AMJ
ISSN: 0005-2523

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

Centella asiatica Extract as Antibacterial Agent against Multidrug Resistant (MDR) *Pseudomonas aeruginosa*

Metta Octora[1,2*], Lina Permatasari[3], Nurmi Hasbi[1], Anggit L. Sunarwidhi[3], Bayu Tirta Dirja[1,2]

Faculty of Medicine Universitas Mataram, Indonesia[1]
Universitas Mataram Hospital, Indonesia[2]
Faculty of Farmacy, Universitas Mataram, Indonesia[3]

Corresponding Author: 1,2*

Keywords:
colistin, tannin, phenolic acid, flavonoid

ABSTRACT

Pseudomonas aeruginosa often causes severe nosocomial infections that is resistant to several antibiotic classes in hospital setting. Multidrug resistant (MDR) *Pseudomonas aeruginosa* infection increases morbidity and mortality as well as hospital expenses. The study was to analyze the antibacterial activity of *Centella asiatica* against MDR *Pseudomonas aeruginosa*. It was a laboratory experimental study. *C. asiatica* was extracted by sonication method. Phytochemical Test was conducted against flavonoid, phenolic acid, alkaloid, tannin, steroid, triterpenoid and saponin. The test groups divided into 5 groups (no treatment, Colistin 10 µg, 1000, 3000 and 5000 ppm of *Centella asiatica* extract) and each group consisted of 4 replications. The antibacterial activity had been tested by Kirby Bauer disc diffusion method. The diameter of inhibition zone was measured and calculated by formula. The best results of zone of inhibition of concentration is a 5000 ppm of *Centella asiatica* extract (means 3.125 mm). The highest inhibition zone of a 5000 ppm of *Centella asiatica* extract was 5 mm (SD ± 1,65) whereas zone of inhibition of Colistin 10 µg as a potent antibacterial therapy against MDR *Pseudomonas aeruginosa* was 8 mm. The ANOVA Test showed a significance difference between zone of inhibition for each 1000 ppm, 3000 ppm, 5000 ppm and Colistin 10 µg as antibacterial therapy for MDR *Pseudomonas aeruginosa* ($p < 0.05$). *Centella asiatica* has antibacterial activity against MDR *Pseudomonas aeruginosa* as indicated by the phenolic acid and tannin compounds. The higher concentration of *Centella asiatica* extract the higher antibacterial activity against MDR *Pseudomonas aeruginosa*.

1. Introduction

Pseudomonas aeruginosa is Gram-negative bacteria that often causes severe nosocomial infections that is resistant to one or several types of certain antibiotics (multiple drug resistant/MDR) in hospital setting. *P. aeruginosa* often produces non-fluorescent bluish or greenish pigments in MHA media. Multidrug resistant (MDR) *Pseudomonas aeruginosa* infection increases morbidity and mortality as well as hospital expenses

[1]. Intrinsic resistance is encoded in the bacterial chromosome. In the case of *P.aeruginosa*, intrinsic resistance is due to low outer membrane permeability, expression of membrane efflux pumps, and inducible chromosomal b lactamase, AmpC [2]. Control of antibiotic resistance against MDR *Pseudomonas aeruginosa* requires potential alternative antibiotics, including natural antibiotics. Natural antibiotics have been widespread in society as traditional medicinal plants, for example *Centella asiatica.*

Centella asiatica extract was known to have the moderate antibacterial activity in methanol against *Staphylococcus aureus, Bacillus cereus*, and *Escherichia coli*, whereas acetone extract was less effective against these organisms. Several studies also stated that the methanol extract of *C. asiatica* leaves showed inhibitory activity against bacteria compared to acetone extract and water [3]. Recent studies have shown that *Centella asiatica* has bioactive compounds (such as tannins, alkaloids, flavonoids and others) that are responsible for antibacterial properties. *Centella asiatica* extract also has bacteriostatic and bactericidal activity, through its ability to block bacterial efflux pump system [4].

The study aimed to analyze the antibacterial activity of *Centella asiatica* against multidrug resistant (MDR) *Pseudomonas aeruginosa*.

2. Materials and methods

2.1 Tools and materials

The materials were *C. asiatica*, technical ethanol 96%, concentrated HCl, Mg powder, $AlCl_3$ 10%, distilled water, P-iodonitrotetrazolium, agar base: Nutrient and Mac Conkey agar. The tools were burner, autoclave, vacuum rotary evaporator, oven, and incubator [5].

2.2 Simplicia extraction

C. asiatica was extracted by sonication method. As much as 100 grams of *C. asiatica* simplicia powder was dissolved in 500 mL of ethanol 96% with a ratio of simplicia powder and solvent was 1:5. Then the dissolved powder was put into a sonicator at 35°C for 30 minutes. The extraction was repeated 2 times with a new solvent replacement. After that the extraction results were filtered and the filtrate was evaporated by a rotary evaporator at 40–50ºC. The extract was evaporated by a water bath to obtain a viscous extract. After that, it was weighed to calculate the yield (%) [6].

The yield (%) of the C. asiatica extract is calculated by the formula (Ministry of Health RI, 2000):

$$\text{Yield (\%)} = \frac{\text{Condensed extract weight}}{\text{Initial simplicia weight}} \times 100\ \%$$

2.3 Phytochemical Test

2.3.1 Flavonoid Test

The flavonoid test was used by the Wilstater method. As much as 1 mL of *C. asiatica* ethanol extract was added to a test tube and 2 drops of concentrated HCl were added. After that, 0.2 mg of Mg powder is added, the result is positive if it will show discoloration to yellow color [7].

2.3.2 Phenolic Test with $FeCl_3$ Reagent

FeCl3 reagent was used to determine the presence of phenolic compounds. As much as 0.5 ml of *C. asiatica* extract was put into a test tube and 2 drops of $FeCl_3$ 5% solution were added, a positive result was indicated by discoloration to bluish green or blackish blue [8].

ISSN: 0005-2523
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2.3.3 Alkaloid Test

The Mayer, Dragendorff, and Wagner methods were used to determine the presence of alkaloid compounds. As much as 2 ml of *C.asiatica* extract were added a test tube and then 1 ml of Meyer's reagent, a positive result is indicated by a pale yellow precipitate. In another test tube, 2 ml of extract had been heated by H_2SO_4 2%, after that a few drops of dragendorff reagent were added, the presence of an orange-red precipitate indicated the presence of alkaloids. *Centella asiatica* extract was dissolved in HCl and the filtrate was taken. Put the filtrate into a test tube and add Wagner's reagent, a positive result is indicated by a brown or reddish precipitate [9].

2.3.4 Tannin Test

As much as 1000 µg/mL of *Centella asiatica* ethanol extract was put into a test tube and then 2-3 drops of 1% $FeCl_3$ were added. A discoloration to black-green indicates a positive sample containing tannin [6].

2.3.5 Steroid Test

The herb extract of *Centella asiatica* that has been put into a test tube is dissolved in chloroform, adding a few drops of anhydrous acetic acid and 1 drop of concentrated H2SO4. Positive results containing steroids are indicated by a change in color to dark green [8], [9].

2.3.6 Triterpenoid Test

As much as 2 ml of C. asiatica extract was added by 8 ml of warm distilled water, after that it was filtered and the filtrate was put into a test tube and then 3 drops of Bouchardat reagent were added. The positivity result of triterpenoids will produce an orange or brownish orange color [10].

2.3.7 Saponin test

As much as 1 g of C. asiatica ethanol extract is added to warm water, shaken vertically for 10 seconds and then left for 10 seconds. Approximate 1–10 cm of a stable foam formation for not less than 10 minutes indicates saponins [8], [9].

2.4 Preparation of extract concentrations (1000 ppm, 3000 ppm and 5000 ppm)

Respectively 10, 30 and 50 mg of methanol extract was weighed, put into a 100 mL each volumetric measuring flask and added dimethyl sulfoxide (DMSO) up to the mark, so that each concentration of 1000, 3000 and 5000 ppm had been obtained.

2.5 Antibacterial test

Pseudomonas aeruginosa isolate were suspended in sterile NaCl and then compared with 0,5 McFarland's standard solution as Kirby Bauer disc diffusion method on MHA media and left for 10 minutes (Fig.1) [11]. Blank disc paper (blank disc) was immersed in the solution at each extract concentrations for 5 minutes. Respectively 1000 ppm, 3000 ppm and 5000 ppm of *C. asiatica* extract disc, blank disc as negative control and Colistin 10 µg disc as positive control placed on the MHA media surface and incubated for 24 hours at 37^0C. We observed and calculated zone of inhibition following formula:

$$\text{Zone of inhibition} = \frac{(\text{vertical diameter} - \text{disc diameter}) + (\text{horizontal diameter} - \text{disc diameter})}{2}$$

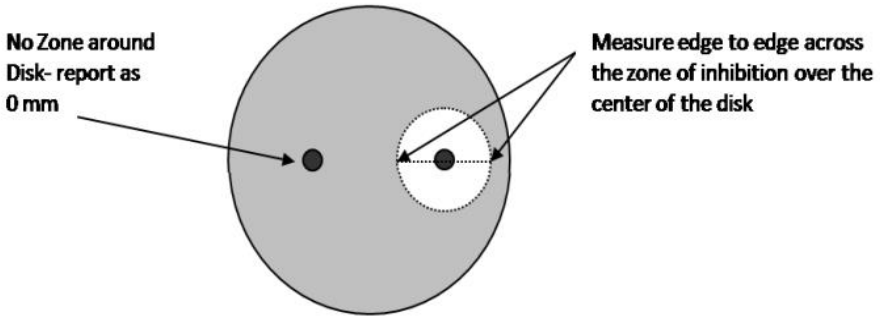

Fig 1. Zone of inhibition in Kirby Bauer disc diffusion method [11]

3. Results and Discussion

3.1 Simplicia Determination Results

Determination of plant simplicia is the initial step before conducting study using simplicia as material. The identification aims to ensure that the plant simplicia was used as an appropriate material [12] In addition, the determination of plant simplicia is used to avoid errors in collecting the test material and to prevent mixing of the test material with other ingredients from other plants. The simplistic determination of *Centella asiatica* was carried out by the Center for Research and Development of Medicinal Plants and Traditional Medicines (B2P2TOOT). The plant simplicia determination showed that the plant was used as a test material was indeed *Centella asiatica.*

3.2 Extraction

The extraction was carried out by sonication method that used ultrasonic frequencies in the range of 20-2000 kHz. This method increased the cell membrane permeability and surface contact between the solvent and sample thereby it could accelerate the diffusion and dissolution of solvent, while the energy used is relatively small, the extraction time is quite short and the temperature is low so that it can be used for the extraction of thermolabile and unstable compounds [12].

The filtrate was evaporated using a vacuum rotary evaporator at 45^{o}C. This method can be carried out at a lower temperature than the boiling point of the solvent because vacuum rotary evaporator uses low pressure, so that it can avoid damage of filtrate compounds. The result of evaporation was ethanol extract of *Centella asiatica* that was a 57.78 g from 300 g of *Centella asiatica* simplicia (yield 19.26%). The ethanol extract is blackish brown in color with a thick consistency.

3.3 Results of phytochemical test

The results of t phytochemical test using a test tube showed that *Centella asiatica* extract positively contained tannin and phenolic compounds, as well as steroid and triterpenoid (Table 1, Figure 2).

Table 1. Tube test of *Centella asiatica*

No.	Test	Reagent	Result	Colour
1.	Phenolic	$FeCl_3$ 5%	+	Blackish blue
2.	Tannin	$FeCl_3$ 1%	+	Blackish green
3.	Flavonoid	Mg + HCl Pekat	-	-
4.	Steroid and Triterpenoid	Chloroform + anhydrous acetic acid + sulfuric acid	+	Dark green
5.	Saponin	HCl 2 N	-	-

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

6.	Alkaloid	Mayer	-	-
		Dragendroff	-	-

Note: result (+) = contains secondary metabolites (-) = does not contain secondary metabolites

Our study in line with study of [13] shows that were 35 polyphenolic compounds that can act as antibacterial against *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella Enteritidis*. His study showed that was an interaction between the polyphenolic compounds and the surface of bacterial cell which causes the bacterial cell lyses.

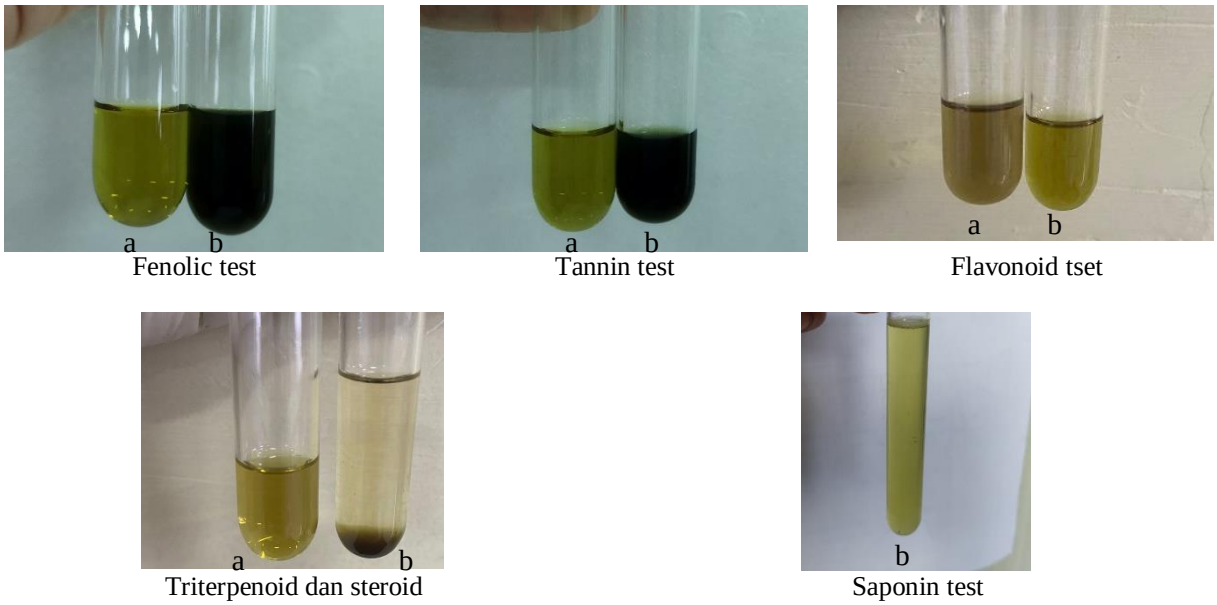

Figure 2. The results of the phytochemical test of *Centella asiatica* extract. (a) before adding the reagents; (b) after adding the reagent

Our study also in line with study of [14] reported that phenolic compounds in gallic acid, quercetin, caffeic acid, coumaric acid, tannin and catechol acid had antibacterial activity against *E. coli, Pseudomonas aeruginosa* and *Bacillus subtilis*.

3.4 Antibacterial Test

P. aeruginosa often produces non-fluorescent bluish or greenish pigments in MHA media (Fig. 3). Multidrug resistant (MDR) *Pseudomonas aeruginosa* infection increases morbidity and mortality as well as hospital expenses

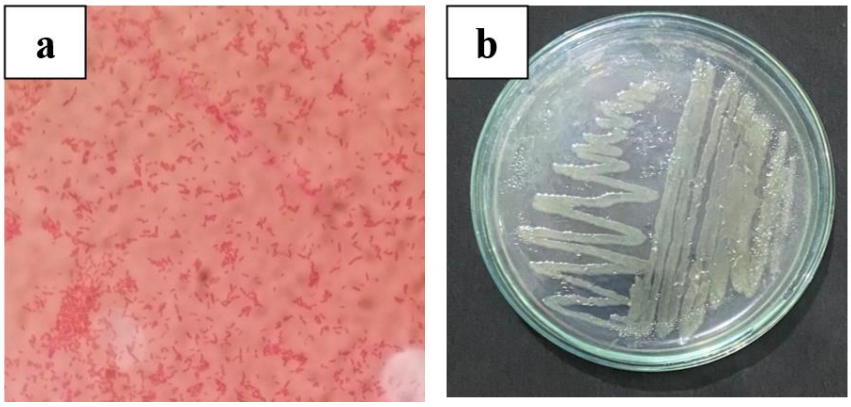

Figure 3 Multi drug resistant *Pseudomonas aeruginosa* isolates; (a) Microscopic observation of Gram stain with 1000x magnification; (b) Greenish pigmentation on Mueller Hinton agar (Source: personal documentation).

The results of antibacterial test with Kirby Bauer disc diffusion at 37^0C for 24 hours was expressed by diameter of the inhibition zone (Figure 4).

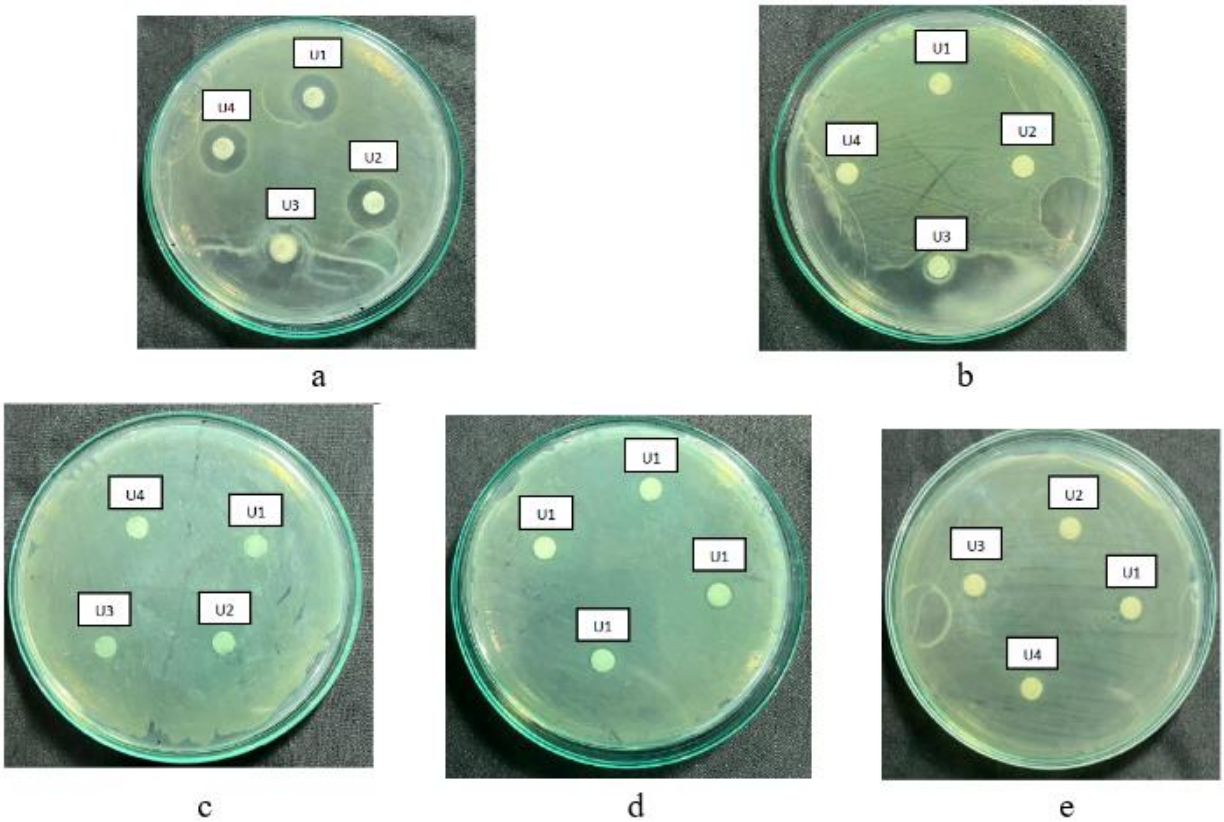

Figure 4. The diameter of inhibition zone of *Centella asiatica* extract against *P. aeruginosa*. (a) positive control of Colistin 10 µg; (b) negative control; (c) *Centella asiatica* extract 1000 ppm; (d) *Centella asiatica* extract 3000 ppm; (d) *Centella asiatica* extract 5000 ppm (source: personal documentation).

The best results of zone of inhibition of concentration is a 5000 ppm of *Centella asiatica* extract (average 3.125 mm). The highest inhibition zone of a 5000 ppm of *Centella asiatica* extract was 5 mm (SD ± 1,65) whereas zone of inhibition of Colistin 10 µg as a potent antibacterial therapy for *Pseudomonas aeruginosa* was 8 mm (SD ± 0). There was a significance difference between zone of inhibition for each 1000 ppm,

ISSN: 0005-2523
Volume 62, Issue 09, November, 2022

3000 ppm, 5000 ppm and Colistin 10 µg as antibacterial therapy for *Pseudomonas aeruginosa*. The diameter of the inhibition zone in three concentrations of *Centella asiatica* extract was increased parallel with increasing concentration of *Centella asiatica* extract. These results indicate the higher concentration of *Centella asiatica* extract the higher antibacterial activity (Table 2)

Table 2 Inhibition zone of *Centella asiatica* extract against MDR *Pseudomonas aeruginosa*

Concentration (ppm)	Inhibition zone (mm)				Average (mm)	p
	Replikasi I	Replikasi II	Replikasi III	Replikasi IV		
1000	1	1	1	1	1	0.00
3000	2	2	2	2	2	
5000	4	1.5	5	2	3.125	
Positive control	8	8	8	8	8	
Negative control	0	0	0	0	0	

The antibacterial activity of *Centella asiatica* extract against MDR *Pseudomonas aeruginosa* was probably due to the active compounds in the *Centella asiatica* extract. The bioactive components of *Centella asiatica* which have antibacterial properties are flavonoids, tannins and saponins [15]. The active antibacterial compounds of *Centella asiatica* extract in this study were tannins, phenolic and triterpenoid. Tannins inhibit bacterial proliferation by inhibiting proteolytic enzymes in bacteria, so that the protein metabolism of pathogenic bacteria will be disrupted. Tannin compounds can also make the bacterial cell wall lysis and rupture [16].

[17], [18] stated that the triterpenoid in C. asiatica are polar compounds that can ionize molecules and join the adsorption of polyphenols onto the bacterial membrane, thereby causing inhibition of bacterial growth by disrupting the bacterial membrane. The antibacterial effect of C. asiatica was higher on Gram-positive bacteria compared to Gram-negative ones. It happens because compared to Gram-positive bacteria, Gram-negative bacteria has an outer membrane permeability that limits the entry of antimicrobial agents into cells and other different resistance mechanisms such as modification of target sites and enzymatic inactivation.

This study was also supported by study of [19]. He stated that phenolic acid demonstrated antimicrobial activity against the bacteria strains such as *Escherichia coli, Staphylococcus epidermidis* (native and drug-resistant), and *Staphylococcus aureus* (native and drug-resistant)) at concentrations suitable for incorporation into polymeric biomaterials. In general, an increase in the number of hydroxyl and methoxy groups was associated with a slight decrease in antimicrobial efficacy. In comparison to unmodified controls, modification had no significant effect on antioxidant or antimicrobial properties, indicating that the carboxylic acid group of phenolic acid can be altered without losing functionality.

4. Conclusion

Centella asiatica has antibacterial activity against MDR *Pseudomonas aeruginosa* as indicated by the phenolic acid and tannin compounds in the *Centella asiatica* extract. The largest diameter of inhibition zone was produced at a concentration of 5000 ppm and there was a significance difference between zone of inhibition for each 1000 ppm, 3000 ppm, 5000 ppm of *Centella asiatica* extract and Colistin 10 µg as antibacterial therapy against MDR *Pseudomonas aeruginosa*. The higher concentration of *Centella asiatica* extract the higher antibacterial activity.

5. Limitations and suggestions

The study was observed in the limited *Centella asiatica* concentrations and replication. The optimal

concentration of *Centella asiatica* extract for MDR *Pseudomonas aeruginosa* isolate have not been known yet. The further in vivo study of steroids effect against multidrug resistant (MDR) *Pseudomonas aeruginosa* infection.

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