (AHA), *Aeromonas salmonicida* (AS), and *Streptococcus agalactiae* (SA). The MIC of EA against AHA and AS was 6.25 mg/mL and 3.125 mg/mL, respectively. All extracts were effective against SA with MIC of 1.5625 mg/mL. Similarly, the EA extract showed better antibacterial activity against all the tested bacteria strains than the H and MeOH extracts. Also, the EA extract exhibited better antioxidant activity compared to others, with IC_{50} values of 52.830 ± 0.271 ppm, 16.923 ± 0.047 ppm and 198.504 ± 1.490 µmol/g in the DPPH, ABTS and FRAP assays, respectively. The study concludes that the EA extract of E. dulcis possesses better antibacterial and antioxidant activities.

***Keywords*:** *Eleocharis dulcis*; Ultrasonic Assisted Extraction, Phenolics Content, Flavonoids Content

Introduction

Aquatic plants are capable of assisting with the discovery of bioactive natural products. Extract and compounds isolated from several aquatic plants have been show to possess valuable biological/pharmaceutical properties.[1] One aquatic plants with biological potential is *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch.[2] *E. dulcis*, or purun rat or Chinese water chestnut, originates from Southeast Asia and has spread to West Africa, Madagascar, India, China, Taiwan, Japan, Australia, and the Pacific Islands. *E. dulcis* is a herbaceous perennial plant usually found in the ground and has partially buried stems. These plants are widely distributed in wetlands and fish habitats, which can enhance water quality and protect the environment.[3] Plants or organisms that survive extraordinary or extreme conditions usually contain active compounds. Studies have shown that plants that withstand extreme climatic and environmental conditions (high temperatures, low or high nutrient, pH and salt soil) are known to express new and unique bioactive molecules.[4]

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In traditional Chinese medicine, *E. dulcis* is used to treat constipation, hypertension, and chronic nephritis. In addition, the tubers of this plant are also used as food by the Chinese people.[3]

E. dulcis is reported to possess antioxidant and antibacterial activities. The methanol and ethyl acetate extracts were also reported to exhibit antibacterial activity against *S. aureus*, *E. coli*, *L. monocytogenes* and *B. subtilis*.[2] *E. dulcis* tubers are also reported to contain phenolic compounds, which act as antioxidants and anti-cancer. Therefore, leaves were observed to also have antioxidant and antibacterial activity. The biological activities shown by this plant are attributed to different secondary metabolites contained in the plant. For instance, the crude methanol crude extract of purun rat leaves positively contains phenolic compounds, flavonoids, tannins and terpenoids.[5] In addition, the ethyl acetate fraction of *E. dulcis* tuber skin was reported to contain fisetin, tektorigenin, quercetin, luteolin, apigenin, diosmetin, jaseosidin, galangin and ramnocitrin, compounds with demonstrable pharmacological activities, such as antioxidant and antibacterial.[6] Natural compounds such as flavonoids and luteolin have been reported to possess antioxidant and antibacterial activities.[7,8] This does not rule out the possibility that this plant has other chemicals that could act as antioxidants and antibacterial agents. However, if a potential compound exists in one part of the plant, it will likely exist in others. So, it is essential to consider this plant in order to gain information about the bioactivity of the whole plant because this plant currently has limited activity profiles.

A previous study evaluated the antioxidant and antibacterial properties of *E. dulcis* leaves methanol extract using the maceration and Ultrasonic Assisted Extraction (UAE) extraction methods. The research showed that extraction using UAE show better yield, with better activity as antioxidant and antibacterial activities.[5] Thus, in this current study,

ultrasonic-assisted extraction using solvents of increasing polarity (hexane, ethyl acetate and methanol) was employed. The various extracts were tested for secondary plant metabolites using standard methods. The extracts were further evaluated for their antioxidant and antibacterial activity against a panel of pathogenic bacterial strains (*Aeromonas hydrophila*, *Aeromonas salmonicida*, and *Streptococcus agalactiae*).

Material and Methods

Chemicals and reagents

Analytical grade solvents and reagents, hexane, ethyl acetat, methanol, Dimetil sulfoksida (DMSO), chloral hydrate, alkaloidal reagent, Bouchardat reagent, Shinoda reagent, Lieberman-Bouchard reagent, gelatin, $FeCl_3$, Folin-Ciocalteu reagent, Quercetin, ascorbic Acid, sodium carbonate, alumunium chlorida, ethanol, DPPH (2-diphenyl-1-picrylhydrazyl), ABTS [2,20-azinobis(3-ethylbenzothiazo-line-6-sulfonic acid) diammonium], potassium persulfate, Ammonium Ferro sulfate, crystal violet, iodine, safranin, chlpramphenicol, Tryptone Soy Agar (TSA), Brain Heart Infussion Agar (BHIA), Tryptone Soy Broth (TSB), and Brain Heart Infusion Broth (BHIB), were used for this study.

Sample preparation

E. dulcis plant was collected on April 2022 from Marullah Swamp in Nanggroe Aceh Darussalam, Indonesia. It was indentified by Yayah Robiah, the Directorate of Scientific Collection Management, BRIN Cibinong, with a herbarium number B-2503/ll.6.2/Dl.05.07/8/2022 (purun).

The plant material was dried, ground into powder form, and extracted successively with hexane, ethyl acetate, and methanol using the Ultrasound-Assisted Extraction (UAE) method. The material (50 g) was ultrasonicated with 500 mL x 4 hexane at 40° C for 30 minutes. The extract was filtered using Whatman No.1 filter paper, and the dried marc was successively re-extracted with 500 mL x 3 ethyl acetate and methanol, respectively. The filtrates from the three solvents were concentrated with a rotary evaporator (Buchi Rotavator R-300, Germany) at 40℃ to obtain thick crude extracts. Subsequently, the hexane, ethyl acetate, and methanol extracts were diluted with 10% DMSO, followed by distilled water (Aqua Pro injection)..

Sample microscopic observation

Microscopic examination of the powder sample was conducted under a light microscope (100 × magnification). Briefly, a small amount of the powdered material was placed in a microscopic slide to which a few drops of chloral hydrate solution was added and then covered with a cover slip. The prepared sample was examined under a light microscope (100 × magnification).

Phytochemical screening

To identify chemical components qualitatively, phytochemical screening was carried out. Different reagents (Bouchardat, Mayer, and Dragendroff) were used to detect the presence of alkaloids, while the Shinoda reagent for flavonoid, Lieberman-Burchard reagent for terpenoids, tannin with gelatin and $FeCl_3$, and saponin with foam index[5].

Total phenolic content determination

The total phenolic content of the hexane, ethyl acetate and methanol extracts were done using the Folin-Ciocalteu reagent test. Briefly, 100 µL of each extract (1000 g/mL) was mixed with 10 mL of the Folin-ciocalteu reagent previously diluted (1:10) with distilled water. The tubes were vortexed for 60 seconds and allowed to stand for 4 minutes. This was followed by 1 mL of 7.5% Na_2CO_3 solution. The mixture was incubated at room temperature for two hours in the dark. The absorbance value was determined with a UV-Vis spectrophotometer at 671 nm. Gallic acid was used as the standard agent to compute the calibration plot. The total phenol content of the extracts was expressed as milligram gallic acid equivalent per gram of the extract (mg GAE/gram).[9]

Total flavonoid content determination

The total flavonoid content was determined by the Aluminium chloride method. Briefly, 2.5 mL of the hexane extract, 1 mL of ethyl acetate and methanol extracts (1000 g/mL) were mixed with 100 µL of a 10% $AlCl_3.6H_2O$ solution followed by 100 µL sodium acetate (1 M) and then ethanol (5 mL). After shaking for 60 seconds, the solution was incubated for 30 minutes at room temperature. The absorbance was measured with a UV-Vis spectrophotometer at 441 nm. Quercetin (1.5 µg/mL, 3 µg/mL, 4.5 µg/mL, 6 µg/mL, 7.5 µg/mL, and 9 µg/mL) was used as the standard to generate a calibration curve. The total flavonoid content of the extracts was expressed as milligram quercetin equivalent per gram of the extract (mg QE/gram).[9]

Antioxidant activity test

DPPH (2-diphenyl-1- picrylhydrazyl) Assay

The free radical scavenging activity of the test samples was determined by the DPPH method. Briefly, ascorbic acid at different concentrations (0.5; 1; 1.5; 2; 2.5, and 3 ppm) and the extracts at (20; 40; 60; 80; 100, and 120 ppm) were used in the DPPH assay. DPPH solution was added to the standard and extracts solutions followed with 1000 µL methanol to make up to 5 mL. The mixture was diluted and incubated for 30 min in the dark, the absorbance value was determined used a UV-Vis spectrophotometer at 515 nm.[10] The following equation was used to compute the percentage of inhibition.

$$\%\text{Inhibition} = \left(\frac{Blank\ abs - sample\ abs}{Blank\ abs}\right) \times 100\%$$

A calibration curve was made based on the inhibition value at each concentration, and a linear regression value was calculated from the equation y=A+BX. Antioxidant activity was assessed by the 50% Inhibition Concentration (IC_{50}), which is the sample concentration that can reduce the concentration of DPP by 50%.[5]

ABTS [2,20-azinobis(3-ethylbenzothiazo-line-6-sulfonic acid) diammonium] Assay

Similarly, the free radical scavenging potentials of the extracts were tested using the ABTS radical assay. The ABTS radical was created by reacting potassium persulphate with ABTS stock solution and letting the mixture stand in the dark at room temperature for 12 - 16 h before use.[11] Ascorbic acid at concentrations of 0.2; 1; 1.5; 2; 2.5, and 3 ppm was used as the standard antioxidant agent. The extract was made into a solution of 10; 15; 20; 25; 30, and 35 ppm for hexane and ethyl acetate extracts and 10; 20; 30; 40; 50 and 60 ppm for methanol extract. The ABTS solution was added to 1000 µL of the standard and extracts solutions, and the mixture was made up to 5 mL with methanol. The mixture was incubated for 30 min in the dark, and the absorbance value was determined with a UV-Vis spectrophotometer at 752 nm.[12] A calibration curve was created in the same way with DPPH method. The following equation was used to compute the percentage of inhibition.

$$\%\text{inhibition} = \left(\frac{Blank\ abs - sample\ abs}{Blank\ abs}\right) \times 100\%$$

FRAP (Ferric Reducing Antioxidant Power) Assay

This test is based on modified method described by Nur et al. (2019). The calibration curve was created using ammonium Ferro sulfate (AFS) solution. In this test, ascorbic acid was employed as a positive control. The extract solution was combined with 1 mL of FRAP reagent and made up to 5 mL of methanol. A similar procedure was done with the Ascorbic acid standard. A UV-Vis spectrophotometer set to 596 nm was used to measured the absorbance value after the mixture was left in the dark for 30 minutes.[13] The antioxidant activity of the test samples and the standard was calculated from the equation below.

$$FeEAC = \frac{\Delta A}{GRAD} x \frac{Av}{Spv} x\ D\ x \frac{1}{Cext} x\ 10^5$$

Antibacterial activity test

The bacteria (*Aeromonas hydrophila*, *Aeromonas Salmonicida* and *Streptococcus agalactiae)* used in this study were obtained from The

Laboratory of Fish Health, Faculty of Marine and Fisheries, IPB (Institut Pertanian Bogor) University.

Gram stain of bacteria
The Gram staining procedure consists of four fundamental phases, including 1. Crystal violet was used as a primary stain; 2. Iodine was used as a mordant to fix the stains; 3. Rapid decolourisation of the microscopic slides with ethanol, acetone, or a combination of both; and 4. Counterstaining with safranin.[14] The slides were examined under a light microscope (100 × magnification).

Inhibition zone
The Agar Diffusion Method (ADM) was used to determine the inhibition zones.[15] Briefly, Tryptone Soy Agar (TSA) medium was used to culture *A. hydrophila and A. salmonicida*, while Brain Heart Infusion Agar (BHIA) was used as the culture medium for *S. Agalactiae*. Agar medium was used poured into the Petri dishes as the base medium. Then, a petri disc containing the base layer was filled with 1 mL of bacterial suspense (1-2 x 10^6 CFU ml^{-1}) and 4 mL of agar medium. A paper disc containing 20 µL of extract solution with serial concentrations 0.05; 0.1; 0.1.5; 0.2; 0.25; and 0.3 g mL^{-1} was then set on the agar plate. The diameter of the inhibitory zone was measured used a calliper after 24 hours of incubation at 30º C for *Aeromonas* and 48 hours for *S. Agalactiae*. Chloramphenicol (30 µg mL^{-1}) and DMSO 5% were used as the positive and negative control agents, respectively.[5]

Minimum inhibitory concentration (MIC) test
The microdilution method was used to determine the MIC of the extracts. In this assay, Tryptone Soy Broth (TSB) medium was used to culture *A. hydrophila and A. salmonicida*, while Brain Heart Infusion Broth (BHIB) was used for *S. Agalactiae*. The extract solution was diluted in 5% DMSO. Chloramphenicol was used as the standard antibiotic agent. The control contained 50 µL of medium mixed with 30 µg mL^{-1} of chloramphenicol diluted with 50 uL of bacterial suspension. The medium with bacterial suspension was used as the untreated control. In contrast, Broth medium and DMSO were used as the negative control. The microplates were incubated for 24 hours for *Aeromonas* and 48 hours for *S. Agalactiae*. The absorbance was measured with a microplate reader set at 600 nm.[5] Inhibition was monitored and calculated based on the absorbance result of OD600 of treated vs control.

Minimum bactericidal concentration (MBC) test
To determine the MBC, the mixture of extract and bacteria used to estimate the MIC described above was streaked on an agar medium to determine the MBC. The plates were incubated for 24 hours at 30º C for *Aeromonas* and 48 hours for *S. Agalactiae*. The concentration that demonstrated no bacterial growth following incubation was used as the indicator for the MBC value.[16]

Statistical analysis
The experimental values of TPC, TFC, IC_{50} of antioxidants activity, and inhibitory zone against bacteria are expressed as mean (±SD) following the triplicates determination. The data were analysed with MS Excel 2019 statistical software.

Results and Discussion

Sample preparation
Sequential extraction is carried out by increasing the polarity of the solvent sequentially. Since the solubility of bioactive chemicals varies with the solvent, this method is appropriate for extracting fractions of natural compounds.[17,18] Ultrasound-assisted extraction (UAE) was used for the extraction and according to earlier investigations on the extraction of *E. dulcis*, higher yield obtained with UAE compared to maceration.[5] The plant material was successively extracted with hexane, a non-polar solvent, followed by ethyl acetate and methanol. Compounds can therefore be generated by grouping them according to their polarity. Non-polar compounds will be separated into the hexane extract, semi-polar compounds into the ethyl acetate extract, and polar compounds into the methanol extract. Table 1 showed the results of the UAE sequential extraction. Table 1 shows the results of the UAE sequential extraction. The results indicated that the methanol extract had the highest yield.

Sample microscopic observation
Microscopical testing is used to characterise plant fragments as cells or tissue to prevent adulteration.[19] The observation of *E. dulcis* powder under a microscope showed fragments including oil gland cell, collenchyma, vascular bundle, spogy parenchyma, cork tissue cell and amylum. The photomicrograph of the examination of *E. dulcis* powder is shown in Figure 1.

Phytochemical screening
The result of the phytochemical screening showed that the plant contained terpenoids/steroids, alkaloids, tannins, flavonoids, saponins, and phenolic compounds (Table 2). The phytoconstituents varied in the different solvent extracts. Only terpenoids/steroids were identified in the hexane extract. Ethyl acetate and methanol extract contained terpenoids/steroid, Tannins, Flavonoids, and saponins.

Total phenolic content
The Folin-Ciocalteu method is based on the principle that phenolic ions will reduce phosphomolybdic-phosphotungstic acid in the reagent due to the phenol oxidation process to produce a blue molybdenum-tungsten complex under alkaline conditions.[20] The result of the determination of total phenolics levels in *E. dulcis* showed in Figure 3. The results showed that the ethyl acetate extract has the highest concentration of phenolic compounds (29.320 ± 0.229 mg GAE/g), followed by the methanol and hexane fractions at 28.715 ± 0.168 mg GAE/g and 6.674 ± 0.453 mg QE/g, respectively. The lowest concentration of phenolics was found in the hexane fraction.

Total flavonoid content
The aluminum chloride technique was used to calculate the total flavonoid content. Flavonoid molecules and aluminum chloride interact to create stable complexes with carbonyl groups. It also react with hydroxyl flavonoids to generate labile acid complexes, which causes the colour to turn yellow when adding sodium acetate. The total flavonoids contain in *E. dulcis* showed in the Figure 4. The results show that the ethyl acetate extract has the highest concentration of flavonoid compounds (14.963 ± 0.133 mg QE/g). Methanol and hexane extracts were 8.459 ± 0.093 mg QE/g and 5.062 ± 0.031 mg QE/g, respectively. The lowest concentration of flavonoids was found in the hexane extract.

Antioxidant activity test
The radical scavenging properties of the extracts were examined by the stable DPPH free radical, which gives specific absorbance at 514 nm. The absorption diminishes as antioxidants provide protons to these radicals.

Table 1: Extraction yield of *E. dulcis* from Ultrasonic Assisted Extraction method

	H	EA	MeOH
Extract (g)	100	100	100
Yield (%)	0.975	1.780	6.40

H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract.

Table 2: Phytochemical screening results of *E. dulcis* extract

Chemical substances	H	EA	MeOH
Terpenoid/steroids	+	+	+
Alkaloids	-	-	-
Tannins	-	+	+
Flavonoids	-	+	+
Saponins	-	+	+

AA: Ascorbic Acid; H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract

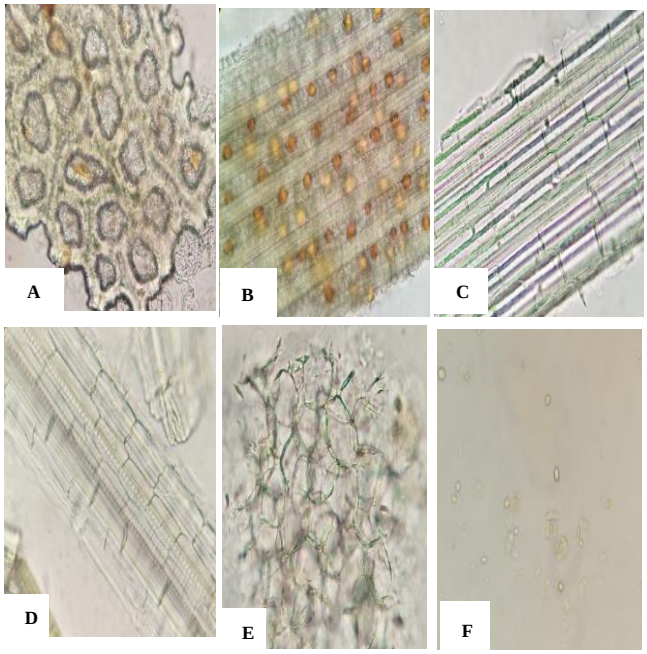

Figure 1: Microscopic result of dried powdered sample of *E. dulcis*.
A: oil gland cell; B: collenchyma; C: vascular bundle; D: spongy parenchyma; E: cork tissue cell; and F: Amylum

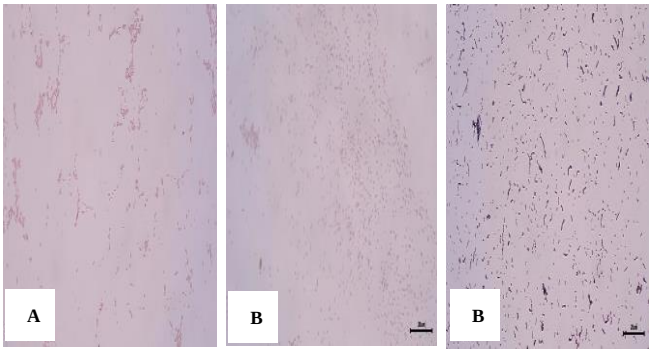

Figure 2: Results from Gram Staining of bacteria strains.
A: *A. hydrophila*; B: *A. Salmonicida*; and C: *S. agalactiae*.

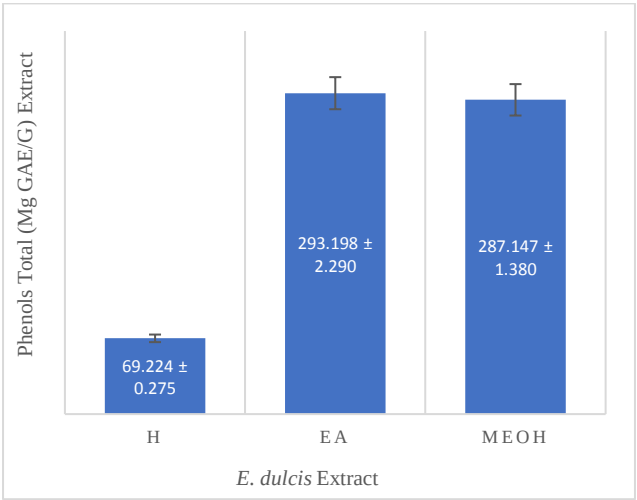

Figure 3: Total Phenolic content of *E. dulcis* extracts.
H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract

The reduction in absorption is used to evaluate the effectiveness of radical scavenging by the test samples.[21] Table 3 shows the various extracts' DPPH free radical scavenging potentials. The IC_{50} is a value that indicates the extract concentration that can inhibit 50% of the DPPH free radical. The results were obtained by plotting a graph with the sample concentration on the ordinate and the free radical inhibition capacity of the extract from *E. dulcis*. As previously mentioned, several sample concentration were prepared. All the extracts showed free radical scavenging activity in all concentrations examined.

Deep purple DPPH radical molecule would turn yellow in the presence of antioxidants species due to its reduction to non-radical DPPH. When the DPPH radical's free electron is paired with electrons from antioxidants, the DPPH radical reduced (DPPH-H), and the stable molecules DPP- Hydrazine is formed.[22] The antioxidant screening results (table 2) showed that the ethyl acetate extract has the highest IC_{50} value of 52.830 ± 0.271 ppm compared to that of methanol (72.060 ± 0.213 ppm) and hexane extract (70.708 ± 0.392 ppm). These values are, however, lower than the standard ascorbic acid (IC_{50} = 2.630 ± 0.001 ppm).

The generation of the ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation forms is one of the fundamental principles of spectrophotometric methods that have been used to determine the overall antioxidant activity of solutions of pure compounds, aqueous mixes, and drinks. ABTS•+ can be created from ABTS by an enzymatic system that uses hydrogen peroxide and the peroxidase enzyme.[23] The extracts' activity to scavenge free radicals was determined used the ABTS test, as shown in Table 4.

The ethyl acetate extract showed the highest IC_{50} value, 16.923 ± 0.047 ppm, followed by hexane extract (28.540 ± 0.117 ppm) and methanol extract (46.345 ± 0.106 ppm). The DPPH test produced an IC_{50} value at a higher concentration than the ABTS. This suggests that the extract's have lower IC_{50} value when it reacted with ABTS radicals. The sensitivity of the DPPH method is lower than that of the ABTS. Unlike ABTS•+, most antioxidants take longer to respond to the DPPH• radical.[24]

In a similar vein, the ability of the sample extract to transmit electrons to the FRAP reagent determines the antioxidant potency of the extract. The ferric tripyridyl triazine salt will be reduced into the blue hue of a ferrous ion in this hydrophilic test at a low pH medium. The Fe^{3+}/ferric cyanide complex is converted from the ferrous form (Fe^{2+}) in the test extracts indicating the presence of antioxidants species. [24,25,26]

The Antioxidant activity with this method can be confirmed by measuring the formation of blue colour at 596 nm. Increased absorbance at 596 nm indicates that test extracts have more reducing power.

Table 3: Results of DPPH free radical scavenging activity of the plant extracts

Sample	IC_{50} (ppm)
AA	2.630 ± 0.001
H	70.708 ± 0.392
EA	52.830 ± 0.271
MeOH	72.060 ± 0.213

AA: Ascorbic Acid; H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract

Table 4: Results of the ABTS radical scavenging activity of *E. dulcis* extracts

Sample	IC_{50} (ppm)
AA	1.915 ± 0.002
H	28.540 ± 0.117
EA	16.923 ± 0.047
MeOH	46.345 ± 0.106

AA: Ascorbic Acid; H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract

Used a polar solvent might be more beneficial as FRAP evaluates the activity of hydrophilic antioxidants. The determination of *E. dulcis* extract's reducing power (antioxidant capacity) results is shown (Table 5). The antioxidant activity of plant extracts in the FRAP assay is based on a linear regression equation from the standard $FeSO_4$ curve.[28] The result revealed that Ethyl acetate extract exhibited a higher FeEAC value than the methanol and hexane extracts. However, when compared with ascorbic acid, the FeEAC value of the ethyl acetate extract was much lower. The antioxidant capacity of the extracts is proportional to the FeEAC value.[5]

The results of the three methods used to measure antioxidant activity were consistent, with the ethyl acetate extract having a more significant antioxidant potential than the methanol and hexane extracts. The ethyl acetate extract has higher TPC and TFC concentrations than other extracts, contributing to its excellent antioxidant activities. It is generally known that phenolic compounds and flavonoids have antioxidant (free radical scavenging) properties.[29] The results in this study were consistent with those presented by Khorasani *et al*.,[30] who reported that phenolic and flavonoid content correlated with antioxidant activity.

Phenolic substances can stabilise free radicals because of their stable chemical structure. This is due to the hydroxyl groups in phenolic substances, which can give free radicals hydrogen atoms or electrons. In addition to phenolic compounds, which function as antioxidants, flavonoid compounds also show antioxidant activity in the presence of hydroxyl compounds, which can decrease free radical reactivity and chelate metal ions to prevent free radical formation.[10]

Antibacterial activity test

Gram stain of bacteria

Bacteria are classified as "gram-positive" or "gram-negative" based on which of two sets of colours they exhibit when stained with a specific set of stains (pink to red or purple to blue). Gram staining separates bacteria based on their cell walls' physical and chemical characteristics.[14] Gram staining can be used to identify bacteria to the cells used in a study are not contaminated. Microscopic examination of the bacteria cells used in this identified *A. hydrophila* and *A. Salmonicida* as gram-negative and *S. agalactiae* as gram-positive. The results of the Gram staining are shown in Figure 2.

Inhibition zone

The bacteria strains used in this study are known to cause diseases in fish. The inhibitory zones of *E. dulcis* extract against the bacteria strains are shown in Table 6. Accordingly, the results revealed that the ethyl acetate extract exhibited a greater inhibition zone diameter at all concentrations tested than the hexane and methanol extracts. Interestingly, methanol extract had the least inhibitory zone for all concentrations. The results of an antibacterial activity assay with the disc diffusion method indicated that each bacteria had different inhibitory zones because of their distinct morphology and structure.[5] Other factors that may impact the extent of the inhibition zone include the microorganisms' metabolic activity, interactions between the active ingredients and the media, incubation temperature, environmental pH, media components, inoculum size, and incubation time.[20]

Table 5: Result of FRAP antioxidant assay of the plant extracts

Sample	Antioxidant Activity FeEAC (µmol/g)
AA	15427.64 ± 28.163
H	57.034 ± 0.098
EA	198.504 ± 1.490
MeOH	167.772 ± 1.713

AA: Ascorbic Acid; H: Hexane Extract; EA: Ethyl Acetate Extract and MeOH: Methanol Extract

Table 6: Inhibition zone of *E. dulcis* extract against the different bacteria strains.

Sample	Concentration	Inhibition zone diameter (mm)		
		AHA	***AS***	***SA***
C	30 ug/mL	25.411 ± 0.083	22.011 ± 0.058	37,033 ± 0.069
DMSO	5%	-	-	-
H	0.05 g/mL	7.267 ± 0.094	7.533 ± 0.377	8.433 ± 0.047
	0.1 g/mL	8.300 ± 0.082	8.000 ± 0.000	8.933 ± 0.094
	0.15 g/mL	8.833 ± 0.125	8.167 ± 0.047	9.733 ± 0.189
	0.2 g/mL	9.667 ± 0.094	9.467 ± 0.094	10.800 ± 0.283
	0.25 g/mL	12.000 ± 0.000	8.867 ± 0.094	12.900 ± 0.082
	0.35 g/mL	14.067 ± 0.094	9.800 ± 0.163	15.167 ± 0.047
EA	0.05 g/mL	8.000 ± 0.082	8.367 ± 0.047	8.200 ± 0.000
	0.1 g/mL	8.400 ± 0.000	8.600 ± 0.000	10.333 ± 0.471
	0.15 g/mL	8.767 ± 0.047	8.800 ± 0.000	12.867 ± 0.094
	0.2 g/mL	8.933 ± 0.047	9.933 ± 0.094	14.000 ± 0.000
	0.25 g/mL	9.300 ± 0.082	10.933 ± 0.094	15.800 ± 0.082
	0.35 g/mL	9.400 ± 0.000	11.667 ± 0.047	17.000 ± 0.000
MeOH	0.05 g/mL	7.000 ± 0.000	7.567 ± 0.047	7.267 ± 0.094
	0.1 g/mL	7.367 ± 0.047	7.800 ± 0.000	8.000 ± 0.000
	0.15 g/mL	8.933 ± 0.047	8.033 ± 0.047	9.100 ± 0.082
	0.2 g/mL	8.300 ± 0.082	8.200 ± 0.000	11.000 ± 0.000
	0.25 g/mL	8.600 ± 0.000	8.600 ± 0.000	12.067 ± 0.094
	0.35 g/mL	9.067 ± 0.094	8.600 ± 0.000	13.000 ± 0.000

C: Chloramphenicol; DMSO: Dimethyl sulfoxide; AHA: *Aeromonas hydrophila*; AS: *Aeromonas salmonicida*; SA: *Streptococcus agalactiae*; H: Hexane Extract; EA: Ethyl Acetate Extract; MeOH: Methanol Extract

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Minimum inhibitory concentration (MIC) test and Minimum bactericidal concentration (MBC) test

MIC was determined by microdilution method at various concentrations (12.5; 6.25; 3.125; 1.5625 mg/mL). The growth inhibition of *A. hydrophila* by hexane extract was at a concentration of 3.125 mg/mL. The hexane extracts MIC against *A. salmonicida* was 1.5625 mg/mL, ethyl acetate extract was 3.125 mg/mL, and methanol extract (6.25 mg/mL). At the lowest concentration, 1.5625 mg/mL, the hexane, ethyl acetate and methanol extract demonstrated MIC against *S. agalactiae*.

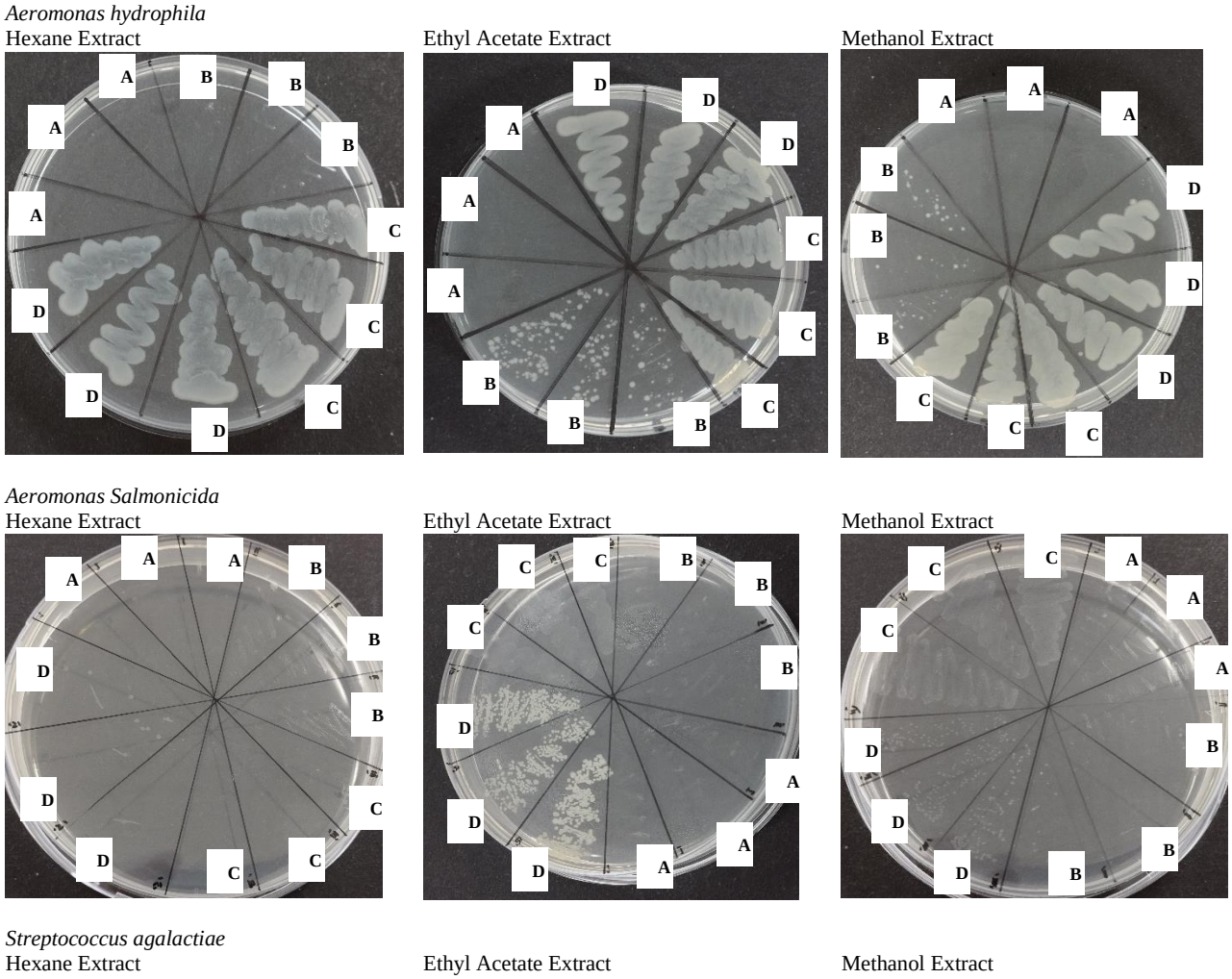
An MBC test was also performed to evaluate each extract's bactericidal activity. The results showed that on *A. hydrophila*, the methanol and ethyl acetate extracts showed MBC at a concentration of 12.5 mg/mL. However, hexane showed MBC at a higher concentration of 6.25 mg/mL. On *A. salmonicida*, hexane extracts exhibited bactericidal activity at 3.125 mg/mL, ethyl acetate extract at 6.25 mg/mL and methanol extract at 12.5 mg/mL. Evaluation on *S. agalactiae*, the hexane and ethyl acetate extract was equally effective with bactericidal activity at 1.5625 mg/mL. However, the methanol extract showed the lowest MBC compared to the hexane and ethyl acetate extracts at a concentration of 3.125 mg/mL. The respective MBC evaluation is shown in Figure 5.

The results showed that the ethyl acetate extract had better antibacterial activity than the hexane and methanol extracts. This could be related to the total phenolic and flavonoid values, where the ethyl acetate extract had the highest value compared to the others. Several physiological systems are known to be affected by polyphenols, which also support vitamin absorption and action, act as antioxidants, and have antimicrobial activities.[31] Phenolic chemicals are claimed to have antibacterial properties because of their capacity to disrupt bacterial membranes, complex proteins, and lipophilic cell walls.[32,33] Furthermore, reductones' antioxidant effect is based on destroying the free radical chain by donating a hydrogen atom. Like reductones, polyphenols may interact with free radicals to convert them into more stable compounds and inhibit free radical chain reactions.[34]

According to Zhang *et al*.[2] *E. dulcis* plants contain Gallocatechin gallate, epicatechin gallate, and catechin gallate compounds from ethanol extract were reported to have potent antioxidant effects. In addition, other flavonoid compounds contained in this plant, namely fisetin, diosmetin, luteolin, and tectorigenin from methanol/ethyl acetate extract, were also reported to have antioxidant activity. Flavonoid compounds from methanol/ethyl acetate extract were also reported to have antibacterial activity. Flavonoid compounds with antioxidant and antibacterial activity are luteolin, diosmetin, fisetin, apigenin, jaceosidin, quercetin, rhamnocitrin, galangin, tectorigenin, and dihydrokaempferide.[2] Some of these compounds were found in the ethyl acetate extract, which is one of the determining factors for the extract's better activity than others. Further research is needed in the future to establish these claims.

Conclusion

The study results concluded that the ethyl acetate extract of *Eleocharis dulcis* has better activity against the antibacterial strains (*Aeromonas hydrophila*, *Aeromonas Salmonicida*, and *Streptococcus agalactiae*) that cause fish disease. The study also established that the ethyl acetate extract of *E. dulcis* showed better antioxidant activity than the hexane and methanol extracts



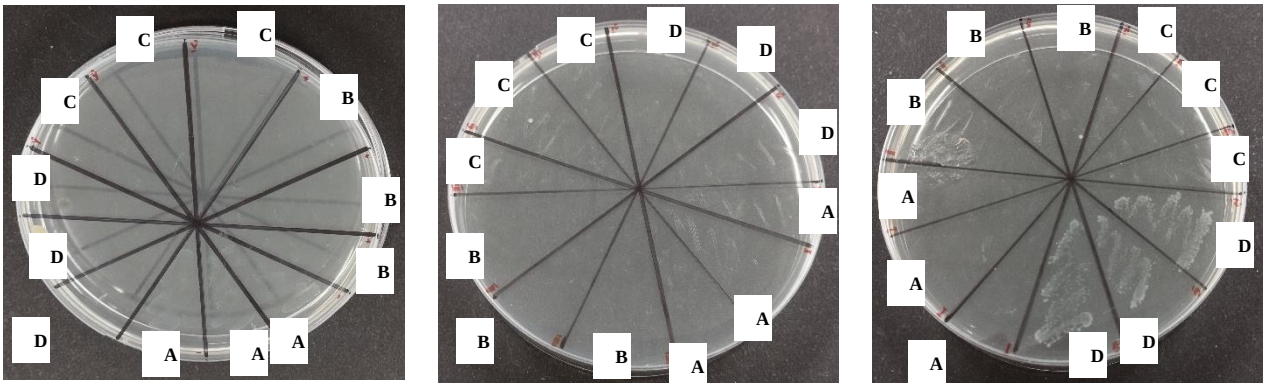


Figure 5: MBC test results of *E. dulcis* extracts against the different bacteria strains.
A: 12.5 mg/mL; B: 6,25 mg/mL; C: 3,125 mg/mL; D: 1.5625 mg/mL

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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