Potency of Ethanol Extract from Berenuk (*Crescentia cujete* L.) Fruit Rind and Flesh as Antibacterial Agents

U Hasanah¹, HT Widhiastuti² and Syaefudin³*

¹ Department of Food Science and Technology, Bogor Agricultural University, Bogor, 16680, Indonesia
² Department of Biochemistry, Bogor Agricultural University, Bogor, 16680, Indonesia
³ Department of Biochemistry, Bogor Agricultural University, Bogor, 16680, Indonesia

*email: syaefudin01@apps.ipb.ac.id

Abstract. Berenuk (*Crescentia cujete* L.) fruit is widely used by the community in East Java as traditional medicine. The objectives of this research were to determine the antibacterial activity and Minimum Inhibitory Concentration (MIC) of ethanol extract of berenuk’s fruit rind and flesh against *Staphylococcus aureus* and *Escherichia coli*. Berenuk’s fruit rind and flesh were extracted with 70% (v/v) ethanol by maceration method. Phytochemical screening was performed by Harborne method. Antibacterial activity and MIC of the extracts were analyzed using disc diffusion method by bacterial concentration of 10⁶ CFU/mL. The result showed that ethanol extract of berenuk’s fruit rind contained alkaloid, saponin, tannin, and flavonoid, while ethanol extract of fruit flesh contained alkaloid and flavonoid. Antibacterial analysis showed no inhibition zone on both extracts against *E. coli*. Ethanol extract of berenuk’s fruit rind showed inhibition zone only on the growth of *S. aureus* at the concentration of 80% (w/v) and 100% (w/v). The MIC of ethanol extract of berenuk’s fruit rind on *S. aureus* growth obtained at a concentration of 65% (w/v).

1. Introduction

Berenuk (*Crescentia cujete* L.) known as a plant that widely available in the tropics and also in Indonesia. This plant, also known as majapahit or calabash, is widely used by the community as a traditional medicine by utilizing its root, leaf, barks or fruit. Berenuk’s fruit juice is often used as medicine for asthma and stomachache in India [1]. These advantages are probably caused by the active compounds contained in berenuk’s fruit.

Flavonoid, saponin, tannin, alkaloid and phenolic has been identified as active compounds in berenuk’s fruit flesh [2]. Saponins, flavonoids, cardenolides, tannins, and phenols as phytochemicals also has been identified in berenuk’s fruit [3]. The fruit extract of berenuk could inhibit the growth of *Aeromonas salmonicida* by in vitro method, but the inhibition activity was lower than 30 µg of kanamycin [4]. Other antibacterial study of berenuk’s fruit was against *Vibrio alginolyticus*, but there was no inhibition zone [5]. These four previous studies were performed by using fruit juice or fruit concentrates, without any extraction by solvent. The other studies were bioactivity from their leaves and barks [5-10].
The aim of this research was to screen phytochemical compounds of the ethanol extract from berenuk’s fruit rind and flesh. This research also performed to determine the antibacterial activity and Minimum Inhibitory Concentration (MIC) of ethanol extract from berenuk’s fruit rind and flesh that could inhibit bacterial growth of Staphylococcus aureus (*S. aureus*) and Escherichia coli (*E. coli*), as pathogenic Gram positive and Gram negative bacteria. Extraction by ethanol was intended to obtain more accurate active compounds than the usage of juice or concentrates.

2. Materials and methods

2.1. Materials

Fresh berenuk’s fruit rind and flesh (4-6 years old tree) were obtained from Tegalwaru, Ciampea Bogor, West Java, Indonesia (6°34'09.9"S 106°41'57.0"E). This plant was identified and authenticated by Herbarium Bogoriense, Research Center for Biology – Indonesian Institute of Sciences (LIPI), Indonesia. Samples were dried by using oven Eyela NDO-700 (JP). Extraction was performed by using chloroform, ammonia, sulfuric acid, Dragendorf reagent, Wagner reagent, Meyer reagent, FeCl₃, methanol 30% (v/v), ethanol 30% (v/v), ether, and anhydrous acetic acid. Antibacterial analysis was performed by using *E. coli* and *S. aureus* wild-type culture (IPBCC, ID). All chemical reagents were purchased from Merck (DE).

2.2. Methods

2.2.1. Sample preparation of berenuk’s fruit rind and flesh. Berenuk’s fruit were washed and peeled, then the fruit rind were separated from the flesh. Each was taken to 5 kg. Fruit rind and flesh sliced into smaller parts, grinded and then oven-dried at a temperature of 40-50°C until the weights were constant. Simpilsia then sieved with a 100 mesh sieve and stored in refrigerator.

2.2.2. Analysis of moisture content. Moisture content was determined using AOAC method No. 925.10 [11]. The porcelain cup was dried in an oven at 105°C for 45 minutes, then the cup was placed in a desiccator for 30 minutes. The empty porcelain cup was weighed. Two grams of sample was weighed using a dried cup. The sample in the cup was oven-dried at 105°C for 3 hours until the weight was stable, then put in a desiccator to cool and weighed along with the cup.

2.2.3. Extraction of berenuk’s fruit rind and flesh. Simpilsia powder of berenuk’s fruit rind and flesh from the sample preparation (25 g for each sample) dissolved in 250 mL of 70% (v/v) ethanol, incubated in a shaking incubator at 120 rpm at room temperature (28°C) for 24 hours. This treatment was repeated 4 times to obtain a clear filtrate. The filtrate was filtered and then concentrated by rotary evaporator at 60°C. The concentrated extract then weighed to obtain a yield of extract [2]. The concentrated extract were used for phytochemical profile analysis and antibacterial and MIC analysis.

2.2.4. Phytochemical analysis. The phytochemical screening was conducted using Harborne method [12]. Detail of the procedures was described as follow.

2.2.4.1. Alkaloid. About 50 mg of extracts was added by 5 mL of chloroform and 3 drops of ammonia. Chloroform fraction was transferred to another tube and added by 10 drops of H₂SO₄ 2 M. The solution was divided into three parts to the plate drops, then tested with three alkaloid reagents (Wagner, Meyer, and Dragendorf). Positive results of the alkaloid by Wagner reagent formed brown precipitation, by Meyer reagent formed yellowish white precipitation, and by Dragendorf reagent formed red to orange precipitation. The positive control used was simpilsia of vinca leaves (*Catharanthus roseus*) [13].
2.2.4.2. **Flavonoid.** About 50 mg of extracts was added by 2.5 mL of 30% (v/v) methanol, then heated at temperature of 50°C for 5 minutes. The filtrate was separated and added by 3 drops of H$_2$SO$_4$. Positive result showed by formation of red color. Simplisia of the red betel leaves used as positive control [14-15].

2.2.4.3. **Saponin.** About 50 mg of extracts was added by 2.5 mL of aquadest, then boiled at temperature of 100°C for 5 minutes. The solution was cooled and shaken vigorously. A positive result indicated by a stable foam for 10 minutes. The positive control used was simplisia of lerak (*Sapindus rarak*) seeds [16].

2.2.4.4. **Steroid and triterpenoid.** About 50 mg of extracts was diluted by 2.5 mL of 30% (v/v) ethanol. The solution was heated at temperature of 50°C until the solvent was evaporated. The residue was added by 1.0 mL of ether. The ether fraction was added by 3.0 mL of anhydrous acetic acid, then heated for 5 minutes. The solution was cooled, then added by a drop of concentrated sulfuric acid. A positive result indicated by a red or purple (triterpenoids) and green or blue (steroid). Som java (*Talinum paniculatum* Willd.) leaves was used as a positive control [17].

2.2.4.5. **Tannin.** About 50 mg of extracts was diluted by 2.5 mL of aquadest, and boiled for 5 minutes. Several drops of sample were moved to the plate drops and added by 5 drops of 1% (w/v) FeCl$_3$. A positive result indicated by formation of blue-black color. Simplisia of tea leaves was used as a positive control [18].

2.2.5. **Antibacterial activity analysis**

2.2.5.1. **Optical Density (OD) measurement.** After culture confirmation by Gram staining, the bacterial culture (24 hours of age) in NB medium was prepared. Six tubes were used to make series of concentration 1:1, 1:2, 1:4, 1:8, 1:16, and blank. Tube 1:1 was filled with 3 mL of bacterial culture, whereas tubes 1:2 to 1:16 were filled with 3 mL of NB medium without bacteria. The tube 1:2 then added by 3 mL of bacterial culture in NB. About 3 mL of solution in tube 1:2 was transferred into the tube 1:4 and continued until the 1:16 tube. Solution of each tube was measured by UV-Vis spectrophotometer at a wavelength of 620 nm [19].

2.2.5.2. **Total Plate Count (TPC).** Bacterial culture (24 hours of age) in NB medium was prepared. A total of 8 tubes was prepared for 10-1, 10-2, 10-3, 10-4, 10-5, 10-6, 10-7, and 10-8 concentration series. Each tube was filled with 9 mL of 0.85% (w/v) NaCl. One mL of bacterial suspension (24 hours of age) was added to 10-1 dilution tube. The dilution continued by taking 1 mL from a tube to the next dilution tube until 10-8. Pour plating were performed on dilution of 10-6 to 10-8 (duplo). The plates were incubated at temperature of 37°C for 24 hours and observed [19].

2.2.5.3. **Antibacterial and Minimum Inhibitory Concentration (MIC) analyses.** Antibacterial analysis was performed by disc diffusion method [20]. One loop of bacterial culture of *E. coli* and *S. aureus* were taken respectively, then dissolved in 10 mL NB and incubated at 37°C for 24 hours. About 20 mL NA media was poured on petri dish, and 0.1 mL of 106 CFU/mL bacterial suspension were spread above after the media solidified. The 10 µL of extract (20%, 40%, 60%, 80% and 100% concentration (w/v) ) was injected into paper disc with a diameter of 6 mm. Distilled water was used as negative control and 0.5% (w/v) of antibiotic amoxicillin was used as positive control. The discs were put on the agar surface, each petri dish contained 5 discs. The samples were incubated at 37°C for 24 hours, and the antibacterial activity was calculated based on the diameter of the inhibition zone. Each treatment was performed three times. Minimum Inhibitory Concentration (MIC) was analysed by lower series of concentrations (65%, 70%, and 75% (w/v)). The lowest concentration that showed clear zone was marked as MIC value.
3. Results and discussion

3.1. Moisture content of simplicia and yield of extracts
The result showed that moisture content of the berenuk’s fruit flesh simpisia was 5.37% (w/w), greater than the berenuk’s fruit rind (4.48% (w/w)). The moisture content was in accordance with good condition of sample in powder form, less than 10% to avoid the growth of microorganisms [21]. The extraction was done by maceration method because it was performed without heating treatment. High temperature in sample extraction or sample preparation might be contribute to destruct bioactive compounds [22]. Ethanol 70% (v/v) was the chosen solvent due to the better extraction of antibacterial compounds such as phenolic and saponins [12]. The yield of extraction by 70% (v/v) of ethanol was 4.44 g (9.30% (w/w)) and 31.52 g (66.62% (w/w)) for berenuk’s fruit rind and flesh, respectively. Berenuk’s fruit flesh had higher yield extract than berenuk’s fruit rind because of the higher glucose content [3]. Higher yield of extract showed higher amount of extracted secondary metabolites [23]. The amount of yield depends on several factors such as harvesting time, solvent, and extraction method.

3.2. Phytochemical compounds
Phytochemical analysis performed to determine the characteristics of bioactive compound of a crude extract that have beneficial pharmacological effects when tested by bioassay. The result of phytochemical analysis showed on Table 1. This research showed that ethanol extract of berenuk’s fruit rind contained more phytochemical compounds than its fruit flesh, either the kind or the intensity. Ethanol extract of berenuk’s fruit rind showed positive results on alkaloid, saponin, tannin, and flavonoid tests. However, the ethanol extract of berenuk’s fruit flesh showed positive results only on alkaloid and flavonoid tests. According to other studies, berenuk’s fruit flesh contained flavonoid, saponin, tannin, alkaloid, and phenols [2], anthraquinone and cardenolide [3]. Differences of the experimental results might be caused by the differences in plant origin, sampling and harvesting time, or sample preparation method. The differences of plant origin, sampling, and harvesting time may lead to difference of nutrients in plants so that secondary metabolites that formed were different [24]. In addition, Obuguagu [2] and Ejelonu et al. [3] used berenuk’s without any solvents, while this research performed by ethanol 70%.

Table 1. Phytochemical compounds of berenuk’s fruit rind and flesh extract.

| No | Sample                        | Phytochemical compounds |
|----|-------------------------------|-------------------------|
|    |                               | Alkaloid | Saponin | Tannin | Flavonoid | Triterpenoid | Steroid |
| 1  | Ethanol extract of berenuk fruit rind | +++      | +++     | +++    | +++       | -           | -       |
| 2  | Ethanol extract of berenuk fruit flesh | +++      | -       | -      | +         | -           | -       |
| 3  | Positive control              | +++      | ++      | +++    | ++        | +++         | +++     |

Note: Positive control: alkaloid (vinca leaves), saponin (lerak seeds), tannin (tea leaves), flavonoid (red betel leaves), triterpenoid & steroid (som java leaves). Negative result indicated by (-), while (+) expressed positive results. The intensity of positive results were indicated by more (+) marks.

3.3. Antibacterial activity and Minimum Inhibitory Concentration (MIC)
Figure 1 showed the correlation between cell number of bacteria and optical density (OD). The R2 number was 0.98, indicated a strong correlation. The antibacterial analysis of ethanol extract of berenuk’s fruit rind showed inhibition zone only on the growth of S. aureus at the concentration of 80% (w/v) and 100% (w/v), i.e. 3.0 mm and 4.2 mm, respectively (Table 2). The inhibition zone
diameters were less than 10 mm thus classified as no inhibition [25]. The other concentrations in this experiment showed no inhibition zone to the growths of *S. aureus* and *E. coli*.

Ethanol extract of berenuk’s fruit rind showed more potency as antimicrobial agent than fruit flesh. Previous research of antibacterial activity of berenuk’s fruit flesh was against *Aeromonas salmonicida*, a bacteria in freshwater fish [4], and *Vibrio alginolyticus*, bacteria that invade the grouper fish [5]. The results indicated that berenuk’s fruit flesh 100% (w/v) was not potential to inhibit *S. aureus* and *E. coli*.

The potency of ethanol extract from berenuk’s fruit rind as antimicrobial agent was supported by the result of phytochemical screening which showed more active compounds than ethanol extract of fruit flesh. Ethanol extract of berenuk’s fruit rind contained alkaloid, saponin, tannin, and flavonoid. Mechanism of alkaloid as antibacterial agent is permeabilising cells and inhibiting efflux pump ATPases [26]. Saponin can interact with the cell membrane through hydrogen bonds. This kind of detergent-like properties can increase membrane permeability’s without destroying the cells and increase nutrient influx [27-28]. It was also reported that flavonoids is effective as antimicrobials, by interacting with the polar head-group of the membrane followed by penetration into the hydrophobic regions [29].

Ethanol extract of berenuk’s fruit rind showed more potency as antimicrobial agent to Gram positive bacteria represented by *S. aureus* than to Gram negative bacteria represented by *E. coli*. Gram-negative bacteria are covered by a thin peptidoglycan with lipopolysaccharide (LPS) around the outer membrane. Gram-positive bacteria has no LPS around the outer membrane but are surrounded by multilayers of peptidoglycans [30-31]. However, this structure make it easier to be penetrated by antibiotics that have membrane cells destruction ability. The cell wall structure of Gram-positive bacteria is easily inhibited by polar phytochemical compounds such as the result (Table 1). Roversi et al. [32] reported that some antimicrobial agents also can kill the bacteria via membrane disruption as an event taking place saturate the bacterial membrane.

Based on the antimicrobial activity result, MIC test was performed to ethanol extract of berenuk’s fruit rind on *S. aureus* growth. Clear zone diameters on the MIC test result were 3.38 mm (65% (w/v)), 3.75 mm (70% (w/v)), and 3.75 mm (75% (w/v)), then the MIC values obtained at a concentration of 65% (w/v). The low activity of both extracts in killing the bacteria can be caused by age, region, and time of sampling plants, resulting in sub-optimum extraction of secondary metabolites [33].

In conclusion, ethanol extract of berenuk fruit rind contained alkaloid, saponin, tannin, and flavonoid, while fruit flesh contained alkaloid and flavonoid. Antibacterial analysis showed no clear zone on both extracts against *E. coli*. Ethanol extract of berenuk’s fruit rind extract showed clear zone only on the growth of *S. aureus* at the concentration of 80% (w/v) and 100% (w/v). The MIC of ethanol extract of berenuk’s fruit rind on *S. aureus* growth obtained at a concentration of 65% (w/v). This research showed that ethanol extract of berenuk’s fruit rind and flesh were less potential as an antimicrobial agent, because the MIC obtained was high.
Figure 1. Correlation of cell number and Optical Density of *S. aureus* (A) and *E. coli* (B).
Table 2. Antibacterial analysis results of ethanol extract from berenuk fruit rind and flesh to S. aureus dan E. coli

| No | Sample                            | Concentration (% (w/v) | Inhibition Zone Diameter (mm) |
|----|-----------------------------------|------------------------|------------------------------|
|    |                                    |                        | S. aureus      | E. coli |
| 1  | Ethanol extract of berenuk’s fruit flesh | 20                     | -              | -       |
|    |                                    | 40                     | -              | -       |
|    |                                    | 60                     | -              | -       |
|    |                                    | 80                     | -              | -       |
|    |                                    | 100                    | -              | -       |
| 2  | Ethanol extract of berenuk’s fruit rind| 20                     | -              | -       |
|    |                                    | 40                     | -              | -       |
|    |                                    | 60                     | -              | -       |
|    |                                    | 80                     | 3.0<sup>a</sup> | -       |
|    |                                    | 100                    | 4.2<sup>b</sup> | -       |

Note: (-) : no inhibition zone
Different alphabets showed significant difference

References
[1] Cano J H and Volpato G 2004 *J. Ethnopharmacol.* 90 293-316
[2] Oguagu N M 2008 *J. Anim. Vet. Adv.* 7 1069-1072
[3] Ejelonu B C, Lasisi A A, Olaremou A G and Ejelonu O C 2011 *African J. Biotech.* 10 19631-19636
[4] Rahardja B S, Sari F Y and Cahyoko Y 2011 *JIPK* 3 109-111 (in Indonesia Language)
[5] Rinawati N D 2011 Antibacterial activity of majapahit plant (*Crescensia cujete* L.) on bacteria *Vibrio alginolyticus*. (Surabaya: Institut Teknologi Sepuluh November) (in Indonesia Language)
[6] Das N, Islam M E, Jahan N, Islam M S, Khan A, Islam M R and Parvin M S 2014 *BMC Complement. Altern. Med.* 14 1-9
[7] Kusumala A M, Sulistyono A N, Susanti and Sabikis 2014 *PSR* 1 134-140
[8] Ardianti A and Kusnadi J 2014 *JPA* 2 28-35
[9] Parvin M S, Das N, Jahan N, Akhter M A, Nahar L and Islam M E 2015 *BMC Res. Notes* 8 1-7
[10] Intan S M 2008 Antibacterial activity of majapahit (*Crescensia cujete* L.) leaf extract to *Staphylococcus aureus* and *Streptococcus pyogenes* bacteria through in vitro (Surabaya: Institut Teknologi Sepuluh November) (in Indonesia Language)
[11] The Association Official Analytical Chemists 2007 *Official Methods of Analysis of AOAC International* (Washington DC: AOAC International)
[12] Harborne J B 1987 *Phytochemical Methods A Guide to Modern Techniques of Plant Analysis* (New York: Chapman and Hall, Inc)
[13] Zhu J, Wang M, Wen W and Yu R 2015 *Pharmacon Rev.* 9 24-28
[14] Safitri M, Kurniawati A and Syaufudin 2016 *IFRJ* 23 1123-1130
[15] Syaufudin, Safitri M and Hasanah U 2016 *J. Nutr. Food* 11 83-90 (in Indonesia Language)
[16] Hamburger M, Slacanin I, Hostettmann K, Dyatmiko W and Sutarjadi 1992 *Phytochem. Anal.* 3 231-237
[17] Ramos M P O, Silva G D F, Duarte L P, Peres V, Miranda R R S, de Souza G H B, Belinelo V J and Filho S A V 2010 *J. Chem. Pharm. Res.* 2 265-274
[18] Savolainen H 1992 *J. Applied Toxicol.* 12 191-192
[19] Hadiotomo R S 1993 *Basic Microbiology in Practice: Basic Technique and Procedure of Laboratory* (Jakarta: Gramedia Pustaka Utama) (in Indonesia Language)
[20] Lv F, Liang H, Yuan Q and Li C 2011 *Food Res. Int.* 44 3057-3064
[21] Cahyono B, Huda M D and Limantara L 2011 *J. Reaktor* 13 165-171 (in Indonesia Language)
[22] Febrianti A, Dwiyanti G and Siswaningsih W 2014 *JSTK* *5* 85-95
[23] Sani R N, Nisa F C, Andriani R D and Maligan J M 2014 *JPA* *2* 121-126
[24] Maser W H, Yuliana N D and Andarwulan N 2015 *J. Liq. Chromatogr. R. T.* *38* 1230-1235
[25] Greenwood 1995 *Antibiotics, Susceptibility (Sensitivity) Test Antimicrobial and Chemoterapy* (New York: Mc Graw Hill Company)
[26] Chusnie T P, Cushnie B and Lamb A J 2014 *Int. J. Antimicrob. Agents* *44* 377-378
[27] Jacob M C, Favre M and Bensa J C 1991 *Cytometry* *12* 550–558
[28] Arabski M, Węgierek-Ciuk A, Czerwonka G, Lankoff A and Kaca W 2012 *J. Biomed. Biotechnol.* *2012* 1-6
[29] He M, Wu T, Pan S and Xu X 2014 *Appl. Surface Sci.* *305* 515-521
[30] Silhavy T J, Kahne D and Walker S 2010 *Cold Spring Harb. Perspect. Biol.* *2* 1-16
[31] Malanovic N and Lohner N 2016 *Biochim. Biophys. Acta* *1858* 936-946
[32] Roversi D, Luca V, Aureli S, Park Y, Mangoni M L and Stella L 2014 *ACS Chem. Biol.* *9* 2003–2007
[33] Azwanida N N 2015 *Med. Aromat. Plants* *4* 1-6