

Research Report

## **ANTIMICROBIAL ACTIVITY OF PINEAPPLE (*ANANAS COMOSUS* L. MERR) EXTRACT AGAINST MULTIDRUG-RESISTANT OF *PSEUDOMONAS AERUGINOSA*: AN IN VITRO STUDY**

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### **ABSTRACT**

*Pseudomonas aeruginosa* is the main cause of nosocomial infection which is responsible for 10% of hospital-acquired infection. *Pseudomonas aeruginosa* tends to mutate and displays potential for development of antibiotic resistance. Approximately, 10% of global bacterial isolates are found as Multidrug-resistant *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* have a quite tremendous severity index, especially on pneumonia and urinary tract infections, even sepsis, which 50% mortality rate. Pineapple (*Ananas comosus* L. Merr) has antimicrobial properties. The active antimicrobial compounds in *Ananas comosus* L. Merr include saponin and bromelain. This research aims to find the potency of antimicrobial effect of pineapple (*Ananas comosus* L. Merr) extract towards Multidrug-resistant *Pseudomonas aeruginosa*. Multidrug-resistant *Pseudomonas aeruginosa* specimen is obtained from patient's pus in orthopaedic department, Dr Soetomo Public Hospital, Surabaya. Multidrug-resistant *Pseudomonas aeruginosa* specimen is resistant to all antibiotic agents except cefoperazone-sulbactam. This research is conducted by measuring the Minimum Inhibitory Concentration (MIC) through dilution test with Mueller-Hinton broth medium. Pineapple extract (*Ananas comosus* L. Merr.) is dissolved in aquadest, then poured into test tube at varying concentrations (6 g/ml; 3 g/ml; 1.5 g/ml; 0.75 g/ml, 0.375 g/ml; and 0.1875 g/ml). After 24 hours' incubation, samples are plated onto nutrient agar plate, to determine the Minimum Bactericidal Concentration (MBC). The extract of pineapple (*Ananas comosus* L. Merr) has antimicrobial activities against Multidrug-resistant *Pseudomonas aeruginosa*. Minimum Inhibitory Concentration (MIC) could not be determined, because turbidity changes were not seen. The Minimum Bactericidal Concentration (MBC) of pineapple extract (*Ananas comosus* L. Merr) to Multidrug-resistant *Pseudomonas aeruginosa* is 0.75 g/ml. Further study of in vivo is needed.

**Keywords:** *Ananas comosus* L. Merr, antimicrobial, Multidrug Resistant *Pseudomonas aeruginosa*, pineapple

### **ABSTRAK**

*Pseudomonas aeruginosa* adalah penyebab utama infeksi nosokomial, dan bertanggung jawab pada sekitar 10% kasus infeksi di rumah sakit. *Pseudomonas aeruginosa* mudah bermutasi dan memunculkan sifat resistensi antibiotik. Diperkirakan 10% isolat bakteri global dinyatakan sebagai *Pseudomonas aeruginosa* Multidrug Resistant (PaMR). *Pseudomonas aeruginosa* memiliki severity index yang cukup tinggi, terutama pada pneumonia dan infeksi saluran kencing, bahkan sepsis, dimana tingkat kematian mencapai 50%. Buah nanas (*Ananas comosus* L. Merr) mempunyai sifat antimikroba. Senyawa aktif yang terkandung dalam *Ananas comosus* L. Merr meliputi saponin dan bromelain. Penelitian ini bertujuan untuk menguji potensi efek antimikroba dari ekstrak buah nanas (*Ananas comosus* L. Merr) terhadap *Pseudomonas aeruginosa* Multidrug Resistant (PaMR). Penelitian ini menggunakan metode true experimental. Specimen *Pseudomonas aeruginosa* Multidrug Resistant diperoleh dari pus pasien bagian orthopedi Rumah Sakit Umum Daerah Dr. Soetomo Surabaya. Specimen *Pseudomonas aeruginosa* Multidrug Resistant dinyatakan resisten pada seluruh golongan antibiotik, kecuali cefoperazone-sulbactam. Penelitian dilakukan dengan mengukur Konsentrasi Hambat Minimal (KHM) melalui uji dilusi dengan media Mueller-Hinton broth. Ekstrak buah nanas (*Ananas comosus* L. Merr) dilarutkan dalam aquades, dan dituangkan

ke dalam tabung uji dengan konsentrasi tertentu (6 g/ml; 3 g/ml; 1.5 g/ml; 0.75 g/ml, 0.375 g/ml; and 0.1875 g/ml). Setelah diinkubasi selama 24 jam, bakteri dalam tabung uji ditanam pada nutrient agar plate, untuk menentukan Konsentrasi Bunuh Minimal (KBM). Replikasi dalam penelitian ini dilakukan empat kali. Konsentrasi Hambat Minimal (KHM) tidak dapat ditentukan, karena kekeruhan larutan yang tidak menunjukkan perubahan. Konsentrasi Bunuh Minimal (KBM) dari ekstrak buah nanas (*Ananas comosus* L. Merr) terhadap *Pseudomonas aeruginosa* Multidrug Resistant adalah 0.75 g/ml. Diperlukan penelitian lebih lanjut, terutama eksperimen *in vivo*.

**Kata kunci:** *Ananas comosus* L. Merr, antimikroba, *Pseudomonas aeruginosa* Multidrug Resistant, nanas

## INTRODUCTION

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative bacterium which is categorized as an opportunistic pathogen that infects impaired immunity individuals.<sup>1</sup> *Pseudomonas aeruginosa* is a major cause of nosocomial infections and it is responsible for 10% of hospital-acquired infections.<sup>2</sup> *P. aeruginosa* infections recurrently lead to serious and life-threatening conditions, generally difficult-to-treat. Multidrug - resistant *Pseudomonas aeruginosa* is *Pseudomonas aeruginosa* which is resistant to at least one agent in three or more categories of antimicrobial, including: aminoglycoside, cephalosporin, fluoroquinolone, penicillin +  $\beta$ -lactamase inhibitor, monobactam, phosphonic acid, carbapenem and polymyxin.<sup>3</sup> Infections by cause of Multidrug - resistant *Pseudomonas aeruginosa* have a quite tremendous severity index, especially on pneumonia and urinary tract infections, even sepsis, which 50% mortality rate.<sup>4</sup>

*Pseudomonas aeruginosa* has intrinsic antimicrobial resistance due to the permeability of the membrane and has a wide range of efflux pumps. Some strains of *P. aeruginosa* show mutations in the fluoroquinolone binding site, loss of porin channels, and increased production of beta - lactamase as well as cephalosporinase. *Pseudomonas aeruginosa* may acquire additional resistance mechanisms through external plasmids and has a high potential to be resistant against antimicrobials used during the treatment.<sup>5</sup> The prevalence of *P. aeruginosa* that is reported to be resistant against various antimicrobial agents continues to increase, with 10% of global isolates are reported to be Multidrug - resistant *Pseudomonas aeruginosa*.<sup>6</sup> Particularly in Indonesia, the prevalence of Multidrug-resistant *P. aeruginosa* was reported to be 23.5%.<sup>7</sup>

A novel antimicrobial is necessary to overcome the increasing resistance of *Pseudomonas aeruginosa*. Pineapple (*Ananas comosus* L. Merr) was reported to have strong antimicrobial activity and should be taken into consideration as antimicrobials.<sup>8</sup> The active antimicrobial compounds in *Ananas comosus* L. Merr include saponin and bromelain.<sup>9</sup> Both saponin and bromelain work as antimicrobial through membranolytic properties.<sup>10</sup> Bromelain particularly works as a proteolytic enzyme once it is bound to bacterial cell membrane, causing damage and inducing bacterial cell death.<sup>11</sup> Antimicrobial activity of the active compounds in *Ananas comosus* L. Merr had been

reported to be notably potent against both Gram-negative and Gram-positive bacteria, including *Pseudomonas aeruginosa*.

Even though pineapple extract (*Ananas comosus* L. Merr) had been reported to be a potent antimicrobial agent against *Pseudomonas aeruginosa*, there was no research regarding its' potential against Multidrug-resistant *Pseudomonas aeruginosa*. Therefore, we seek to test the antimicrobial potency of pineapple extract (*Ananas comosus* L. Merr) against Multidrug - resistant *Pseudomonas aeruginosa*.

## MATERIAL AND METHOD

### Preparation of Plant Extract

The pineapples were taken from Pesanggrahan Village, Batu, East Java at the age of  $\pm$  1.5 years. Its surroundings on the basis of ethnopharmacological information during December 2015. The plant materials were identified and authenticated by East Java Department of Health, Indonesia.

Samples of 1 kg pineapple fruit were washed, cut into small parts and dried under 40°C for 72 hours. The dried plant materials were processed into subtle powder. The dried powder of any of pineapple fruit were soaked with 800 ml – 1 L of (96%) ethanol and cold distilled water, respectively for 3 x 24 hours. The pulverized materials were extracted by maceration. The filtrates were collected in a beaker and concentrated in a vacuum at a temperature below 40°C using a rotary evaporator. The resulting raw extracts obtained were stored at 4°C.

### Preparation of Bacterial Specimen

The specimen of Multidrug-resistant *Pseudomonas aeruginosa* was obtained from a patient in Orthopaedic Department, Dr. Soetomo Public Hospital Surabaya. The bacterial specimen is resistant to several antibiotics agents includes: aminoglycoside, fluoroquinolone, penicillin +  $\beta$ -lactamase inhibitor, cephalosporin, monobactam, phosphonic acid, carbapenem, polymyxin, and only sensitive to cefoperazone-sulbactam. Multidrug-resistant *Pseudomonas aeruginosa* cultures were maintained on the sterile nutrient agar plate media and incubated at 37 ° C for 24 hours, following refrigeration storage at 4°C.

### Inoculation Preparation

Isolated colonies of the same type from a culture agar plate were selected and grabbed the surface of colony with a loop and relocated to a tube containing 4 ml of a compatible medium like nutrient broth. The suspension was incubated at 37°C and the proportion was adjusted to the 0.5 MacFarland definitive turbidity, approximately  $1.5 \times 10^8$  Colony Forming Unit (CFU)/cc or 250-300 colonies in solid medium.

### Preparation of Antimicrobial Test

Antimicrobial activity test was conducted using dilution method in Mueller-Hinton broth media. Pineapple (*Ananas comosus* L. Merr.) extract was diluted with distilled water and divided into several tubes with particular concentrations (6 g/cc; 3 g/cc; 1.5 g/cc; 0.75 g/cc; 0.375 g/cc, and 0.1875 g/cc). Bacterial specimens were diluted in isotonic solution (NaCl 0,9%) to reach concentration of 0.5 McFarland or equal to bacterial count of  $1.5 \times 10^8$  Colony Forming Unit (CFU)/cc or 250-300 colonies in solid medium. Afterwards the bacterial suspension was added to each tube. After 24 hours of incubation, bacterias grown in tubes were planted into a solid medium to obtain the Minimum Bactericidal Concentration (MBC). Repetition was done for four times, according to Federer formula.

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

Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration that totally inhibited the growing of bacteria after 24 hours of incubation at 37°C. Minimum Bactericidal Concentration (MBC) was determined as the lowest concentration that revealed no visible bacterial growth after sub-culturing in 37°C incubation for 24 hours, from a portion of liquid (5 µl) each tube. Positive and negative cultures were also prepared.

## RESULT AND DISCUSSION

Each process used 8 tubes, including one tube for positive control group and one tube for negative control group. The pineapple extract concentrations of the treatment

group are: 6 g/cc; 3 g/cc; 1.5 g/cc; 0.75 g/cc; 0.375 g/cc; 0.1875 g/cc (sorted from first tube to the sixth tube). The concentration was obtained from serial dilutions of the first tube (6 g/cc). Negative control was obtained by mixing 1 cc of liquid medium (Mueller-Hinton broth) with 1 cc of Multidrug-resistant *Pseudomonas aeruginosa* specimen, while positive control tube was obtained by mixing 1 cc of liquid medium (Mueller-Hinton broth) with 1 cc of pineapple (*Ananas comosus* L. Merr.) extract at 6 g/cc concentration.

Observation of bacterial growth was conducted in two methods: (1) serial dilution test to determine the Minimum Inhibitory Concentration (MIC); and (2) planting the bacteria into nutrient agar plate to determine the Minimum Bactericidal Concentration (MBC). In the serial dilution test, Minimum Inhibitory Concentration (MIC) could not be determined because the medium was discoloured by pineapple extract which made the visual observation difficult. Each serial dilution tubes were planted to nutrient agar plates. The 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> replication showed varying result of Minimum Bactericidal Concentration (MBC) in Table 1, which are: 0.75 g/cc; 0.1875 g/cc; 0.1875 g/cc; and 0.375 g/cc respectively. The negative control group showed bacterial growth on nutrient agar plates in all replication while the positive control group did not show bacterial growth on nutrient agar plates in all replication. The absence of bacterial growth in the positive control group indicates that there were no bacterial contaminants.

### Antibacterial Mechanism of Pineapple Fruit Extract

In the immense definition, antibacterial agents interfered with the growth and replication of bacteria. Nowadays, antibacterial agents are generally defined as the agents which disinfect and eliminate adverse bacteria.<sup>12</sup> Antibacterial agents are classified into 2 groups, which are: inhibiting the growth of bacteria and killing the bacteria.

Mechanism of antibacterial agents confide in the structure and composition of the bacterial cell. Destruction of bacterial structure led to a change in bacterial metabolism which causes cell death. Antibacterial mechanism may include: cell walls destruction; disruption or injury to cell membrane; or synthesis inhibition of proteins and nucleic acids.<sup>13</sup>

**Table 1.** Bacterial growth in nutrient agar plates

Tube	Pineapple Fruit Extract Concentration (g/cc)	Bacterial Growth of <i>Multidrug Resistant Pseudomonas aeruginosa</i>			
		1 <sup>st</sup> Replication	2 <sup>nd</sup> Replication	3 <sup>rd</sup> Replication	4 <sup>th</sup> Replication
C1 (+)	6	-	-	-	-
C2 (-)	-	+	+	+	+
T 1	6	-	-	-	-
T 2	3	-	-	-	-
T 3	1,5	-	-	-	-
T 4	0,75	-	-	-	-
T 5	0,375	+	-	-	-
T 6	0,1875	+	-	-	+

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

Multidrug - resistant *Pseudomonas aeruginosa* specimen in our study had categorized as resistant to several antibiotic class like aminoglycosides, beta-lactam penicillin, sulfa - trimethoprim, tetracycline, chloramphenicol, macrolide, and quinolones. These bacterial specimens are sensitive only to the beta-lactam class of 3rd generation cephalosporin, such as cefoperazone - sulbactam.

Various antibiotics had been tested against the Multidrug - resistant *Pseudomonas aeruginosa* specimen which used in our experiment. We conclude that the bacteria showed resistance to diverse mechanisms of antibiotics such as: protein synthesis inhibition (aminoglycosides, tetracycline, chloramphenicol, macrolide); cell wall synthesis inhibition (beta-lactam penicillin); inhibition of folic acid pathway metabolism (sulfa - trimethoprim); and nucleic acid synthesis inhibition (quinolones).<sup>14</sup> The only mechanism that is effective against this specimen was bacterial cell wall synthesis inhibition by binding to the penicillin binding protein (cefoperazone), coupled with  $\beta$ -lactamase irreversible inactivation of sulbactam.<sup>15</sup>

Resistance mechanism in *Pseudomonas aeruginosa* can be classified into 2 types: intrinsic resistance and acquired resistance. Intrinsic resistance is an innate mechanism which cannot be changed and unmodifiable. *Pseudomonas aeruginosa* intrinsically possessed resistance against several classes of antibiotics such as ampicillin, amoxicillin, amoxicillin-clavulanate, as well as first and second generation of cephalosporin such as cefotaxime and ceftriaxone. Intrinsic resistance is difficult to intervene; hence it is necessary to find gaps in the mechanism of acquired resistance to overcome the antibiotic resistance in *Pseudomonas aeruginosa*.

Acquired resistance mechanism in *Pseudomonas aeruginosa* includes: efflux pumps, impermeability mutation,  $\beta$ -lactamases, carbapenemase, and aminoglycoside-modifying enzymes.<sup>16</sup>

The phytochemical analysis of pineapple has revealed several compounds such as polyphenols, saponins, and flavonoids which are known antimicrobial agents. Also, pineapple fruit contains a protease called bromelain. The exact antimicrobial mechanism of pineapple (*Ananas comosus* L. Merr) extract against gram-negative bacteria is still not clearly known. Previous studies suggested the active compounds working against gram-negative bacteria are majorly bromelain and saponin, while flavonoids and polyphenols are more potent in inhibit gram-positive bacteria. Flavonoids and polyphenols are phenolic compounds which have polar properties, so these compounds mostly work in the peptidoglycan layer in Gram-positive bacteria, which also have polar properties, rather than the non-polar lipid layer.<sup>17</sup>

Both of bromelain and saponin, act on bacterial cell walls and membranes. Their antimicrobial activity against *Multidrug-resistant Pseudomonas aeruginosa* in our study indicates two things. First, the active compounds in the extract of pineapple might have similar mechanism with antibiotics like cefoperazone - sulbactam which is

also sensitive against *Multidrug-resistant Pseudomonas aeruginosa*. Second, the active compound in pineapple extracts might have antimicrobial mechanism which unrecognized by *Multidrug - resistant Pseudomonas aeruginosa* bacteria.

Bromelain is a proteolytic enzyme. Proteolytic enzymes play a role in the breakdown of proteins, as we know protein is one of the essential component in bacterial membrane. Bromelain is hypothesized to induce protein breakdown in bacterial membrane, causing injury and cell death.<sup>18</sup> The exact mechanism of how bromelain inhibits the growth of gram-negative bacteria is still not fully identified. Studies suggest that bromelain operates through associated mechanism in weakening of outer membrane in gram-negative bacteria which contain proteins. Bromelain are thought to disintegrate protein in surface membrane which eventually weakens the cell wall, leads to cell leakage, swells the cell, and damages the cell. The numbers of amino acids in the bacterial cell wall are thought to determine the antibacterial activity of proteolytic enzymes.<sup>11</sup>

Saponins increases the permeability of the bacterial cell membrane, causing alteration of structure and function of the membrane, disrupting the surface tension of the cell wall, then allowing antibacterial substances to easily enter the cells and interfere the cell metabolism while denaturates proteins on the membrane so the cell membrane will be lysis.<sup>19</sup> Saponin selectively interacts with cholesterol on cell membrane, leaving a hole in the membrane.<sup>20</sup>

#### Minimum Bactericidal Concentration (MBC) Determination

Other studies that are similar was reviewed, and taken as considerations in determining the Minimum Bactericidal Concentration (MBC). In this study, we used g/cc in each extract concentration, namely 6 g/cc; 3 g/cc; 1.5 g/cc; 0.75 g/cc; 0.375 g/cc; and 0.1875 g/cc. Respectively, which mean the concentration of 6 grams of pineapple extract in 1 cc of distilled water; 3 grams of extract in 1 cc of distilled water; 1.5 grams of extract in 1 cc of distilled water; 0.75 grams of extract in 1 cc of distilled water; 0.375 gram of extract in 1 cc of distilled water; and 0.1875 grams of extract in 1 cc of distilled water.

Our study was compared with earlier studies on antimicrobial activity of pineapple (*Ananas comosus* L. Merr) extract. The result of three earlier studies was compared with our research in Table 2.

Study by Ajibade et al,<sup>21</sup> reported pineapple extract eliminate non - Multidrug Resistant *Pseudomonas aeruginosa* at concentration of 0.2 g/cc. At these concentrations, pineapple extract also exhibits antibacterial activity against other bacteria, including *Streptococcus pneumoniae* & *Staphylococcus aureus*. In our study, bactericidal effect of pineapple fruit extract showed in ranged concentration from 0.1875 g/cc to 0.75 g/cc. Compared to Ajibade et al study,<sup>21</sup> the 0.1875 g/cc is the closest concentration to 0.2 g/cc.



**Table 2.** Other studies of antimicrobial activity of pineapple fruit extract

Bacteria	MC	Reference
<i>Enterotoxigenic Escherichia coli</i>	1 g/cc	8
<i>MDR Pseudomonas aeruginosa</i>	0,75 g/cc	Author's
<i>Pseudomonas aeruginosa</i>	0,2 g/cc	21
<i>Staphylococcus aureus</i>	0,2 g/cc	21
<i>Streptococcus pneumonia</i>	0,2 g/cc	21
<i>Aggregatibacter actinomycetemomitans</i>	16,6 mg/cc	22
<i>Porphyromonas gingivalis</i>	4,15 mg/cc	22
<i>Streptococcus mutans</i>	2 mg/cc	22

MBC: Minimum Bactericidal Concentration

Green color: Author's result

Minimum bactericidal concentration (MBC) was determined differently across previous studies. This difference is highlighted especially in studies with different MBC on each replication. Prihantoro et al<sup>23</sup> mentioned that minimum bactericidal concentration (MBC) is determined when bacterial colonies growth in the media as few as <0.1% of the original inoculum. Raharjo<sup>24</sup> determined MBC as the smallest concentration with insignificant difference to negative control and significant difference to positive control. Christiawan & Perdanakusuma<sup>25</sup> determined MBC as the smallest concentration with no bacterial growth on the growing media. Other attention is the potency of bacterial resistance to antibacterial agents. *Pseudomonas aeruginosa* is bacteria with a high mutation rate and easy to show resistant properties against antibiotic agent.<sup>5</sup> Based on this study, the MBC of pineapple fruit extract (*Ananas comosus* L. Merr.) against Multidrug-resistant *Pseudomonas aeruginosa* is starting to show at the concentration of 0.1875 g/cc. However, to guarantee the emergence of resistance not being returned, the concentration of 0.75 g/cc is considered to be a minimum bactericidal concentration (MBC).

## CONCLUSION

The extract of pineapple (*Ananas comosus* L. Merr) has antimicrobial activities against Multidrug-resistant *Pseudomonas aeruginosa*. The Minimum Bactericidal Concentration (MBC) is 0.75 g/cc, which is the lowest concentration of pineapple extract (*Ananas comosus* L. Merr) that eliminate the Multidrug - resistant *Pseudomonas aeruginosa*. The researcher suggests that further study of in vivo is needed.

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