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Phytochemical analysis and antibacterial activities of *Eleutherine bulbosa* (Mill.) Urb. extract against *Vibrio parahaemolyticus*

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ABSTRACT

Objective: To analyze compounds in *Eleutherine bulbosa* (*E. bulbosa*) (Mill.) Urb. extract and to determine its antibacterial capability against *Vibrio parahaemolyticus* (*V. parahaemolyticus*).

Methods: *E. bulbosa* bulb extract was preprared using 96% ethanol by the maceration method. Phytochemical investigation of *E. bulbosa* extract was analyzed using GC-MS, spectrophotometry and titrimetry methods. The zone of inhibition was identified by the diffusion agar method. The minimum inhibitory concentration and minimum bactericidal concentration were determined using the plate count method. The inhibitory rate against *V. parahaemolyticus* was determined by the microdilution method. Cellular leakage was evaluated by spectrophotometry and cellular damage was observed by scanning electron microscopy.

Results: GC-MS analysis showed the high compound of the *E. bulbosa* extract was securixanthone E (7-hydroxy-1,2-dimethoxyxanthone). The compound groups also included fatty acid esters, isoquinolines, naphthalenes, and phenolics. The total phenolic content was (2.50 ± 0.00) mg/g, flavonoid (6.61 ± 0.00) mg/g, and tannin (0.03 ± 0.00)%. The greatest zone of inhibition and inhibitory rate were (11.83 ± 0.06) mm and (91.32 ± 2.76)%, respectively, at 10 mg/mL. The minimum inhibitory concentration was 0.156 mg/mL, while the minimum bactericidal concentration was 10 mg/mL. The *E. bulbosa* extract caused leakage and cellular damage to *V. parahaemolyticus*.

Conclusions: The *E. bulbosa* extract possesses inhibitory activities against *V. parahaemolyticus* and causes cellular leakage and damage.

1. Introduction

Vibrio parahaemolyticus (*V. parahaemolyticus*) is a normal (opportunistic) flora in the aquacultural environment, but it is pathogenic to both cultivated organisms and humans[1]. This bacterium is found in marine environments and in seafood[2,3]. Controlling opportunistic pathogenic bacteria in aquaculture using measures such as prophylactic chemotherapy or antibiotics is commonly known. However, the use of antibiotics has been banned because it could lead to an increased number of antibiotic-resistant bacteria and it leaves antibiotic residue in cultivated organisms which could potentially harm both humans and the aquatic environment[4,5]. Application of medicinal plants is one of the bacterial disease control methods[6]. Natural compounds found in

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plants or medicinal herbs are not dangerous and could potentially be an alternative to antibiotics in cultivation[7,8]. Secondary metabolites from plants are potential antimicrobial substances[9].

Eleutherine bulbosa (*E. palmifolia*) (Mill.) Urb. (in previous publications, it was called *E. palmifolia*, but after being identified, it includes the species *E. bulbosa*) is a medicinal plant that is known for its antimicrobial properties; it can inhibit the replication of viruses and has anti-inflammatory and anti-hypertension activities[10]. Extracts of *E. palmifolia* have antidiabetic and antioxidant properties[11]. The potential of *E. bulbosa* (Mill.) Urb. extract as an antimicrobial has been proven to inhibit a number of bacteria species such as *Staphylococcus aureus*[12,13], and *Vibrio harveyi*[14].

The evaluation of antimicrobial plant extracts *in vitro* could provide information on the inhibitory effect of the extract, for example, the bactericidal or bacteriostatic effect and the cellular damage against microorganism[15]. In addition, an important characteristic that needs to be evaluated in plant extracts is their hydrophobic capability on the lipids in the bacterial cell membrane, *i.e.*, their capability to damage cellular structure to ultimately cause death[16]. The inhibitory effect and damage to bacterial cells depend on the time and concentration of the extract[15]. Therefore, this study aimed to analyze the compounds found in *E. bulbosa* (Mill.) Urb. extract and to determine its capability as an antibacterial against *V. parahaemolyticus*.

2. Materials and methods

2.1. E. bulbosa extract preparation

E. bulbosa was obtained from Kanawa Village, Buton Regency, South East Sulawesi, Indonesia, in April 2017. The *E. bulbosa* was then identified at the Botany Division, Reseach Center for Biology, Indonesian Institute of Sciences (No. 1000/1PH.1.01/11.07/V/2018). Extraction of the *E. bulbosa* was carried out with 96% ethanol using the maceration method[14]. *E. bulbosa* bulbs aged 3-4 months or those that have flowered were cleaned, thinly sliced and then dried in an oven for 48 h at a temperature of 60 ℃. The *E. bulbosa* was powdered using a blender, then extracted using 96% ethanol at a ratio of 1:4 (w/v) and macerated for 24 h at room temperature using a magnetic stirrer. The maceration results were filtered using Whatman's filter paper's No 41, and then re-macerated twice using the same method. The maceration results were thickened using a vacuum evaporator at 40 ℃ and the extract was then stored in a freezer at -20 ℃ for further analysis.

2.2. Bacterial preparation

The strain of *V. parahaemolyticus* MPL was collected from the Research Institute for Coastal Aquaculture and Fisheries Extention, Maros, South Sulawesi, Indonesia. This isolate was originally from vaname shrimp when an outbreak occurred in a shrimp farm in Lampung Province, Indonesia. *V. parahaemolyticus* was grown at 37 ℃ and reared at 4 ℃ on sea water complete (SWC) agar slant (1 g of yeast extract, 3 mL of glycerol, 5 g of bactopeptone, 250 mL of distilled water, and 750 mL of seawater). The mutant cell of *V. parahaemolyticus* was prepared to be resistant using 50 μg/mL of the antibiotic rifampicin (0.25 g of rifampicin, 9.5 mL of absolute ethanol, 0.5 mL of aqua bidestilata).

2.3. Analysis of the E. bulbosa extract with gas chromatography-mass spectrometry (GC-MS)

The *E. bulbosa* extract was dissolved in ethanol at a ratio of 1:1 (b/v). The equipment employed was a GC-MS 5973 Pyrolysis, Agilent Technology. The sample injection volume was 2 μL, and the type of column was HP-5MS (30 m long, 0.5 mm in diameter, 0.25 μm wide). Helium gas (99.999%) was used as the carrying gas at a total flow of 104 μL/min, for a run time of 30 minutes at an oven temperature of 50 ℃, injector temperature of 290 ℃, and aux temperature of 290 ℃. The number of compounds obtained was reflected by the number of peaks in the chromatogram. The names of the compounds found were interpreted based on the mass spectra data of each peak matched to the GC-MS Pyrolysis database.

2.4. Total phenolic, flavonoid and tannin analysis

The total phenolic content in the *E. bulbosa* extract was measured using the spectrophotometry method[17]. An amount of 5 mg of the *E. bulbosa* extract was dissolved in 2 mL of 95% ethanol. This was then dissolved in 5 mL of distilled water and homogenized, then 0.5 mL of 50% (v/v) Folin-Ciocalteu reagent was added. The solution was allowed to rest for 5 min, then 1 mL of 5% sodium carbonate (b/v) was added. The solution was homogenized and incubated in a dark room at room temperature (28-29 ℃) for 1 h. The absorbance was measured using a spectrophotometer at a wavelength of 725 nm. The standard solution used was gallic acid. The total phenolic content obtained was stated as gallic acid equivalent (GAE) in mg per gram dry extract.

The analysis of flavonoid content was conducted using the spectrophotometry method[18]. Ten μL of the *E. bulbosa* extract, 60 μL of methanol, 10 μL of aluminum chloride (10% w/v), 10 μL of potassium acetate (1 mol/L), and 120 μL of distilled water were mixed thoroughly and incubated at room temperature for 30 min. The absorbance was measured at 415 nm using a spectrophotometer. The flavonoid content was stated in quercetin equivalent (QE) in mg per gram dry extract.

The analysis of tannin content was conducted using the titrimetric method[18]. An amount of 0.2 g of the *E. bulbosa* extract was dissolved in hot water, boiled for 30 min and then allowed to settle. An amount of 2.5 mL of this solution was mixed with 2.5 mL of indigo carmine solution (0.01 grams of indigo carmine dissolved in 0.25 mL of sulphuric acid). This was then titrated with 0.1 N $KMnO_4$ and the resulting tannin content percentage was calculated.

2.5. Antibacterial activity

The antibacterial activity of the *E. bulbosa* extract was evaluated using the diffusion agar method[19]. Extract of *E. bulbosa* was diluted using PBS solution at concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 mg/mL (b/v). An amount of 100 μL of the *V. parahaemolyticus* which had been cultured for 14 h in liquid SWC medium (1 g of yeast extract, 3 mL of glycerol, 5 g of bactopeptone, 250 mL of distilled water, 750 mL of seawater) at a temperature of 28-29 ℃ and a speed of 140 rpm (10^8 CFU/mL) was collected and spread on to SWC agar medium. Sterile paper discs (Whatman filter paper No. 41, 5.5 mm in diameter) that had *E. bulbosa* extract with different concentrations were affixed to the SWC agar medium. The negative control used PBS solution, while the positive control used 30 μg/mL of chloramphenicol, each in triplicate. The medium was then incubated for 24 h at 37 ℃. The antibacterial activity of the *E. bulbosa* extract was measured based on the diameter of the zone of inhibition surrounding the paper discs.

2.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of the MIC and MBC was done using the plate count method[20] with minor modifications. A serial dilution of the *E. bulbosa* extract stock at a concentration of 20 mg/mL (1:1 dilution factor) was conducted in test tubes using liquid SWC medium until the treatment concentrations reached 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 mg/mL. The negative control used PBS solution, while the positive control used 30 μg/mL of chloramphenicol, each in triplicate. Each test tube was then inoculated using 100 μL of the *V. parahaemolyticus* suspension (10^8 CFU/mL) which had been rinsed twice using PBS solution (phosphate buffer saline: 8 g of NaCl, 1.5 of g Na_2HPO_4, 0.2 g of KCL, 0.2 g of KH_2PO_4, 1 000 mL of distilled water). The number of colonies in the negative control was counted before the incubation (data for 0 h as the C* treatment). All the treatment test tubes were then agitated in a shaker at a temperature of 28-29 ℃ and a speed of 140 rpm for 24 h. The suspension was counted by re-culturing 100 μL of each treatment in thiosulphate citrate bile-salt sucrose medium and then incubated for 24 h. MIC was the lowest concentration which could inhibit the growth of bacteria (bacteriostatic) using negative control prior to incubation (C*) as the comparison. The MBC was calculated as the lowest concentration which could kill 99% of the bacteria (bactericidal).

2.7. Inhibition of V. parahaemolyticus

The inhibition rate was calculated based on the microdilution method using microplates[20] with minor modifications. The dilution factor was 1:1, and liquid SWC medium was used to produce treatment concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 mg/mL. Each well was filled with 100 μL of the treatment liquid and inoculated with 10 μL of *V. parahaemolyticus* (10^8 CFU/mL). The treatment suspensions were agitated in a shaker at a temperature of 28-29 ℃ at a speed of 140 rpm for 24 h and then measured in a 630 nm microplate reader to determine the inhibitory rate. The sample blank was the absorbance of the treatments prior to inoculation with the *V. parahaemolyticus* suspension. The equation used to calculate the percentage of the inhibition of *V. parahaemolyticus* was as follows[20]:

Inhibitory rate (%) = (ODr – ODs)/(ODr - ODb) × 100

Where ODr was the negative control's absorbance, ODs was the sample's absorbance, and ODb was the sample blank's absorbance.

2.8. V. parahaemolyticus cell leakage and damage

The method used for analyzing *V. parahaemolyticus* cell leakage in the present study was a modification of the studies by Oonmetta-aree *et al.*[21] and Bunduki *et al.*[22] using spectrophotometry, whereas observations of the *V. parahaemolyticus* cell damage were conducted using scanning electron microscopy (SEM)[23]. The concentrations of the *E. bulbosa* extract used in the observations of *V. parahaemolyticus* cell leakage were 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 mg/mL (b/v), whereas the concentration for observing the *V. parahaemolyticus* cell damage was the concentration which resulted in the smallest (0.156 mg/mL), middle-sized (1.25 mg/mL), and largest zones (10 mg/mL) of inhibition in the antibacterial test.

Test tubes were filled with 90 μL of *E. bulbosa* extract. The negative control used PBS solution, and the positive control used 30 μg/mL of chloramphenicol. Each treatment was directly exposed to 10 μL of the *V. parahaemolyticus* bacterial suspension which had been cultured in liquid SWC for 14 h at 28-29 ℃ and rinsed twice using PBS solution (at a density of 10^8 CFU/mL). These were then homogenized and incubated for 24 h. The suspension was centrifuged for 10 min at a speed of 10 000 rpm. The supernatant was used for analyzing cellular leakage, while the pellet for *V. parahaemolyticus* cellular damage. The supernatant was filtered using a 0.20 μm acrodisc, and then the optical density of the supernatant was analyzed using a spectrophotometer UV-200-RS at a wavelength of 260 nm to determine the cellular nucleic acid while the 280 nm wavelength was used to determine the cellular protein. Observations of the cellular leakage were conducted for 0, 2, 6, 12, and 24 h.

The pellet was rinsed twice with PBS solution, then the supernatant was disposed. The remaining pellet was mixed with 2.5% glutaraldehyde (pH 7.3) and allowed to stand for 2 h. This was then fixated for 2 h using 1% osmium tetraoxide and rinsed three times with aqua bidestillata. The dehydration process was conducted in stages using 25%, 50%, 75%, and 100% alcohol, respectively, each for 10 min. Then, this was affixed to aluminum stubs and coated with gold using a vacuum process for 20 min. The preparation was then observed and documented using SEM (JSM-5310LV, Japan).

Table 1. Compounds of *Eleutherine bulbosa* extract by GC-MS pyrolysis.

Retion time (min)	Area (%)	Name of compound	Compound group	Function	References
14.27	9.06	Hexadecanoic acid, ethyl ester	Fatty acid esters	Antibacterial; antioxidant	[24,25]
15.37	1.75	Isoquinoline-1-carbonitrile	Isoquinolines	Antibacterial; anticancer; anti-inflammatory; Antidepressant; antimalarial; anti-HIV	[26–31]
15.46	23.76	Linoleic acid, ethyl ester	Fatty acid esters	Hypocholesterolemic, nematicide, antiarthritic, hepatoprotective, anti-androgenic, 5-alpha reductase inhibitor, antihistaminic, anticoronary, insectifuge, anti-eczemic, Anti-acne	[25]
15.61	2.81	Octadecanoic acid, ethyl ester	Fatty acid esters	Antimicrobial activity	[32]
16.31	31.42	Securixanthone E (7-hydroxy-1,2-di methoxyxanthone)	Xanthones	Antioxidant	[33]
16.69	19.20	2(1H)-Phenanthrenone		Antioxidant & antiinflammatory activities	[34]
18.10	8.89	2,3-dihhydro-2,2,3,3-tetramethyl-6-Butyl-1,4-dimethoxynaphthalene	Naphthalenes	Antibacterial; viral replication inhibitor; Antioxidant	[35–37]
19.50	3.14	2H-1-Benzopyran-2-one	Phenolics	Antioxidant; Bacteriostatic and anti-tumor activity; Cancer-preventive and used as flavours	[38–40]

2.9. Data analysis

The data were analyzed qualitatively and quantitatively and expressed as mean ± standard deviation (SD). Statistical analysis was conducted using Analysis of Variance (ANOVA), then followed by Duncan's test using the SPSS (Statistical Program Software System) program version 16.0. Significant differences were those where $P<0.05$ or $P<0.01$.

3. Results

3.1. Analysis of the E. bulbosa extract with GC–MS

GC-MS showed that the high compound found in the *E. bulbosa* extract was securixanthone E (7-hydroxy-1,2-dimethoxyxanthone) (31.42%) (Table 1). A number of compounds found in this study were new compounds that had not been discovered in previous studies of *E. bulbosa*; therefore, the functions of these compounds were taken from other studies of different plants.

3.2. Quantitative analysis of phytochemicals of E. bulbosa extract

The results of the phytochemical quantitative analysis revealed that the total phenolic content of the *E. bulbosa* extract in the present study was (2.50 ± 0.00) mg/g, flavonoid (6.61 ± 0.00) mg/g, and tannin (0.03 ± 0.00)%.

3.3. Antibacterial activity

The results on zone of inhibition test revealed that *E. bulbosa* extract could inhibit *V. parahaemolyticus* (Table 2). *E. bulbosa* extract at 10 mg/mL had the largest zone of inhibition [(11.83 ± 0.06) mm, $P<0.05$)]. This concentration was more bactericidal compared to the other concentrations which were bacteriostatic. The inhibitory zone diameters of *E. bulbosa* extract at all concentrations were significantly different ($P<0.05$) from the positive and negative control.

3.4. MIC and MBC

The MIC test revealed that the *E. bulbosa* extract at 0.156 mg/mL was not significantly different ($P>0.05$) from the negative control prior to incubation (C*); therefore, the MIC of the *E. bulbosa* extract on *V. parahaemolyticus* was 0.156 mg/mL (Figure 1). The MBC was 10 mg/mL. The *E. bulbosa* extract at 10 mg/mL and the positive control (30 μg/mL of chloramphenicol) inhibited *V. parahaemolyticus* to 0 log CFU/mL after 24 h of incubation.

3.5. Inhibition to V. parahaemolyticus

E. bulbosa extract at 0.156 mg/mL inhibited the growth of *V. parahaemolyticus* at (12.79 ± 2.68)%, whereas 10 mg/mL inhibited at (91.32 ± 2.76)% (Figure 2). All the treatment concentrations of the *E. bulbosa* extract were significantly different ($P<0.05$) from the negative control.

Table 2. Antibacterial activity of *Eleutherine bulbosa* extract at different concentrations against *Vibrio parahaemolyticus*.

Treatment	Inhibitory zone diameter (mm)
0.156 mg/mL	2.83 ± 0.06^{b}
0.313 mg/mL	3.17 ± 0.06^{b}
0.625 mg/mL	4.50 ± 0.10^{c}
1.25 mg/mL	5.50 ± 0.10^{cd}
2.5 mg/mL	6.17 ± 0.06^{d}
5 mg/mL	9.83 ± 0.06^{e}
10 mg/mL	11.83 ± 0.06^{f}
Chloramphenicol (30 μg/mL)	15.83 ± 0.06^{g}
PBS solution	0.00 ± 0.00^{a}

Data (mean ± SD) with different letters indicate significant difference ($P<0.05$).

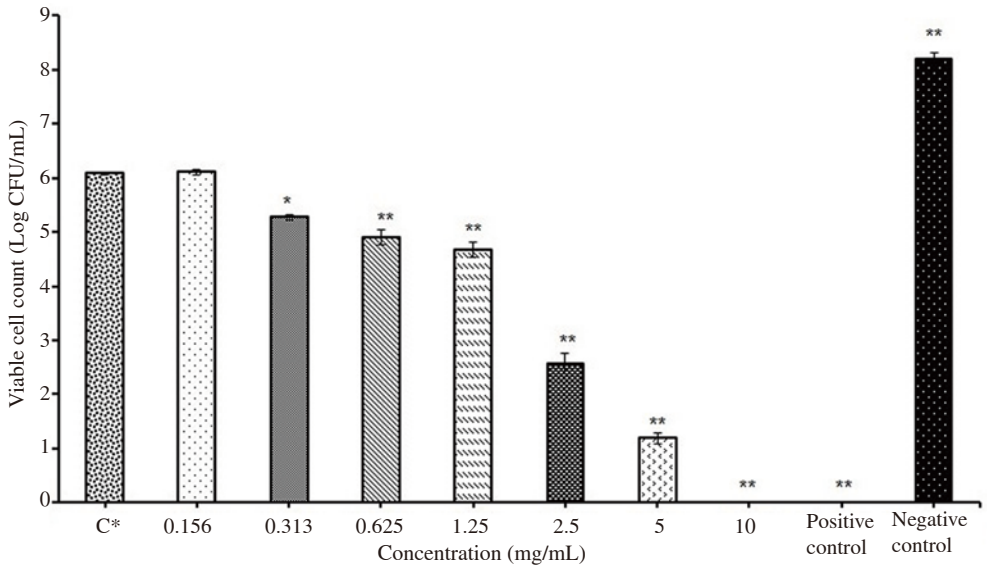

Figure 1. Effects of *Eleutherine bulbosa* extract on growth of *Vibrio parahaemolyticus* for 0 and 24 h. Data are expressed as mean ± SD. **P*<0.05; ***P*<0.01 when compared with the negative control without the addition of *Eleutherine bulbosa* extract for 0 h (C*).

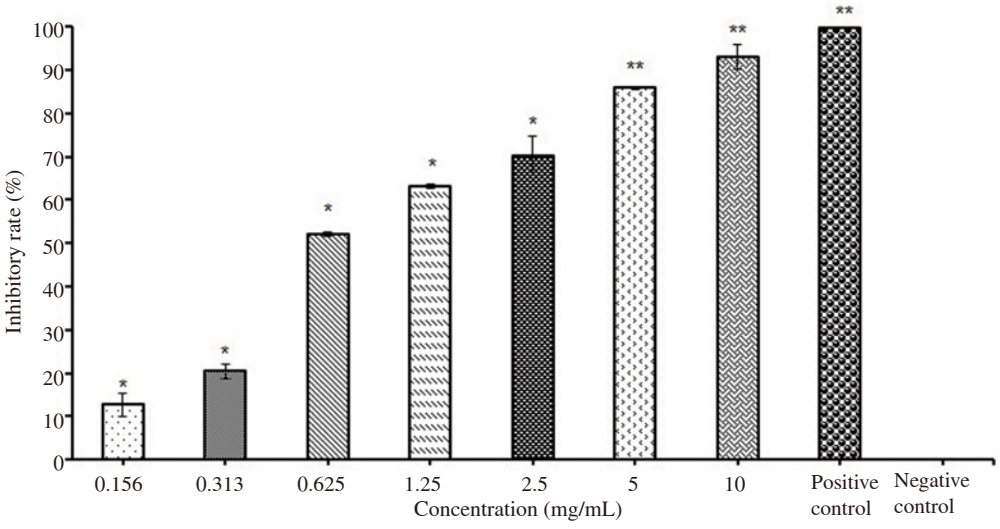

Figure 2. Inhibitory rate of *Eleutherine bulbosa* extract at different concentrations against *Vibrio parahaemolyticus*. Data are expressed as mean ± SD. **P*<0.05; ***P*<0.01, when compared with the negative control (PBS solution).

3.6. V. parahaemolyticus cell leakage and damage

The results of cell leakage demonstrated that after treatment with *E. bulbosa* extract for 2 h, the absorbance values were increased for both 260 nm and 280 nm, and there was no significant increase after 24 h of incubation in all the treatments except for the positive control (Figure 3). The higher the concentration of the *E. bulbosa* extracts was, the greater the damage to *V. parahaemolyticus* cells was.

Figure 4A shows that without the administration of the *E. bulbosa* extract, *V. parahaemolyticus* cell was short, curved rod with the cells flocking together and with smooth and whole cell surface. After treatment with *E. bulbosa* extract at 0.156 mg/mL, most cells were still whole and the surface was smooth, even though some cells had altered shapes. Some cells shrank with cellular leakage as indicated by the red arrow in Figure 4. At the concentration of 1.25 mg/mL, the *E. bulbosa* extract caused more cellular leakage and morphological damage than at the concentration of 0.156 mg/mL. *E. bulbosa* extract at 10 mg/mL caused most serious damage compared to the other concentrations. The positive control treatment using 30 μg/mL of chloramphenicol caused the destruction of *V. parahaemolyticus* cells into small parts.

4. Discussion

The antibacterial capability of *E. bulbosa* extract was attributed to the activity of the secondary metabolite compounds. A previous study revealed that the secondary metabolite compounds found in *E. bulbosa* extract include flavonoids, tannins, saponins, quinones, steroids, and triterpenoids[14]. This study also found fatty acid esters, isoquinolines, naphthalenes, and phenolics in *E. bulbosa* extract. Plant extracts contain many flavonoid compounds and phenolics, which could inhibit the growth of Gram-negative and Gram-positive bacteria[41]. Naphthalene-derivate compounds are

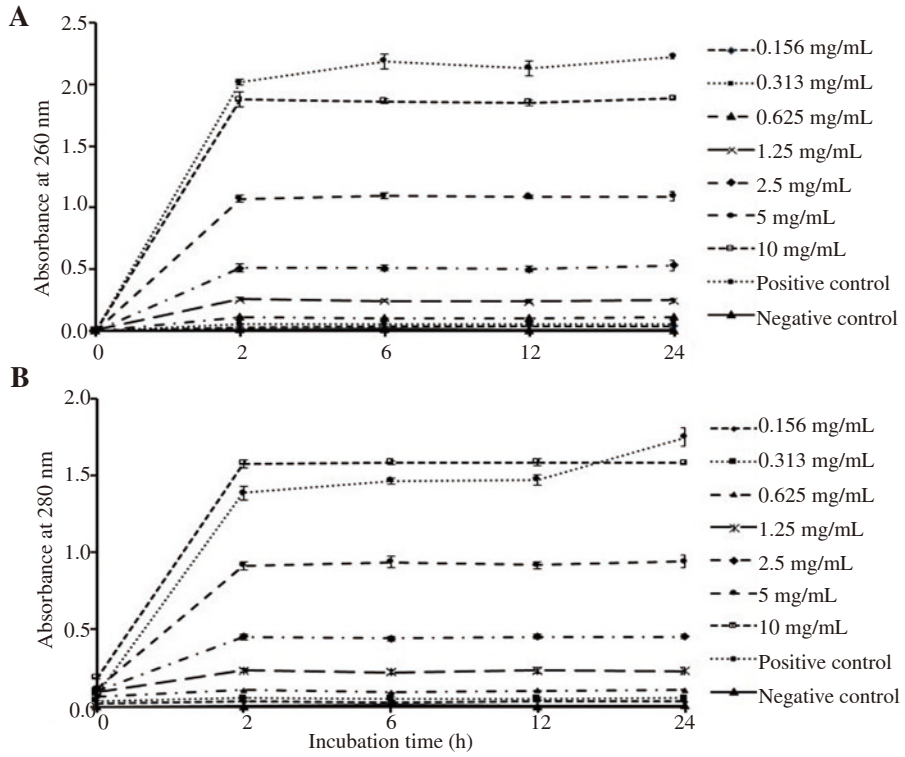

Figure 3. Absorbance of 260 nm (A) and 280 nm (B) after exposure to *Eleutherine bulbosa* extract at different concentrations. Positive control (30 μg/mL of chloramphenicol), negative control (PBS solution).

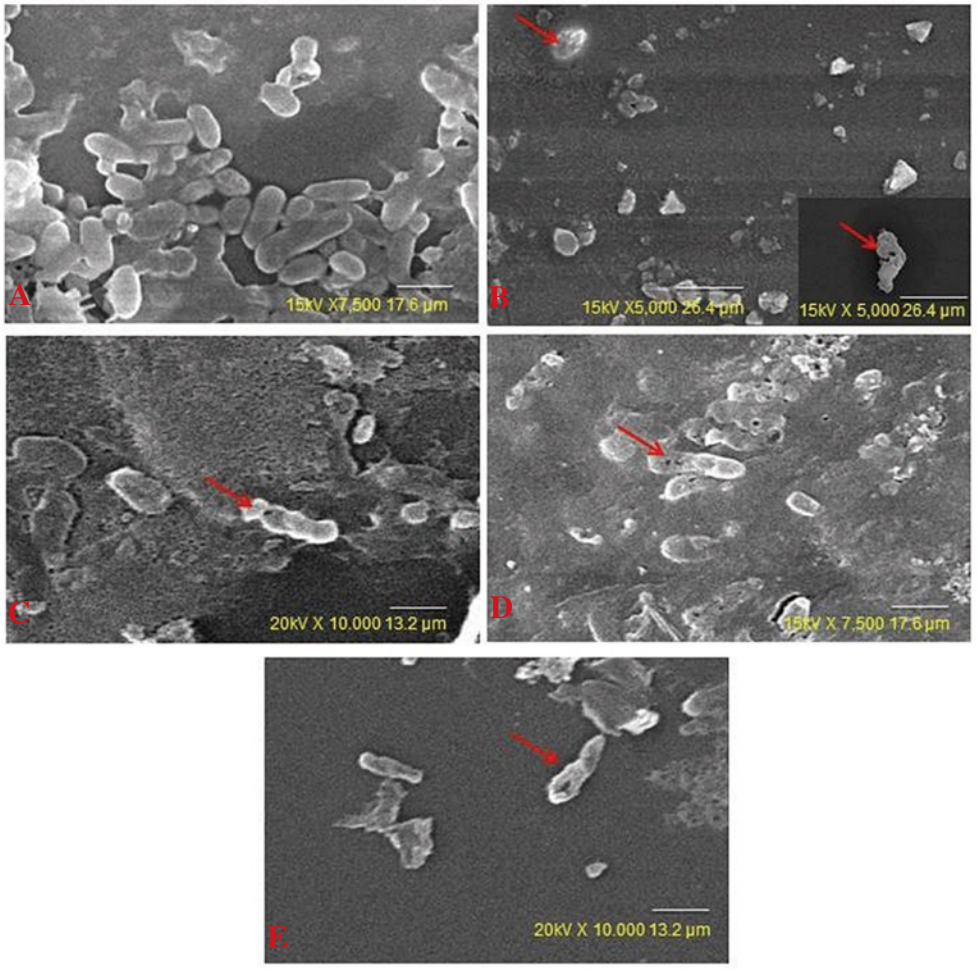

Figure 4. Morphology of *Vibrio parahaemolyticus* exposed to *Eleutherine bulbosa* extract. Arrows indicate damage or leakage to the cell membrane. A: negative control (PBS solution), B: positive control (30 μg/mL of chloramphenicol), administration of *Eleutherine bulbosa* extract: 0.156 mg/mL (C), 1.25 mg/mL (D), 10 mg/mL (E).

bioactive metabolites commonly found in the genus Eleutherine[42]. The naphthalene-derivate compounds in *Ewingella americana* are antibacterial[35]. In addition to antibacterial activity, the compounds of the *E. bulbosa* extract possess antioxidant, antivirus, and anticancer activities.

Our study showed that the zone of inhibition, the inhibition rate toward *V. parahaemolyticus*, the cellular leakage and damage rate are all concentration dependent. The *E. bulbosa* extract at 10 mg/mL inhibited *V. parahaemolyticus* to 0 log CFU/mL after 24 h of incubation, and was bactericidal. Another study found that *Ginkgo biloba* leaf extract could inhibit the growth of *Shewanella putrefaciens* to 0 log CFU/mL after 24 h with an inhibition rate of 100%, whereas the inhibition rate of the MIC dose toward *Saprophytic staphylococcus* was 22.78%[20]. The increase of absorbance in the cellular leakage test after 2 h of incubation demonstrated the presence of cellular leakage in the *V. parahaemolyticus*. Another study reported that the 4×MIC concentration of the *Polygonum minus* Huds. leaf extract could cause cellular leakage in *Escherichia coli* and *Staphylococcus aureus* after 30 min of incubation[43]. SEM displayed changes in the morphological changes due to the antibacterial activity of *E. bulbosa* extract against *V. parahaemolyticus*. The function of cell membrane is to maintain the balance of materials and energy in the cell in order to maintain the bacteria's activities[44]. Our study shows that without the administration of the *E. bulbosa* extract, *V. parahaemolyticus* cell was short, curved rod with the cells flocking together and with smooth and whole cell surface. *E. bulbosa* extract induced morphological and structural damage to *V. parahaemolyticus*. *E. bulbosa* extract at 10 mg/mL caused most serious damage compared to the other concentrations. The *V. parahaemolyticus* cell displayed morphological changes in shape; the cells shriveled and shrunk, causing leakage in the cell wall, and destroyed cell parts were also observed. Another study also demonstrated that extracts of medicinal plants can infiltrate the outer membrane of bacteria, disrupt cellular and metabolic functions, and cause loss of cellular contents, which ultimately causes the death of the bacteria cells[45]. *Ginkgo biloba* leaf extract could also cause damage to the cell membrane and cell wall, the SEM revealed that the bacterial cell structure altered, shriveled, adhered to each other, and some parts of the cells disintegrated and shrunken[20]. In conclusion, our study indicated that the secondary metabolite compounds found in the *E. bulbosa* extract could inhibit the growth of *V. parahaemolyticus*, result in damage to cellular morphology and cause cellular leakage.

Conflict of interest statement

Authors declare that there are no competing interests.

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