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RESEARCH ARTICLE

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Antimicrobial Activity of Ethanol Extract of *Centella asiatica* Leaves on *Proteus mirabilis*, *Proteus vulgaris*, and *Yersinia enterocolitica in vitro*

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Background: *Centella asiatica* leaves ethanol extract (CALEE) has higher concentration compared to other structures within the plant. The extract contains alkaloids, saponins and flavonoids, which play an active role as antioxidant and antibacterial. Current study aimed to determine the effect of CALEE on *Proteus mirabilis*, *Proteus vulgaris*, and *Yersinia enterocolitica*, Gram-negative bacteria that cause diarrhea.
Materials and methods: Simplicia of *C. asiatica* leaves was dissolved in 96% ethanol and macerated to get condensed extract, which then produced a concentration of 1 g/mL. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined to assess the effectiveness of CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*. MIC was determined through serial dilution test with Mueller Hinton broth media. After incubation, the bacteria were streaked on nutrient agar or McConkey agar to determine the MBC.
Results: The MIC value of CALEE could not be determined since the color of CALEE was dark, hence the turbidity could not be compared. CALEE had the same MBC value (0.25 g/mL) in all bacteria species used in this study.
Conclusion: CALEE is effective against Gram-negative bacteria, such as *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*. Further research is needed, especially *in vivo* experiments and evaluation of the cytotoxicity effect of CALEE.

Keywords: *Centella asiatica, Proteus mirabilis, Proteus vulgaris, Yersinia enterocolitica, antibacterial*

Introduction

Numerous plant species are known to have potential as alternative herbal medicines.[1-5] *Centella asiatica* or Indian pennywort is a traditional plant that has the potential to treat infectious diseases, indicated by anti-inflammatory and antioxidant activities of the extract obtained from this species.[6,7] *C. asiatica* leaves ethanol extract (CALEE) has

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higher concentration of flavonoids, alkaloids, and saponins, which have an active role in killing bacteria, compared to other structures within the plant.[8,9]

Microbes such as bacteria can cause symptoms and signs of an infectious disease in the digestive tract called diarrhea.[10] Indonesia is one of the developing countries in the tropics that are prone to diarrhea with a case fatality rate (CFR) of 1.97% in 2017.[11,12] Study on the pattern of aerobic bacteria that cause diarrhea in children in Manado shows that several Gram-negative bacteria, such as *Proteus vulgaris*, *Proteus mirabilis*, *Shigella* sp., *Enterobacter aerogenes*, *Salmonella arizonae*, are able to cause diarrhea.[13] The main cause of diarrhea is *Escherichia coli*.[13,14] *Yersinia enterocolitica* is also one of the *Yersinia* members that cause food-borne diarrhea.[15]

Gram-negative bacteria have three layers on its surface with lipopolysaccharide in the middle layer. Lipopolysaccharide in particular can resist the entry of antibacterial bioactive substances.[15] This layer can select certain drugs and antibiotics, making bacteria resistant to those compounds.[16] However, not all Gram-negative bacteria have been tested with ethanol plant extracts. Several *in vitro* experiments show that methanol extracts obtained from several plant species, including *C. asiatica*, have antimicrobial activities on Gram-negative bacteria, such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella* spp.[16-18] This study aimed to determine the effect of the CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*. This research is expected to provide an alternative herbal medicine for antibacterial drugs development.

Materials and methods

Preparation of CALEE

C. asiatica leaves were obtained from Balai Materia Medika Batu, East Java, Indonesia using random sampling technique. One thousand g of *C. asiatica* leaves were cleaned and dried at room temperature. The leaves were dried again at 50°C in a hot air oven for 3 h. Then, the dry leaves were grounded and put into a grinder machine to obtain uniform powder (simplicia). One kg simplicia was dissolved in 1 L 96% ethanol (ratio 1:1) and extracted with maceration technique at 50°C for 24 h. Maceration result was filtered and evaporated using an evaporator for 3 h to get condensed extract, which then produced a concentration of 1 g/mL.

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

To assess the effectiveness of CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[19] MIC was determined through serial dilution test with Mueller Hinton (MH) broth media. Test tubes were filled with MH broth, bacterial suspension with a turbidity of 0.5 McFarland (equal to 1.5×10^8 CFU/mL), and various concentrations of CALEE (0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 g/mL). Untreated bacterial suspension was used as a negative control. Meanwhile, bacterial suspension treated with 1 g/mL CALEE was used as a positive control, since bacterial growth was not observed at this concentration. MIC was determined at the lowest concentration that could inhibit microbes by observing the turbidity of media after incubation. All incubation processes were conducted at 37°C for 24 h. After incubation, *Y. enterocolitica* was streaked on nutrient agar (NA). Meanwhile, *P. mirabilis* and *P. vulgaris* were streaked on MacConkey agar to prevent swarming.[20] MBC was determined at the lowest concentration of CALEE which kills the bacteria by observing the growth of bacterial colonies on agar media after incubation.[19] Each experimental group was measured in four replicates.

Data Analysis

This research was a post-test only control group design study. Data analyses were carried out descriptively.

Results

The MIC value of CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica* could not be determined since the color of CALEE was dark, hence the turbidity could not be compared. All replicates in each experimental group showed similar results (Figure 1). The description of CALEE concentrations used in each tube was described in Table 1, Table 2, and Table 3. CALEE had the same MBC value (0.25 g/mL) in all bacteria species used in this study. The inoculation results were shown in Figure 2, while the growth of bacterial colonies on agar media after incubation were shown in Table 1, Table 2, and Table 3. Based on these results, CALEE was proven to have an antibacterial effect against *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*.

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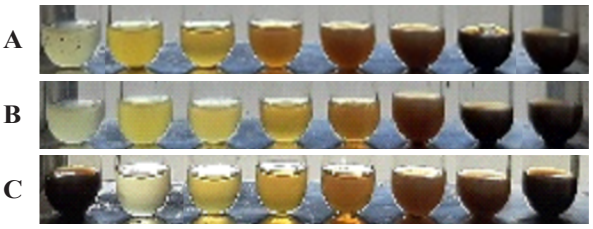

Figure 1. Results of serial dilution test of CALEE. A: *P. mirabilis* (Pm; left to right: C-; Pm-6; Pm-5; Pm-4; Pm-3; Pm-2; Pm-1; C+). B: *P. vulgaris* (Pv; left to right: C-; Pv-6; Pv-5; Pv-4; Pv-3; Pv-2; Pv-1; C+). C: *Y. enterocolitica* (Ye; left to right: C+; C-; Ye-6; Ye-5; Ye-4; Ye-3; Ye-2; Ye-1). MIC could not be determined since the turbidity of experimental group could not be compared with the control group. C-: negative control group; C+: positive control group.

Discussion

Selection of C. asiatica Leaves and Ethanol Solvent in Extraction Process

Leaves of *C. asiatica* are the best choice for the extraction of bioactive compounds, since the leaf of *C. asiatica* was reported to have a higher concentration of bioactive compounds compared to the roots and leaves. There are notable differences in flavonoids and alkaloids content between the plant parts. The leaves have the highest saponins content, although there are no notable differences compared to the stems and roots.[21]

C. asiatica leaves extracted with ethanol has higher yield compared to those extracted with water. Ethanol extracts more secondary metabolites in *C. asiatica* leaves due to differences in polarity between the hydroxyl and methyl groups.[9] Another study demonstrates that the yield of CALEE is 6 times higher compared to *C. asiatica* leaves water extract.[22] Therefore, ethanol is an effective solvent to extract bioactive compounds from *C. asiatica* leaves.

Table 1. *P. mirabilis* growth on MacConkey agar.

Tube	CALEE Concentration (g/mL)	Growth on MacConkey Agar			
		Replication			
		1	2	3	4
C-	-	+	+	+	+
C+	1	-	-	-	-
Pm-1	1	-	-	-	-
Pm-2	0.5	-	-	-	-
Pm-3	0.25	-	-	-	-
Pm-4	0.125	+	+	+	+
Pm-5	0.0625	+	+	+	+
Pm-6	0.03125	+	+	+	+

Pm: *P. mirabilis*; C-: negative control group; C+: positive control group; +: bacterial growth; -: no bacterial growth.

Table 2. *P. vulgaris* growth on MacConkey agar.

Tube	CALEE Concentration (g/mL)	Growth on MacConkey Agar			
		Replication			
		1	2	3	4
C-	-	+	+	+	+
C+	1	-	-	-	-
Pv-1	1	-	-	-	-
Pv-2	0.5	-	-	-	-
Pv-3	0.25	-	-	-	-
Pv-4	0.125	+	+	+	+
Pv-5	0.0625	+	+	+	+
Pv-6	0.03125	+	+	+	+

Pv: *P. vulgaris*; C-: negative control group; C+: positive control group; +: bacterial growth; -: no bacterial growth.

Mechanisms of Antibacterial Compounds Contained in CALEE

Flavonoids, alkaloids, and saponins contained in *Centella asiatica* leaves extract have been shown to have antibacterial properties.[23,24] This is confirmed in a previous study shows that *Centella asiatica* leaves extract affected enteric bacteria *in vitro*.[25]

Flavonoids work as antibacterial agents in various ways. Its lipophilic characteristic can disrupt microbial

Table 3. *Y. enterocolitica* growth on NA media.

Tube	CALEE Concentration (g/mL)	Growth on NA			
		Replication			
		1	2	3	4
C-	-	+	+	+	+
C+	1	-	-	-	-
Ye-1	1	-	-	-	-
Ye-2	0.5	-	-	-	-
Ye-3	0.25	-	-	-	-
Ye-4	0.125	+	+	+	+
Ye-5	0.0625	+	+	+	+
Ye-6	0.03125	+	+	+	+

Ye: *Y. enterocolitica*; C-: negative control group; C+: positive control group; +: bacterial growth; -: no bacterial growth.

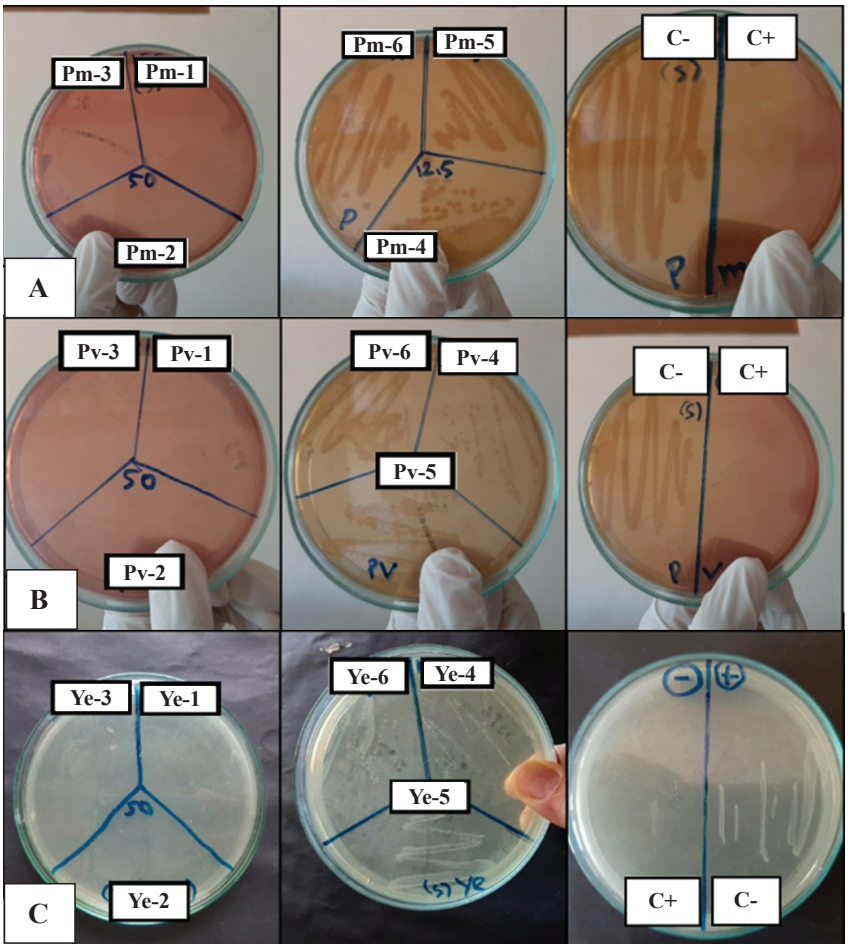

Figure 2. The results of bacteria inoculation. A: *P. mirabilis* (Pm). B: *P. vulgaris* (Pv). C: *Y. enterocolitica* (Ye). C-: negative control group; C+: positive control group.

membranes. Flavonoids also deactivate adhesins, enzymes, and transport proteins in the cell walls of microbes, hence inhibit energy metabolism. In addition, flavonoids impair bacterial DNA synthesis by inhibiting DNA gyrase.[26,27]

Saponins are active glycosides that are widely distributed in plants.[28] The properties of saponins are similar to soap which have hydrophilic and lipophilic parts in their chemical structure, hence they can inhibit bacterial growth by damaging the bacterial cell wall and cell membrane.[29,30]

Alkaloids are natural compounds which exist in plants and have bacteriostatic and bactericidal properties against Gram-positive and Gram-negative bacteria. Alkaloids have many skeleton structures that play a role in their antibacterial activity. Alkaloids inhibit nucleic acid synthesis and damage surface adhesins in bacteria.[31]

In the current study, the MBC values of CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica* were 0.25 g/mL, while the MIC values could not be determined since the color of CALEE was dark. It has been reported that aqueous extract of *C. asiatica* is dark yellow and black, even when other solvents are added.[32] In a previous study on the effect of CALEE towards *Staphylococcus aureus* using the disc diffusion method, the MIC and MBC values are 8 and 16 mg/mL, respectively.[22] Moreover, in another *in vitro* research on the effect of CALEE towards *Streptococcus pyogenes* and *P. aeruginosa* using the disc diffusion method, MIC and MBC values are 60% and 40% respectively.[33]

Ethanol, petroleum ether, and chloroform extracts of *C. asiatica* leaves have been reported to have the best effects against several bacteria, including *P. vulgaris* using the disc diffusion method compared to other extracts.[17] Current study has the same result with a previous study on the methanol extract of *C. asiatica,* which is tested on *P. vulgaris* bacteria and has the best effect at a concentration of 250 mg/mL.[34] Methanol extract of *C. asiatica* is also able to inhibit *P. mirabilis* and *P. vulgaris* from autoimmune disease samples with a MIC value of 0.672 mg/mL.[35]

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Antibacterial Activity of CALEE Against Gram-negative and Positive Bacteria

CALEE has different effects on Gram-negative and Gram-positive bacteria since these types of bacteria are different in the composition and structure of the cell wall and cell membrane. All bacteria have a cell membrane. However, in particular, the cell wall of Gram-positive bacteria has a simpler structure with a low lipids content. This assumes why antibacterial bioactive materials easily enter Gram-positive bacterial cells.[15]

Gram-negative bacteria have a lipopolysaccharide layer in the middle of their cell wall that can resist the entry of antibacterial substances.[15,16] This layer may be the target of CALEE on *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*, since these bacteria are Gram-negative bacteria. Each bioactive compound present in CALEE may attack bacteria on several targets.

Flavonoids are phenolic compounds that have polar properties and mostly target the bacteria on the peptidoglycan layer.[36] After attacking the outer membrane of bacterial cells, some flavonoid contents then inhibit DNA gyrase, cytoplasmic membrane function, and energy metabolism.[27] Although it has been reported that flavonoids are more efficient at inhibiting Gram-positive bacteria[16,30], flavonoids are able to inhibit Gram-negative bacteria, as shown in the current study.

Saponins have been reported to have a greater role in fighting Gram-negative bacteria, which are characterized by having a cell membrane rich in lipids.[15] Saponins form complexes with water to form micelles, which destroy the bacterial cell wall by changing their permeability.[29,36,37] These compounds have also been reported to be found in various plant extracts. In pineapple extract, it was reported that saponins were more efficient in attacking Gram-negative bacteria.[30]

Alkaloids have been recognized as antibacterial compounds. Alkaloids from various plant extracts have been reported to intercalate DNA and inhibit β-lactamase in Methicillin-resistant *S. aureus* (MRSA).[38] The bis-indole alkaloid may have an antibacterial effect by inhibiting the pyruvate kinase enzyme, which is important for the Krebs cycle, resulting in bacterial metabolic disorders.[39] In general, alkaloids can inhibit Gram-positive and negative bacteria. Alkaloids extracted from *Terminalia arjuna* plant have been reported to have the best activity on *E. aerogenes*, a Gram-negative bacteria. However, these extracts also have an effect on other tested bacteria (*E. coli*, *P. aeruginosa*, and *S. aureus*). Therefore, further research is needed to compare the alkaloids activity of plant extracts on Gram-positive and Gram-negative bacteria.[40]

Conclusion

CALEE is effective against Gram-negative bacteria, such as *P. mirabilis*, *P. vulgaris*, and *Y. enterocolitica*. The MBC values are 0.25 g/mL, while the MIC values cannot be determined. Further research is needed to determine MIC with spectrophotometry. *In vivo* experiments and evaluation of the cytotoxicity effect of CALEE are also needed to be involved in the future research.

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