In Vitro test: antimicrobial activity potential from Ciplukan fruit (physalis minima L.) extract in Methicillin-resistant Staphylococcus aureus (MRSA)

by Muhammad Mahyuddin

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Literature Review

In vitro test: antimicrobial activity potential from Ciplukan fruit (*Physalis minima* L.) extract in *Methicillin-resistant Staphylococcus aureus* (MRSA)

Muhammad Hanun Mahyuddin[1], Arifa Mustika[1,2*], Gwenny Ichsan Prabowo[1,3], Deby Kusumaningrum[1,4]

1) Faculty of Medicine, Airlangga University
2) Pharmacology Department, Airlangga University
3) Biochemistry Department, Airlangga University
4) Microbiology Department, Airlangga University

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Correspondence:
arifa-m@fk.unair.ac.id

Abstract

In Indonesia, in 2006, the prevalence of infections due to MRSA was 23.5%. *Physalis minima* L. plants are known to have antimicrobial activity because they contain compounds withaferin A, which can induce programmed cell death. This research was to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ciplukan (*Physalis minima* L.) extract in Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria. Dilution test with Mueller-Hinton broth medium used for measuring the minimum inhibitory concentration (MIC). Ciplukan fruit extract was dissolved in distilled water, and poured into a test tube with a certain concentration (0.9 g/mL (90%); 0.3 g/mL (45%); 0.15 g/mL (22.5%); 0.075 g/mL (11.25%) and 0.0375 g/mL (5.625%). After being incubated for 24 hours, the bacteria in the test tube were plated on nutrient agar plates to determine the MBC. The MIC cannot be determined, because the medium in the dilution test tube is disturbed by the color of the extract so that turbidity cannot be observed. From the observations of the minimum bactericidal concentration, MBC of the Ciplukan (*Physalis minima* L.) fruit extract against MRSA was in the P1 tube or equivalent to 0.9 g / ml (90%).

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INTRODUCTION

The carelessly use of antibiotics against the *Staphylococcus aureus* can cause resistance, such as *Methicillin-resistant Staphylococcus aureus* (MRSA). From 2004 to 2006, around 77% of nosocomial infections in Korea were caused by MRSA, as well as in Taiwan as many as 55% (Chen & Huang, 2014). As many as 29%-35% of *Staphylococcus aureus* isolates in all hospitals in America, and Europe has MRSA strains (Haddadin et al., 2013). In Indonesia, in 2006, the prevalence was 23.5% (Sulistiyaningsih, 2010). Conducted from Antimicrobial Resistance (AMR), surveillance showed that from a total 250 *Staphylococcus aureus* isolated found that 45 (18%) of them were are MRSA (Hadi et al., 2013).

The MRSA has beta-lactamase, which makes the MRSA have resistance to all beta-lactam antibiotics, which means that antibiotics that have beta-lactam rings cannot be used to treat infections due to MRSA (Rasigade & Vandenesch, 2013). The MRSA is also known to have the mecA gene and fem gene. *Staphylococcus aureus* is also often found to have additional resistance mechanisms from plasmids, prophages, and transposons that have the potential to spread MRSA strains to other *Staphylococcus aureus* bacteria (Lowy, 1998).

Until now, there has been no research on the potential for Ciplukan (*Physalis minima* L.) as an antibacterial agent such as MRSA. It is necessary to test the antimicrobial potential of the Ciplukan (*Physalis minima* L.) plant against MRSA.

METHODS

Preparation of Plant Extract

The sample used was Ciplukan fruit (*Physalis minima* L.) obtained from the Gresik area. Samples that have been collected will be identified in the Materia Medica Batu to find out the type and species of the sample.

Samples obtained from Gresik areas were collected and made in the form of Simplicia. Simplicia obtained as much as 300g. Simplicia will be soaked with 70% alcohol as much as 750 ml liters in the tube for 3 x 24 hours. The filtrate obtained from the immersion will be concentrated with a rotary evaporator. The extract obtained was 180mg. The extract is stored at 4 ° C.

Ethical Clearance

This research was carried out based on a certificate of ethics made by the Health Research Ethics Committee of the Faculty of Medicine, Airlangga University, Surabaya, with certificate number No.141 / EC / KEPK / FKUA / 2019.

Preparation of Bacterial Specimen

The MRSA bacterial specimens were obtained from bacterial storage in the Department of Microbiology, Faculty of Medicine, Airlangga University.

Inoculation Preparation

The existing stock is cultured in advance so that healthy growth is obtained (it thrives and in the logarithmic growth phase or does not experience mutations or lag or die phase). The bacterial colony is taken using a loop and transferred to a tube that was previously filled with nutrient broth. Bacteria were incubated for 24 hours at 37ºC. The turbidity proportion is equivalent to 0.5 *McFarland*.

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Preparation of Antimicrobial Test

Antimicrobial activity tests were carried out by the dilution method in the Mueller-Hinton media. Ciplukan (*Physalis minima* L.) fruit extract was diluted with distilled water (*aquadest).* Then, Ciplukan fruit extracts divided into five test tubes with different concentrations. P1 tube 90% (g/mL), P2 45% (g/mL) tube, P3 tube 22.5% (g/mL), P4 tube 11.25% (g/mL), and P5 tube 5.625% (g/mL). Each tube will be given 1 mL of bacterial suspension except the control tube. Repetition is done five times.

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

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibits bacterial growth after being incubated for 24 hours at 37 ° C.

The minimum bactericidal concentration (MBC) is the concentration of an antimicrobial agent needed to kill certain bacteria. The minimum bactericidal concentration can be determined from the antimicrobial agent, which has been determined the minimum inhibitory concentration after incubation for 24 hours at 37 ° C.

RESULTS

Observations on bacterial growth are made through two methods: 1) Dilution test to determine the MIC; 2) cultured bacteria on nutrient agar plates to determine the MBC.

Dilution test was carried out using seven tubes, including positive and negative control tubes. Each tube was filled with extract concentrations of 90% (g / mL), 45% (g / mL), 22.5% (g / mL), 11.25% (g / mL), and 5.625% (g / mL). For positive control containing MRSA colonies and negative control containing 90% (g / mL) extract without MRSA.

Table 1. Observation of MIC in Bacterial Dilution Test

Tube	Concentration	Observation of the Medium Color in the Tube				
		Replication				
		1	2	3	4	5
K 1	90% (g/mL) extract	Dark Purple	Dark Purple	Dark Purple	Dark Purple	Dark Purple
K 2	MRSA	Yellow	Yellow	Yellow	Yellow	Yellow
P 1	90% (g/mL) extract + MRSA	Dark Purple	Dark Purple	Dark Purple	Dark Purple	Dark Purple
P 2	45% (g/mL) extract + MRSA	Dark Brown	Dark Brown	Dark Brown	Dark Brown	Dark Brown
P 3	22,5% (g/mL) extract + MRSA	Brown	Brown	Brown	Brown	Brown
P 4	11,25% (g/mL) extract + MRSA	Light Brown	Light Brown	Light Brown	Light Brown	Light Brown
P 5	5,625% (g/mL) extract + MRSA	Dark Yellow	Dark Yellow	Dark Yellow	Dark Yellow	Dark Yellow

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Table 2. Observation of Bacterial Growth in Agar Plate Nutrient

Tube	Concentration	Observation of Bacterial Growth in Agar Plate Nutrient				
		Replication				
		1	2	3	4	5
K 1	90% (g/mL) extract	-	-	-	-	-
K 2	MRSA	+	+	+	+	+
P 1	90 % (g/mL) extract + MRSA	-	-	-	-	-
P 2	45 % (g/mL) extract + MRSA	-	+	+	+	-
P 3	22,5 % (g/mL) extract + MRSA	-	+	+	+	-
P 4	11,25 % (g/mL) extract + MRSA	+	+	+	+	+
P 5	5,625 % (g/mL) extract + MRSA	+	+	+	+	+

'+' show bacterial growth, while '-' no bacterial growth

MRSA : Methicillin-resistant *Staphylococcus aureus*

Observation of dilution results found that each tube has a different color. The difference in color was due to differences in concentration between the tubes. Dilution test results are carried out visually by comparing the level of turbidity between the treatment tube and the control tube. In Table 1. we can see the colored medium in the tube so that the MIC cannot be determined. The medium color is determined subjectively.

Then Each tube will be cultured on nutrient agar plates to determine the MBC. The result of the MBC showed in Table 2. In the first and fifth replications, minimum bactericidal concentration was observed at a concentration of 22.5% (g/mL), and at the second to fourth replication, the MBC was observed at a concentration of 90% (g/mL). In the positive control, found bacterial growth on nutrient agar plates and in the negative control tube found no bacterial growth. The absence of bacterial growth in the negative control also indicated the extract was not contaminated.

DISCUSSION

Antibacterial Mechanism of Ciplukan Fruit Extract

Methicillin-resistant *Staphylococcus* damages the structure of β-Lactam antimicrobials by producing beta-lactamase and has structural changes in the binding protein penicillin. MRSA strains have two resistance mechanisms, namely intrinsic (innate) and extrinsic (acquired). Intrinsic resistance will continue to be attached to bacteria and never change. The intrinsic mechanism of MRSA is the presence of the mecA gene that encodes PBP2a. In addition to the mecA gene, MRSA also has a fem gene. Fem gene, which can make MRSA has resistant to methicillin, penicillinase-resistant penicillins, and cephalosporin (Jansen et al., 2006).

Staphylococcus aureus peptidoglycan has a long characteristic in the form of a woven structure (cross-linkage) with a flexible pentaglycine side chain. This peptidoglycan is an antimicrobial target of β-lactam. Resistance occurs when there is a change of structure in PBP2a because of the production of the beta-lactamase enzyme that can catalyze the β-Lactam antimicrobial so that the antimicrobial β-Lactam cannot damage

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the structure of peptidoglycan. Penicillin-binding protein is a group of proteins involved in peptidoglycan biosynthesis, which catalyzes the transpeptidation reaction (Memmi et al., 2008). The transpeptidation reaction by PBP is required at the time of bacterial cell wall synthesis. Penicillin-binding protein is also a target of the β-Lactam protein so that if there is a change in the structure of PBP, the β-Lactam protein will be inactive and cannot interfere with cell wall synthesis. Some of the mechanisms that cause acquired resistance are efflux pumps, impermeability mutations, carbapenemases, and aminoglycoside-modifying enzymes.

The active compounds in Ciplukan are within one, withanolide A, withaferin A, dihydroxyphysalin B2-4, and physalin A (Chothani & Vaghasiya, 2012). Withaferin A is a steroidal lactone isolated from a medicinal plant from India, *Withania somnifera*. Withaferin A is widely known as an anti-inflammatory, anticancer and antimicrobial. Withaferin A can inhibit cell growth and induce apoptosis (Sail & Hadden, 2012).

The mechanism of action of withaferin A is to bind the metabolic enzymes that have amino acids with the SH group. Amino acids that have an SH group are methionine and cysteine. Methionine is an essential amino acid containing sulfur. Methionine is the precursor of any metabolite protein such as succinyl-CoA, homocysteine, cysteine, creatine, and carnitine. Methionine also helps the biosynthesis of glutathione, which functions to fight oxidative stress on cells (Martínez et al., 2017). The withaferin A bond to the SH group that will inactivate the metabolic enzymes, induce oxidative stress because glutathione is not formed and cut the glycolysis pathway because it destroys succinyl-CoA so the cells will start to die.

The content of the active substance Withaferin A as an antibacterial is also aided by the action of flavonoids, which are lipophilic substances which can damage bacterial cell membranes that will cause the release of metabolites from the cell and the entry of fluid from the outside into the cell. Alkaloids are heterocyclic nitrogen compounds that are known to be able to interfere with nucleic acid production and cell division (Cushnie et al., 2014; Narasimha Rao & Raman Venkatachalam, 1999)

Minimum Bactericidal Concentration (MBC) Determination

Table 3. Antimicrobial Activity of Ciplukan Extract From any Studies

Microba	MBC	Reference	Part of the Plant
B. Subtilis, E.coli, P. solanacearum	2mg/mL	(Shariff et al., 2006)	Leaf
X. Vesicatoria	4mg/mL	(Shariff et al., 2006)	Leaf
B. subtilis, P. solanacearum, and *X. Axonopodis*	2mg/mL	(Shariff et al., 2006)	Callus
E.coli and *X. vesicatoria*	4mg/mL	(Shariff et al., 2006)	Callus
Methicillin-resistant *Staphylococcus aureus*	70% (ml/ml)	(Fitrianti et al., 2011)	Leaf
Methicillin-resistant *Staphylococcus aureus*	90% (g/mL)	Author's	Fruit

MBC: Minimum Bactericidal Concentration

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Minimum Bactericidal Concentration (MBC) Determination

From table 3, the research has varying results from the first replication to replication five. In the first replication, MBC was observed in P3 tube (22.5% (g / mL)). But in the second replication, MBC can be observed in P1 tube (90% (g / mL)), third replication in P1 tube (90% (g / mL)), fourth replication in P1 tube (90% (g / mL)), and the fifth replication, MBC can be observed in P3 (22.5% (g / mL)). This study was compared with previous research on antimicrobial activity testing of Ciplukan (*Physalis minima* L.) fruit extracts.

Ciplukan (*Physalis minima* L.) leaf extracts have antimicrobial mechanism against *B. Subtilis, E.coli, P. solanacearum,* and *X. axonopodis* at a concentration of 2mg/mL while *X. Vesicatoria* at a concentration of 4mg/mL. Also, Ciplukan (*Physalis minima* L.) callus extracts also showed the minimum bactericidal concentration of *B. subtilis, P. solanacearum,* and *X. axonopodis* at a concentration of 2mg/mL whereas in *E.coli* and *X. vesicatoria* at a concentration of 4mg/mL (Shariff et al., 2006).

A similar study conducted with *Physalis angulata* L. leaf extract found that at a concentration of 70% could kill (MBC) MRSA bacteria (Fitrianti et al., 2011).

In this study of Ciplukan fruit extract against the MRSA bacteria, the bacteria could not grow on the media at a concentration of 22.5% (g / mL) in the first and fifth replications, but in the second, third and fourth replication bacteria could not grow at a concentration of 90% (g/mL).

Other studies have also shown similar results, namely differences in the rates of MBC in replication. The differences in the MBC ethanol extract of *Zingiber cassumunar Roxb*, between concentrations of 12.5%; 25%; and 50%. At a concentration of 50% already, no bacterial growth was found in all replications, a concentration of 25% obtained bacterial growth in 1 of 7 replications, a concentration of 12.5% was obtained growth in 4 of 7 replications. The MBC is determined at the smallest concentration where there is no significant difference compared with negative controls, and there are significant differences when compared with positive controls so that the MBC is determined at a concentration of 25% (Raharjoyo & Gunardi, 2009). From that, we conclude that the MBC in this study was 90% (g/mL).

This study could not determine the MIC because the solution in the Ciplukan (*Physalis minima* L.) fruit extract was colored, so the turbidity level to determine the MIC was not possible. Therefore, it is necessary to filtrate the solution to make it colorless. Another way is to make an extract with another method so that the extract will be colorless. Further research in vivo is needed to determine the dosage and side effects of the Ciplukan fruit.

CONCLUSION

Ciplukan fruit extract (*Physalis minima* L.) has an antibacterial effect against MRSA. The minimum bactericidal concentration (MBC) in this study was 90% g/mL. The MIC cannot be determined.

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