

***In Vitro* Activity Of *Eleutherine Palmifolia* L. Merr. Ethanolic Extract Against *Bacillus Cereus* Isolate**

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ABSTRACT

Aim: This research was conducted to determine the antibacterial activities, determine the value of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the *Eleutherine palmifolia* extract against isolate of *Bacillus cereus*.

Methods: Extraction of *E. palmifolia* was done by maceration method with ethanol 96%, phytochemical content and profile determination of *E. palmifolia* was done by phytochemical screening and thin layer chromatography. The antibacterial activity carried out by agar diffusion method, MIC and MBC value determined by dilution method using the test tube.

Results: The result showed that ethanolic extract of *E. palmifolia* has antibacterial activity against *Bacillus cereus* bacteria. This extract has MIC and MBC value of the extract in the concentration range of 1.25 – 2.5% (w/v).

Conclusion: The antibacterial activity of this extract probably derived from flavonoids, polyphenols, monoterpene and sesquiterpene compound contained. From the results suggest that the extract gave antibacterial effect and can be used to discover new drugs to control diarrhea infection and foodborne illness caused by *Bacillus cereus*.

Keywords: Antibacterial, *Bacillus cereus*, *Eleutherine palmifolia* L. Merr., Foodborne illness

INTRODUCTION

Bacillus cereus is an opportunistic member of the *Bacillus cereus* group, aerobic Gram-positive, spore-forming that exists ubiquitously in soil, marine environments, vegetables, and human skin. The bacteria exist as a spore former and as a vegetative cell when colonizing in the human body [1]. The most human pathogen in the *B. cereus* group is *B. cereus* [2]

B. cereus can cause a wide range of human diseases, including foodborne illness and systemic infection [3]. *B. cereus*-induced gastroenteritis, bloody diarrhea and emetic poisoning [4]. The diarrhea syndrome is caused by enterotoxins produced by *B. cereus*, the emetic syndrome is caused by cereulide during the growth phase in the food [5,6]. The ability of *B. cereus* to colonize and to form biofilms required for the persistence in various environments such as the host intestinal tract and subsequently invade the tissues, food, or hospital facilities [7].

Broad-spectrum antibiotics are usually used for treatment of *B. cereus* infection. Isolates *B. cereus* showed sensitive to vancomycin, gentamicin, and carbapenem. However, the isolates 48.3-100% resistant to cephalosporins and almost all to ampicillin, 65.5% resistant to clindamycin, 10.3% resistant to levofloxacin and trimethoprim [2,8]. Antibiotics resistance is

causing an increase in mortality and morbidity in the world, and also another side effects of antibiotics include kidney problems, abnormal blood clotting and blood disorder [9].

Plant extract for centuries have been utilized for human health, particularly in Asia [10]. Among those medicine that came from higher-plant origins, only a few are utilized as antimicrobials. However, toxicity and resistance issues have promoted interest in plant secondary metabolites as antimicrobials sources such as polyphenol, flavonoid, terpenoids, and essential oils [11].

One of the potential plant used for diarrhea treatment is *Eleutherine palmifolia* from Iridaceae family. *E. palmifolia* used by Dayak people in Borneo empirically to treat several disease, including diarrhea [12]. Previous study showed that *E. palmifolia* extracted with different polarity solvent (n-hexane, ethyl acetate, and ethanol 96%) gave an antimicrobial activity against several bacteria causing diarrhea such as methicillin-resistant *Staphylococcus aureus*, *B. cereus*, *Shigella* sp., and *Pseudomonas aeruginosa* [13]. Another study showed that *E. palmifolia* active against *Escherichia coli*, *Shigella sonnei*, *Shigella flexneri*, and *Shigella boydii* [14]. Hence, the purpose of this research was to determine antimicrobial activity from *E. palmifolia* ethanolic extract against isolate *B. cereus*.

MATERIALS AND METHODS

Materials

The chemical materials used consist of solvents and reagents. The solvents and reagents used are ethanol (Merck, Germany), ammonia (Merck, Germany), chloroform (Merck, Germany), hydrochloric acid (Merck, Germany), iodide mercury (Merck, Germany), potassium iodide (Merck, Germany), magnesium powder (Merck, Germany), amyl alcohol (Merck, Germany), ferric chloride (Merck, Germany), gelatin solution (Merck, Germany), diethyl ether (Merck, Germany), sodium hydroxide (Merck, Germany), dimethyl sulfoxide (DMSO) (SigmaAldrich, Germany), Mueller-Hinton Agar (Oxoid, Basingstoke, UK), Mueller-Hinton Broth (Oxoid, Basingstoke, UK), and distilled water.

The plant materials of *E. palmifolia* L. Merr. species was obtained from Tasikmalaya, West Java, Indonesia.

Plant Collection and Determination

Whole fresh bulb of *E. palmifolia* were obtained from Tasikmalaya region – West Java province, Indonesia. Plant determination was done in Plant Taxonomy Laboratory, Universitas Padjadjaran with number of determination letter: 135/HB/03/2017.

Extraction of *E. palmifolia* L. Merr.

Fresh bulb was extracted with ethanol 96% with sample-solvent ratio of 1:5 by maceration method in triplicate. Macerate obtained from extraction process was concentrated with rotary evaporator and then further concentrated with waterbath to obtained crude extract of *E. palmifolia*.

Phytochemical Screening

Phytochemical screening was done by Farnsworth method to determine the presence of alkaloid, polyphenolic, tannin, flavonoid, quinone, saponin, monoterpene & sesquiterpene, and steroid & triterpenoid compound in *E. palmifolia* bulb [15].

Thin Layer Chromatography Analysis

Thin layer chromatography (TLC) analysis was performed for *E. palmifolia* extract with silica gel GF₂₅₄ plate as stationary phase and *n*-hexane-ethyl acetate (7:3) as mobile phase. The chromatogram was observed under visible light, UV 254 and 366 nm light, and sprayed with vanillin sulphate spray reagent.

The preparation of the Bacteria Culture

Bacillus cereus isolate used in this study was obtained from rotten food, isolated at Pharmaceutical Microbiology and Biotechnology Laboratory, Pharmaceutical Biology Department, Universitas Padjadjaran. The bacteria maintained on the Mueller-Hinton Agar (MHA) slant. The preparation of the culture for assay was carried out by isolating a single colony of *B. cereus* aseptically from the MHA slant and planted in Mueller-Hinton Broth (MHB) medium, then incubated at 37°C for 16-18 hours. The turbidity of bacterial suspension was adjusted to 0.5 McFarland standards (1.5×10^8 CFU/mL) [16].

Antibacterial Activity Assay

Twenty μ L of bacterial suspension was placed and in 20 mL of molten agar (40-45°C) were poured into sterile Petri dishes (diameter 90 mm), then homogenized until the media and bacterial suspension mixed well, after that left a few minutes to allow the bacteria to adapt with the medium. Once the agar plates were aseptically dried, the agar plates have been perforated using a perforator 9 mm. The extract (10 mg/mL) was dissolved with dimethyl sulfoxide (DMSO) : water (1:9). The amount of 100 μ L extract solution in various concentrations were then transferred into each well and allowed to set. After that, all the plates were incubated at 37°C for 16-18 hours. Then, measured the diameter of bacterial inhibition using calipers [17,18].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Preparation of MIC sample solution made with some variation of the smallest concentration still has activity. After dilution of the extract, added an overnight culture of *B. cereus* and incubate at 37°C for 16-18 hours. After that, a subculture from each MIC tube was incorporated into the MHA plates and dispersed using a spreader aseptically. It was incubated at 37 °C for 18-24 hours [17,18].

RESULTS AND DISCUSSION

Extraction of *E. palmifolia* L. Merr.

The *E. palmifolia* bulb used for maceration method was 1000.14 g and 55.32 g crude ethanolic extract was obtained from the triplicate maceration. The yield of crude extract was 5.53%. *E. palmifolia* crude extract characteristic were brown to red color, bitter taste, and specific odor.

Phytochemical Screening

The phytochemical screening results showed that *E. palmifolia* bulb contain alkaloid, flavonoid, tannins polyphenolic, quinone, steroid and triterpene, and then monoterpene and sesquiterpene (Table 1). In another study, *E. palmifolia* bulb ethanolic extract was known to contain phenols, sterols, steroids, tannins, terpenoids, and flavonoids, thus the results were quite similar [19,20].

Table 1: *E. palmifolia* phytochemical screening result

Secondary Metabolite	Result
Alkaloids	+
Flavonoids	+
Polyphenolic	+
Tannins	+
Quinone	+
Saponins	-
Monoterpenes & Sesquiterpenes	+
Steroids & Triterpenoids	+

*(+): detected

(-) : absence

Thin Layer Chromatography

TLC was performed to determine the phytochemical profile in *E. palmifolia* extract. The results are shown in Table 2.

Table 2: Thin layer chromatography profile of *E. palmifolia* extract

Spot	Rf	Observation			
		Visible light	UV 254 nm	UV 366 nm	Vanillin sulphate
1	0.18	Pale yellow	Blue	Bright yellow	Brown
2	0.35	Pale yellow	-	Blue	Pale yellow
3	0.40	Yellow	Yellow	Blue	Brown
4	0.48	Pale yellow	-	Pale yellow	Yellow
5	0.55	Yellow	Yellow	Bright blue	Red
6	0.62	Yellow	Yellow	Bright blue	Red

*(-) : absence

The chromatogram profile resulted in 6 spots with varied Rf observed at visible light, UV 254 and 366 nm, and vanillin sulphate spray reagent (Table 2). It was also showed that *E. palmifolia* extract contained monoterpene or sesquiterpene compound spotted in spot 5 and 6 that react with vanillin sulphate reagent and gave a red colored spot, as mobile phase used was *n*-hexane-ethyl acetate (7:3) would desorpt less polar compound such as monoterpene or sesquiterpene.

Antibacterial Activity Assay Results

The extract of *E. palmifolia* L. Merr. were tested for its antibacterial activity against *B. cereus* isolate. The results showed that the extract had antibacterial activity against *B. cereus* isolate with various concentration are shown in Table 3, indicating that the antibacterial activity observed was due to the activity of secondary metabolites compound present in the extract and not caused by the solvent (DMSO) used in the extract solution. The amount of 1% (v/v) DMSO were also tested as a control solvent and no activity against *B. cereus* isolate.

This assay was also qualitatively evaluated by agar diffusion method with perforation technique. After 16-18 hours of incubation, the growth of *B. cereus* was inhibited by all concentration of the extract. The diameter of bacterial inhibition was measured with calipers.

Table 3: The Inhibition Diameter of The Extract Against *Bacillus cereus* Isolate Result

Sample	Concentration	Diameter Zone of Inhibition (mm)*
The ethanolic extract of <i>E. palmifolia</i> L. Merr	60% w/v	19.80 ± 0.02
	40% w/v	18.14 ± 0.02
	20% w/v	16.31 ± 0.02
	10% w/v	15.20 ± 0.02
Dimethyl sulfoxide (DMSO)	1% v/v	-

* data are the average of three replicates.

The diameter zone of bacterial inhibition of extract was directly proportional to increase as indicated by increase of the extract concentration. The inhibition zone (the presence of the clear zone around the well in the plates test) indicated that the secondary metabolites compound present in the extract can inhibit the growth of *B. cereus* isolate was probably derived from flavonoids, and polyphenols that contained in the extract, which are phenolic substances, and monoterpene or sesquiterpene as essential oils in plants that have antibacterial activity [11,21]. Related studies of antibacterial activity from crude extract containing flavonoids, and terpenoids had antibacterial activity against various bacteria [22]. The sites and number of hydroxyl group (-OH) on phenolic group are thought to be related to their relative toxicity to microorganism, with evidence that increased hydroxylation result in increased toxicity. The toxicity mechanism thought to included enzyme inhibition by the oxidized compound, possibly through reaction with sulfhydryl groups or through more nonspecific interaction with the proteins [11]. Flavonoids are known to have antimicrobial activity, probably due to their ability to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes [11]. Terpenoids and essential oils are also active against bacteria. The mechanism of action is not fully understood but it speculated to involve membrane disruption by the lipophilic compounds, as some study showed that increasing the hydrophilicity would drastically reduced their antimicrobial activity [11]. Tannins will disrupting the permeability of the cell membrane itself and the cell cannot perform life activities [23].

Determination of MIC and MBC Results

Table 4: The MIC and MBC Results

Extract Concentration (%w/v)	Bacterial Growth of <i>B. cereus</i> *
10	-
5	-
2.5	-
1.25	+
0.625	+
0.3125	+

* (-): absence; (+): presence.

To evaluate the effectiveness of the concentration extract on the growth of *B. cereus* isolate, the MIC and MBC test was conducted using various extract concentration by tube dilution method. The lowest concentration of the extract solution from antibacterial activity assay that is still active to inhibit bacterial growth of *B. cereus* isolate, and then this concentration was used for the highest concentration to determine MIC and MBC of the extract against *B. cereus* isolate. Determination of MIC and MBC was performed using macrodilution or tube dilution method with various concentration as shown in Table 4. Determination of MIC was aimed to find the lowest concentration of the extract which can inhibit bacterial growth, while MBC has no bacterial growth.

The results (Table 4) showed that the extract has MIC and MBC value in concentration range between 1.25% w/v to 2.5% w/v. The results can be seen from turbidity level of solution in tube, the suspension in tube becomes clearly visible in concentration 2.5% w/v indicated that concentration has no bacterial growth.

CONCLUSIONS

The results suggest that the extract gave antibacterial effect against *Bacillus cereus* with MIC and MBC value of the extract in the concentration range of 1.25% (w/v) – 2.5% (w/v). This could be used to discover new drugs to control diarrhea infection and foodborne illness caused by *Bacillus cereus*. The antibacterial activity of this extract probably derived from flavonoids, polyphenols, monoterpene and sesquiterpene compound.

ACKNOWLEDGEMENTS

We would like to express our gratitude to all members of Pharmaceutical Biology Department and Pharmacology and Clinical Pharmacy Department at Faculty of Pharmacy Universitas Padjadjaran for supporting this research.

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