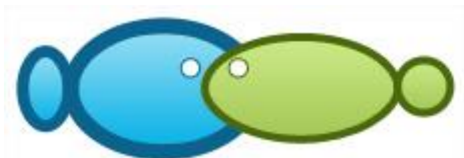

Chemical compounds screening of leaves extract from *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch and *in vitro* antibacterial pathogenic test for fish

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Abstract. *Eleocharis dulcis* (local name: 'purun tikus') is an aquatic plant endemic to Sulawesi, Indonesia. It is commonly found in the swamps of Southeast Asia. The plant presents antioxidant and antibacterial activities. The objective of this study was to evaluate the compound content and antibacterial activity of *E. dulcis* extracted with 70% ethanol using conventional maceration methods and with ultrasound-assisted extraction (UAE). Antibacterial activity assays were carried out using the disc diffusion method and microdilution. The evaluation of chemical compounds was conducted using GC-MS. The results showed that UAE extract had better antibacterial activity compared with extracts using traditional methods, with an inhibition zone diameter of 8.8 mm against *Aeromonas hydrophila* and 7 mm against *Flavobacterium columnare*. Minimum inhibitory concentration (MIC) values were 1.5625±0.015 mg mL^{-1} against *A. hydrophila* and 3.125±0.011 mg mL^{-1} against *F. columnare* and *Edwardsiella ictaluri*. GC-MS results determined 19 compounds in extract by the UAE method and 15 compounds by the maceration method. UAE provides better total phenolic content, antioxidant activity, and antibacterial activity. In addition, the number of compounds identified by GC-MS is more than that of the maceration method.
Key Words: antioxidant, GC-MS, maceration, MIC, UAE.

Introduction. Fish disease is one of the serious problems in fish farming. Fish disease can cause a decrease in the quality of fish flesh, growth inhibition, and even death. Fish diseases can be caused by infectious factors such as bacteria, viruses, fungi and parasites. They can also be caused by non-infectious factors, such as environmental conditions, nutrition and congenital abnormalities (Idowu et al 2017).

Fish disease can be caused by contaminated environmental conditions. In many cases, this is connected to an overload of free radicals. Free radicals can cause a decrease in immune responses and protein and lipid degradation (Lushchak 2016). This can be overcome by administering antioxidant compounds to fish. Antioxidant compounds can scavenge free radicals that can play a role in healing and in prophylaxis, increasing fish growth and preventing the degradation of several types of nutrients from fish (Kiron et al 2004).

Diseases in fish can be caused by several pathogenic bacteria that often attack fish, i. e. *Aeromonas hydrophila*, *Flavobacterium columnare* and *Edwardsiella ictaluri*. Fish infected with *A. hydrophila* can be diagnosed with Motile Aeromonas Septicemia (MAS), which is characterized by hemorrhage in internal organs, causing death. *F. columnare* can cause lesions of the fins and necrosis of the gills, while *E. ictaluri* can cause Edwarsiellosis, which is characterized by tissue necrosis and lesions of the head. Therefore, it is necessary to administer antibacterial compounds to fish (Austin & Austin 2012).

The use of synthetic antioxidants such as vitamin E in fish can increase the cost of cultivation because the price is relatively high. Synthetic antibacterial compounds from fish are reported to cause the accumulation of residues in humans consuming the fish, thus triggering resistance to antibiotics (Okocha et al 2018). Consequently, natural antioxidant and antibacterial compounds are needed. An example of natural antibacterial and antioxidant compounds contained in plants are phenolic compounds. Phenolic compounds have antioxidant activity based on their ability to donate single hydrogen atoms or electrons to free radicals (Gupta 2014). In addition, phenolic compounds can also damage the cytoplasmic membrane of bacterial cells (Rempe et al 2017).

Eleocharis dulcis is a water plant commonly found in swamps. *E. dulcis* is also known as 'purun tikus' or Chinese water chestnut, and originates from Southeast Asia. According to previous research, the methanol extract of *E. dulcis* leaves contains tannin, terpenoids, phenols, and flavonoids, which can play an important role in antioxidant and antibacterial activities (Rosyidah & Rohman 2018). Therefore, this study was conducted to determine the antioxidant and antibacterial activities from *E. dulcis* leaves extract against fish pathogenic bacteria, and to compare the results of activities obtained from 2 extraction methods, conventional and modern, maceration and ultrasound-assisted extraction (UAE), respectively.

Material and Method

Plant material. *E. dulcis* leaves was collected from Towuti Lake in South Sulawesi, Indonesia in October 2018 and this study was conducted from January to June 2019, using 850 g of simplicia. The leaves were sorted from contaminant/dirt, dried using direct sunlight and oven then powdered with blender until fine powder was obtained (40 mesh) (Ministry of Health Indonesia 2011). The identification of *E. dulcis* was conducted by the Botanical Division, Research Center for Biology, Indonesian Institute of Sciences (No. 253/IPH.1.01/If.07/1/2018).

Chemical compounds. Quercetin (Sigma-Aldrich, Q4951, USA), ascorbic acid (Sigma-Aldrich, A5960, USA), gallic acid (Sigma-Aldrich, USA), chloramphenicol, solvents and other chemicals were purchased from E. Merck (Germany).

Pathogenic bacteria. *A. hydrophila*, *E. ictaluri*, and *F. columnare* were obtained from the collections of the laboratories for fish disease research and development of the Ministry of Marine and Fisheries of the Republic of Indonesia.

Microscopic observation. Microscopic observations were carried out on dried leaf powder of *E. dulcis*. A scanning electron microscope (SEM) Model: JSM – IT 200 was used by the Zoology Field of the Biology Research Centre, Indonesian Institute of Sciences (LIPI), Cibinong, and a light microscope was used in the Faculty of Pharmacy, University of Indonesia.

Extraction. The dried leaves of *E. dulcis* were powdered and extracted with 2 extraction methods: maceration and UAE. The maceration extraction method was carried out by soaking simplicia in ethanol 70% (1:10) for 24 h in a closed vessel with occasional stirring. This step was repeated 3 times. The UAE method was carried out by soaking simplicia in 70% ethanol (1:10) for 1 h using UAE machine and repeating 3 times. After that, both liquid extracts were evaporated using a rotary vacuum evaporator and water bath at 50°C. The extraction yield (%) was calculated by dividing the weight of the thick extract obtained by the weight of the initial simplicial (Ministry of Health Indonesia 2011).

Antioxidant activity assay with DPPH method. For qualitative antioxidant activity test, quercetin solution and extract (1000 μg mL^{-1}) in methanol was loaded on silica TLC plates. It was sprayed with DPPH 150 μmol L^{-1} reagent and the TLC profile was observed using UV rays λ254 nm. The quantitative antioxidant activity assay was based on Bobo-García et al (2015) with minor modifications. An aliquot (20 μL) of a dilution series of extracts and

quercetin positive were added into the well. After that, 180 μL of DPPH was added and stirred for 60 seconds in the well. The mixture was incubated for 30 min in the dark at 37°C. Absorbance was measured using a microplate reader at 516 nm. The percentage of inhibition was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100\%$$

Based on the value of inhibition of each concentration, a calibration curve was determined and a linear regression value was calculated using the equation y=A+Bx. Antioxidant activity was expressed by 50% Inhibition Concentration (IC50), which is the concentration of samples that can reduce 50% of the DPPH concentration. IC50 values can be calculated by substituting y in the linear regression with 50 and obtaining x values as IC50.

Antioxidant activity assay with FRAP method. This assay was based on a method described by Benzie & Strain (1999). The calibration curve is constructed from the standard ammonium ferro sulphate (AFS) in methanol. Ascorbic acid was used as positive control. An aliquot (20 μL) of the test solution was mixed with 280 μL of FRAP reagent. Then it was incubated for 30 min in the dark at 37°C. Absorbance was measured using a microplate reader at 593 nm. Antioxidant activity assay with FRAP method was expressed as Ferrous Equivalent Antioxidant Capacity (FeEAC). The FeEAC value was calculated using the following equation:

$$\text{FeEAC}(\mu\text{mol/gr}) = \frac{\Delta A}{\text{GRAD}} \times \frac{\text{Av}}{\text{Spv}} \times \text{D} \times \frac{1}{\text{C sample}} \text{ x } 10^5$$

FeEAC is the equivalent antioxidant activity of iron ions (μmol g^{-1}), where A is absorbance change corrected by blank, GRAD (M^{-1}) is the gradient of the AFS calibration curve, Av is total volume of test solution (300μL), Spv is sample solution voulume (20μL), C sample is the sample concentration and D is the dilution factor.

Antibacterial activity assay with the disc diffusion method. This method is based on measuring the diameter of the inhibition zone formed around the paper disc. This diameter is related to the sensitivity of bacterial isolates and the rate of diffusion of the test sample through agar media. *A. hydrophila, F. columnare,* and *E. ictaluri* bacteria were cultured in tryptone soy agar (TSA) medium. The base medium was prepared in a petri dish with 10 mL Muller Hinton agar (MHA), which was frozen. Then a mixture of 1 mL of bacterial suspense (1-2 x 10^6 CFU mL^{-1}) in 4 mL of liquid MHA was poured into a petri dish containing the base layer. Afterwards, the paper disc with 20 μL of extract solution (200 mg mL^{-1} in 5% DMSO) was placed on the surface of the agar plate. It was incubated for 24 h at 30°C and the diameter of the inhibition zone was measured using a caliper. Chloramphenicol (30 μg mL^{-1}) was used as a positive control and DMSO was used as negative control (Novitaningrum 2018).

Antibacterial activity assay with the microdilution method. This assay was carried out 3 times using 96-well microplates. Serial 2-fold dilutions of extract solution were made in 5% DMSO. Chloramphenicol was used as antibiotic control. The control consisted of 50 μL dilution of chloramphenicol (30 μg mL^{-1}) with medium and 50 μL bacterial suspension. Germ control consisted of 50 μL medium and 50 μL bacterial suspension. Media control consisted of 100 μL medium. Negative control consisted of 50 μL medium and 50 μL 5% DMSO. The microplate was incubated for 24 hours at 30°C and the absorbance was measured at 600 nm using a microplate reader (Balouiri et al 2016).

The assay result was expressed as minimum inhibitory concentration (MIC). The determination of the MIC value was based on measuring turbidity by spectrophotometry. The principle of turbidimetry is based on increasing turbidity along with the increase in the number of bacteria in the medium. As the number of bacteria increases, less light will reach the detector. The change in the amount of light is considered as the percentage of

transmission (%T) and will be read on the instrument in logarithmic form, called absorbance or optical density (OD). OD values were measured at 600 nm with a microplate reader (OD600) (Tortora et al 2010).

Phytochemical screening. Phytochemical screening was performed to determine alkaloids with Bouchardat, Mayer and Dragendorff reagents. Flavonoid content was determined with Shinoda reagents and $AlCl_3$ spray; tannin with gelatin and $FeCl_3$; terpenoids with Lieberman-Burchard reagents; saponins with foam index; glycosides with Molisch reagents and anthraquinone with the Borntrager test.

GC-MS analysis. The final residue obtained from *E. dulcis* was subjected to GC-MS analysis. GC-MS analysis was performed with an Agilent Technologies 7890 Gas Chromatograph with auto sampler, 5975 Mass Selective Detector and Chemstation Data System. Compounds were separated on HP Ultra 2, with a capillary column of 30 m × 0.20 mm I.D × 0.11 (m) film thickness. Samples were injected with ionisation mode electron impact with an electron energy of 70 eV. Other conditions are 80°C for 0 min, rising with 3°C per min to 150°C, hold for 1 min and finally rising with 20°C per min to 280°C and hold for 26 min. Ion source temperature was maintained at 230°C, interface temperature at 280°C, quadrupole temperature at 140°C; the carrier gas was helium, column mode was at constant flow, flow column was at 1.2 mL min^{-1}, and the injection volume was 5 mL split 8:1. This method was developed by the Indonesian Spice and Medicinal Crops Research Institute (ISMCRI).

Determination of total phenol content. This assay was based on Bobo-García et al (2015) with modifications. An aliquot (25 μL) of standard gallic acid solution (1.25; 2.5; 3.75; 5; 6.25 and 7.5 μg mL^{-1}) and extract sample (500 μg mL^{-1}) was added into each well, then 100 μL of dilute Folin-Ciocalteu in aquadest (1:4) was added and stirred for 1 min in the microplate reader. The mix was incubated for 4 min at room temperature. 75 μL of Na_2CO_3 10% b/v was added to the well and shaken for 1 min in the microplate reader and re-incubated for 2 h at room temperature. Absorbance was measured using a microplate reader (Versa Max ELISA Microplate Reader, USA) at λ750 nm.

The absorbance and concentration of dilution series of standard gallic acid solution are plotted to obtain a calibration curve and a linear regression equation which will be used to calculate the total phenol content of the sample. Total phenolic contents were expressed as mg gallic acid equivalents (GAE) for each gram of extract sample that was calculated using the following equation:

$$\text{mg GAE/gram sample } = \frac{\text{total phenol } (\mu\text{g mL}^{-1}) \text{ x dilution factor x volume (mL)}}{\text{sample mass (gram)}}$$

Results and Discussion

Microscopic observations. The use of SEM was aimed to provide a 3-dimensional picture of the object being observed. The results of the microscopic identification of the dry powder from the simplicial leaves of *E. dulcis* are presented in Figure 1.

Extraction. Results showed that the extraction yield by the maceration method was 13.21%, while by the UAE method it was 14.76%. These results are in accordance with a previous study that showed that the extraction yield of the UAE method is higher than the maceration method, because ultrasonic waves cause cavitation, increasing the surface contact between the solvent and the simplicia. It can also increase the permeability of the cell wall, so that the phytochemical compounds can easily get out of the cell and dissolve in the solvent used (Azwanida 2015). Although the maceration method has a lower extraction yield than the UAE method, maceration is often chosen because it is easy to perform and only requires simple equipment (Azwanida 2015).

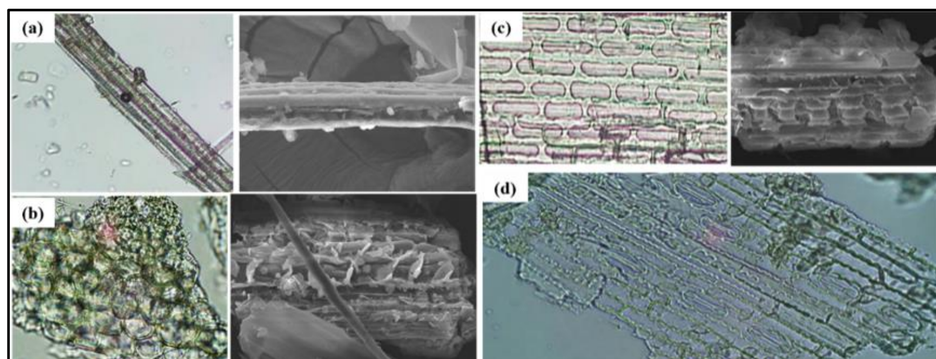

Figure 1. Microscopic results of *Eleocharis dulcis* leaves simplicia powder; a - vascular bundle; b - spongy parenchyma; c - collenchyma; d - epidermis with stomata.

Antioxidant activity test with the DPPH method. From the assay results, IC50 quercetin was 2.9809 mg L^{-1}. For *E. dulcis* leaves extract, IC50 values were 46.9118 mg L^{-1} for the maceration method and 41.0015 mg L^{-1} for the UAE method. Compared to a previous study, *E. dulcis* leaves ethanol extract had higher antioxidant activity than methanol extract with same extraction method (maceration) (Rosyidah & Rohman 2018). Based on previous research, methanol extract has antioxidant activity with an IC50 value of 58.58 ppm (Rosyidah & Rohman 2018).

Antioxidant activity assay with the FRAP method. The results are presented in Table 1. Extract from the UAE method had higher FeEAC value, meaning it had higher antioxidant activity than that of the maceration method. Based on a previous study, extract from the UAE extraction method had better antioxidant activity than that of the maceration method. This occurred because ultrasonic cavitation generates currents in the solvent, leading to elevate the mass transfer rate of antioxidant phytochemical compound to solvent medium (Safdar et al 2016).

Table 1

FRAP antioxidant activity assay results

Sample	*FeEAC (μmol g^{-1})*
Ascorbic acid	2539.241
Eleocharis dulcis leaves extract from maceration method	223.107
Eleocharis dulcis leaves extract from UAE method	317.951

Note: FeEAC - ferrous equivalent antioxidant capacity; UAE - ultrasound-assisted extraction.

Antibacterial activity assay with the disc diffusion method. The inhibitory zone of *E. dulcis* leaves extract against *A. hydrophila*, *F. columnare* and *E. ictaluri* bacteria are presented in Table 2. The results of antibacterial activity assay using the disc diffusion method show differences in the inhibitory zones against each bacteria due to difference of morphology/structure of each bacteria. In *E. ictaluri* bacteria, both *E. dulcis* leaves extracts did not formed inhibitory zones. This was caused by the very complex structure of gram-negative bacteria. The outer membrane of gram-negative bacteria consists of phospholipids, lipoproteins and lipopolysaccharides. Lipopolysaccharides consist of lipid-A, which is an endotoxin, and polysaccharide groups called O-polysaccharides, which function as specific antigens (Radji 2010). The core role of O-polysaccharide is that of a permeation barrier for come antibacterial compounds, such as hydrophobic antibacterial compounds (Radji 2010). However, the absence of inhibition zones in the disc diffusion method does not mean that the compound does not have any antibacterial activity, especially for less polar compounds, which diffuse more slowly into the culture media. The disc diffusion method is not suitable for antimicrobial compounds from natural materials that are difficult to dissolve or cannot dissolve in water, preventing the optimal diffusion through the agar media (Klančnik et al 2010).

Table 2

Antibacterial activity assay with disc diffusion method results

Sample	Inhibition zone (mm)		
	Aeromonas hydrophila	*Flavobacterium columnare*	*Edwardsiella ictaluri*
Positive control (Chloramphenicol 30 μg mL^{-1})	27	18	55
Negative control (DMSO)	-	-	-
Extract from the maceration method	7±0.17	6.4±0.16	-
Extract from the UAE method	8.8±0.15	7±0.23	-

Note: DMSO - dimethyl sulfoxide; UAE - ultrasound-assisted extraction; negative (-) value means that there was no inhibition zone.

Antibacterial activity assay with the microdilution method. The results from measuring the OD and MIC values are presented in Table 3. The MIC value was chosen when the OD value was close to the OD value of the comparative antibiotic. When the condition was compared, the antibiotic could inhibit bacterial growth. Antibacterial activity of plant extracts can be considered strong, if the MIC value is below 100 μg mL^{-1}; medium, if the MIC value is between 100-625 μg mL^{-1}; and weak, if the MIC value is above 625 μg mL^{-1} (Kuete 2010). *E. dulcis* extracts are classified as very weak antibacterial compounds against the bacteria *A. hydrophila, E. ictaluri* and *F. columnare*. This might be caused by extraction solvents using 70% ethanol, so that the extracted metabolites were mostly polar, whereas all test bacteria were gram negative. The outer membrane of gram negative bacteria consists of phospholipids, composed of lipid A, which is nonpolar. Thus, it is difficult for antibacterial compounds in extracts to penetrate the bacterial cell wall. In addition, this can also be caused by inaccurate optical density readings because of dead bacterial cell debris and high extract concentrations affecting the turbidity of the test solution and the optical density value.

Table 3

The results of antibacterial activity assay using microdilution method

Tested bacteria	*Sample*	*Optical density (mg mL^{-1})*					*MIC (mg mL^{-1})*
		12.5	*6.25*	*3.125*	*1.5625*	*0.78125*	
Aeromonas hydrophila	Maceration extract	0.56	0.652	0.766	0.853	0.962	1.5625
	UAE extract	0.58	0.636	0.748	0.807	0.935	1.5625
	Positive control			0.819			-
Flavobacterium columnare	Maceration extract	0.436	0.489	0.764	0.964	1.365	6.25
	UAE extract	0.395	0.441	0.48	0.778	0.912	3.125
	Positive control			0.489			-
Edwardsiella ictaluri	Maceration extract	0.383	0.469	0.583	0.627	0.816	6.25
	UAE extract	0.335	0.344	0.42	0.598	0.706	3.125
	Positive control			0.44			-

Note: UAE - ultrasound-assisted extraction; MIC - minimum inhibitory concentration.

Phytochemical screening. The results from phytochemical screening showed that the extract contains flavonoids, terpenes, tannins, glycosides and anthraquinone, while the results were negative for alkaloids and saponins.

GC-MS analysis. The results pertaining to the GC-MS analysis are presented in Tables 4 and 5. 15 compounds were detected in the ethanolic extract with the maceration method, and 19 compounds were detected in the ethanolic extract with the UAE method. All compounds identified from the maceration method in GC-MS analysis were different, except for 4H-Pyran-4-one, 2.3-dihydro, 3.5-dihydroxy, and 6-methyl-3.41, in which the percentage of the extract from UAE was higher. In addition, the result of maceration was 3.41% with molecular formula $C_6H_80_4$ and MW of 144.1253.

The GC-MS analysis results indicated that there were differences in the percentage of components detected in the oleoresin extracted by various techniques. UAE showed

substantial advantages over conventional maceration. Similar results were obtained by Morsy (2016) on oleoresin extract from nutmeg (*Myristica fragrans*) seeds, where oleoresin and a stronger aroma were obtained from the UAE technique. The difference in the types of compounds resulted from the maceration and UAE methods was caused by ultrasonic waves from UAE, which broke the cell walls and increased the solvent penetration in the cells. It caused enlargement in the pores of the cell wall, which increased the diffusion process and accelerated mass transfer, improved cell structure and resulted in an increase in mass diffusion (Morsy 2016).

Table 4

Components from *Eleocharis dulcis* leaves extract using the maceration method

No	*Chemical compounds*	*Concentration (%)*	*Quality*	*Retention time*
1	Pentitol	3.73	64	4.049
2	Eryhritol	3.44	64	4.235
3	4 (1H)-Pyrimidinone, 2.6-Diamino	2.70	58	5.000
4	4H-Pyran-4-one, 2.3-dihydro-3.5-dihydroxy-6-methyl	16.89	95	6.048
5	4H-Pyran-4-one, 2.3-dihydro-3.5-dihydroxy-6-methyl-3.41	3.41	43	6.2216
6	Octanal	1.19	46	7.821
7	5-(Hydroxymethyl)-2-Furaldehyde	41.65	87	8.931
8	acetin	8.22	50	9.538
9	Butyric acid, 3-hydroxy	1.30	27	26.066
10	d-Mannitol, 1.4-anhydro	1.97	38	26.162
11	imidazole, 2-fluoro-5-hydroxy-1-ribofuranosyl	1.99	35	26.431
12	6-Desoxy-1-altirol	2.73	46	26.555
13	Palmitic acid	2.14	98	28.120
14	Cyclopentadecanone, 2-hydroxy	3.38	96	29.210
15	Oxacyclotetradecan-2-one	1.15	59	29.327

Table 5

Components detected in *Eleocharis dulcis* leaves extract using the UAE method

No	*Chemical compounds*	*Concentration (%)*	*Quality*	*Retention time*
1	Methyl 2-furoate	2.34	30	4.573
2	Cyclopentanone, dimethylhydrazone	3.10	50	5.069
3	4H-Pyran-4-one, 2.3-dihydro-3.5-dihydroxy-6-methyl-3.41	11.37	96	6.124
4	Butanal, dimethylhydrazone	1.01	53	8.214
5	2-Furancarboxaldehyde, 5-(Hydroxymethyl)	39.90	87	9.289
6	Butanedioic acid	3.18	32	9.841
7	2-Methoxy-4-Vinylphenol	1.18	95	10.517
8	3H-1.2.4-Triazole-3-Thione, 1.2-Dihydro-4-Methyl	1.08	35	11.572
9	2.5-Cyclohexadiene-1.4-Dione, 2-methoxymethyl)-3, 5-Dimethyl	1.35	90	19.480
10	Methyl beta-d-galactopyranoside	3.25	43	25.466
11	2H-Pyran-2-One, 3.6-Dimethyl	1.66	38	26.617
12	3-Deoxy-d-mannoic lactone	2.74	49	27.114
13	2R, 3S-9-[1.3.4-Trihydroxy-2-butoxymethyl] guanine	1.67	38	27.196
14	1-Gala-1-ido-octanic lactone	1.25	43	27.293
15	Propene-3.3.3-D3	2.62	38	27.417
16	Hexadecanoic acid, methyl ester	1.68	98	27.569
17	Hexadecanoic acid	5.20	99	28.169
18	Cis-13-Octadecenoic acid	4.50	99	29.230
19	Octadecanoic acid	1.39	95	29.341

Total phenol content. Based on the calibration curve of gallic acid standard solution, the linear regression equation was obtained: y=0.066x + 0.2038 (r=0.99654). This was used to calculate the total phenol content of the sample. The total phenol content of *E. dulcis* leaves extract by the maceration method was 79.0797 mg GAE g^{-1}, while by the UAE method was 85.0154 mg GAE g^{-1}. Similar to the results of Safdar et al (2016), mango peel extract with UAE method had higher phenol content than that obtained by the maceration method because the phenomenon of cavitation leads to cell disruption and elevates phenol compound rate extraction.

Conclusions. *E. dulcis* leaves extract obtained using the UAE extraction method shows higher antioxidant activity (IC^{50} 41.0015 µg mL^{-1}) and antibacterial activity against fish pathogenic bacteria (MIC values are 1.5625 mg mL^{-1} against *A. hydrophila* and 3.125 mg mL^{-1} against *F. columnare* and *E. ictaluri*) compared to the extract obtained by the maceration method. Based on the GC-MS analysis, *E. dulcis* leaves extract has 19 chemical compounds with the UAE and 15 compounds with the maceration extraction method.

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