

## ANTIBACTERIAL ACTIVITY OF MALACCA FRUIT (*PHYLLANTHUS EMBLICA* L.) ETHANOLIC EXTRACT AND FRACTION AGAINST *BACILLUS CEREUS* FNCC0057 AND *SHIGELLA DYSENTERIAE* ATCC13313

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### ABSTRACT

**Background:** Diarrhea is a symptom of a disease, which is characterized by increased frequency of defecation (more than 3 times a day) with a more fluid of feces. In ayurvedic medicine, the fruit of Malacca (*Phyllanthus emblica* L.) is often used as an antibacterial and antiviral against various infectious diseases. **Objectives:** This study aims to determine: (i) The antibacterial activities of ethanol extract and its fraction of Malacca fruit and (ii) determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against diarrhea-causing bacteria such as *Bacillus cereus* FNCC0057 and *Shigella dysenteriae* ATCC13313. **Methods:** The study was conducted through several processes: (i) The determination of the plant and preparation of dried fruit, (ii) extraction of dried fruit, fractionated extract, antibacterial activity test of ethanol extract, and its fractions, (iii) determination MIC and MBC from the most active fractions, and (iv) phytochemical screening of fraction. **Results:** The results showed that ethanol extract, water fraction, and ethyl acetate fraction of Malacca fruit has antibacterial activities against *B. cereus* FNCC0057 and *S. dysenteriae* ATCC13313, where the greatest activity was shown by the fraction of ethyl acetate. This fraction has MIC and MBC values of 0.187-0.375% (w/v) and 0.09375-0.1875% (w/v) against *B. cereus* FNCC0057 and *S. dysenteriae* ATCC13313. The antibacterial activity of this fraction was probably derived from alkaloid, flavonoid, tannin, quinone, saponin, monoterpene, or sesquiterpene compounds. **Conclusions:** The results of the suggested that the extracts of the studied plants can be used as potential leads to discover new drugs to control some enteric-bacterial infections.

**Keywords:** Antibacterial, *Phyllanthus emblica* L, *B. cereus* FNCC0057, *Shigella dysenteriae* ATCC13313.

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### INTRODUCTION

Diarrhea is defined as bowel movements with amorphous or liquid stools which occur for more than 3 times a day. Based on its etiology, acute diarrhea can be caused by infections, intoxication, allergies, adverse drug reactions, as well as other psychological factors. Mostly, diarrhea occurs due to infection from microorganism. Infection with diarrhea symptom can be caused by viruses, bacteria, and parasites [1].

In Ayurvedic medicine, *Phyllanthus emblica* is used to prevent the effects of tumors, inflammatory, and also gastric ulcer [2]. According to Bandyopadhyay *et al.* (2000), *P. emblica* provides cytoprotective action on gastric ulcer formation. *P. emblica* is usually known as gooseberry (in English) or amla, in which this fruit belongs to phyllanthaceae family. Preliminary research on this fruit also demonstrated that this fruit also has antiviral and antimicrobial activity [3]. This plant also contains abundant tannins as its secondary metabolites [4].

Malacca fruit contains polysaccharides, proteins, and high on calcium [5]. This fruit contains tannins as secondary metabolites in excess amount [4].

The aim of this study was (i) to test antibacterial activity of Malacca fruit ethanolic extracts and fractions of fruit Malacca (originating from Bogor, Indonesia), (ii) to determine minimum inhibitory concentration (MIC), and (3) to determine phytochemicals compound that were contained by this Malacca fruit.

### METHODS

#### Chemicals and materials

Materials used in this study were Malacca fruit (*P. emblica* L.), ethanol, toluene, dimethyl sulfoxide (DMSO), n-hexane, ethyl acetate, chloroform, hydrochloric acid, ammonia, Mayer reagents, Dragendorff reagents,

Liebermann–Burchard reagents, iron (III) chloride, gelatin, magnesium powder, amyl alcohol, ether, sulfuric acid, vanillin, acetic acid, sodium hydroxide, silica gel, potassium hydroxide, cellophane, and distilled water.

#### Sample preparation

Malacca fruit was washed with running tap water and then rinsed by distilled water to remove any adsorbed contaminant from sample surface. The cleaned sample was chopped and dried then placed in an oven at 40°C for 12 hrs to remove any remaining moisture. The dried material was ground by a blender into smaller parts and was collected for extraction.

#### Extraction

Maceration is a method that commonly used for extraction of bioactive components from natural products. This extraction method was chosen for the first preliminary study because of its simplicity and manageability [6]. Small parts of dried Malacca fruit was macerated with 70% ethanol (1:20, w/v) at room temperature for 4 days and filtered through a Whatman no.1 filter paper [7]. The extraction process was repeated until the last extract was colorless. Ethanol was then removed using rotary evaporator at 68°C and water was removed by putting the crude extract on top of 40°C water bath for 24 hrs. The percentage of crude dry extract was determined as follows:

$$Y \text{ extract (\%)} = \left( \frac{M \text{ extract}}{m \text{ feed}} \right) \times 100$$

Where Y extract is the extraction yield, M extract is the crude extract mass (g), and m feed is the feed mass (g).

#### Phytochemicals screening

The crude ethanolic extracts of Malacca fruit were tested for the presence of alkaloids, steroids, tannins, saponins, and glycosides.

The qualitative results are expressed as + for the presence and - for the absence of phytochemicals.

#### Test for alkaloids

About 15 mg of extract was separately stirred with 1% HCl (6 ml) on a water bath for 5 min and filtered. These filtrates were divided into three equal parts.

- Dragendorff's test: To one portion of the filtrate, Dragendorff's reagent (potassium bismuth iodide solution) (1 ml) was added; an orange-red precipitate shows the presence of alkaloids [8].
- Mayer's test: To one portion of filtrate, Mayer's reagent (potassium mercuric iodide solution) (1 ml) was added. Formation of cream-colored precipitate gives an indication of the presence of alkaloids [8].
- Wagner's test: Potassium iodide (2 g) and iodine (1.27 g) were dissolved in distilled water (5 ml) and the solution was diluted to 100 mL with distilled water. Few drops of this solution were added to the filtrate; a brown-colored precipitate indicates the presence of alkaloids [8].

#### Tests for steroids and terpenoids

- Salkowski test: The crude extract (about 100 mg) was separately shaken with chloroform (2 ml) followed by the addition of concentrated  $H_2SO_4$  (2 ml) along the side of the test tube, a reddish brown coloration of the interface indicates the presence of terpenoid [9].
- Liebermann-Burchard test: Extract (100 mg) was shaken with chloroform in a test tube; few drops of acetic anhydride were added to the test tube and boiled in a water bath and rapidly cooled in iced water. Concentrated  $H_2SO_4$  (2 ml) was added alongside the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green shows the presence of steroids while formation of deep red color indicates the presence of triterpenoids [9].

#### Test for tannins

Malacca fruit extract (0.5 g) was separately stirred with distilled water (10 ml) and then filtered. A few drops of 5% ferric chloride were then added. Black or blue-green coloration or precipitate was taken as a positive result for the presence of tannins.

#### Test for saponins

Malacca fruit extracts (0.5 g) was separately shaken with distilled water (10 ml) in a test tube. The formation of frothing, which persists on warming in a water bath for 5 minutes, shows the presence of saponins.

#### Tests for glycosides

- Antraquinone glycoside (Borntrager's test): To the extract solution (1 ml), 5%  $H_2SO_4$  (1 ml) was added. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with an equal volume of chloroform and kept to stand for 5 minutes. Then, lower layer of chloroform was shaken with half of its volume with dilute ammonia. The formation of rose pink to red color of the ammoniacal layer gives indication of anthraquinone glycosides [10].
- Cardiac glycoside (Keller-Killiani test): Extract (0.5 g) was shaken with distilled water (5 ml). To this, glacial acetic acid (2 ml) containing a few drops of ferric chloride was added, followed by  $H_2SO_4$  (1 ml) along the side of the test tube. The formation of brown ring at the interface gives positive indication for cardiac glycoside and a violet ring may appear below the brown ring [10].

#### Antibacterial activity

Test organisms

Bacteria samples used in this study were *Bacillus cereus* ATCC13313 and *Shigella dysenteriae* FNCC0057, which originated from the Laboratory of Pharmaceutical Microbiology, Faculty of Pharmacy, Universitas Padjadjaran.

#### Preparation of stock and working solutions

The Malacca fruit ethanolic extract stock solutions were prepared at a concentration of 50 mg/ml, respectively, in 100% dimethyl sulfoxide

(DMSO, Sigma-Aldrich). The working solutions were prepared by diluting stock solutions in Mueller-Hinton broth.

#### Determination of MIC

The MIC was determined by broth microdilution method and as per the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2006, M7-A7), with some modifications. Briefly, 200  $\mu$ l of the test sample was added to the wells of column 1. All the remaining wells from column 2 to column 10 initially received 100  $\mu$ l of MHB. Then, two-fold serial dilutions were performed by transferring 100  $\mu$ l from column 1 to column 2 and continued through column 10. 100  $\mu$ l of excess medium was discarded from the wells in column 10. All the wells from column 1 to 10 received further 100  $\mu$ l of drug-free MHB, whereas both columns 11 and 12 received 200  $\mu$ l of drug-free MHB. For the preparation of bacterial inocula, 24 hrs cultures were suspended in 5 ml of sterile normal saline. The turbidity of each bacterial suspension was adjusted to 0.5 McFarland standards ( $1.5 \times 10^8$  CFU/ml). The bacterial suspension was further diluted in MHB, and 50  $\mu$ l of the same was added to each well of microtiter plate to obtain a required inoculum of  $5 \times 10^5$  CFU/ml in the well. The final concentration of ant-plant ethanolic extract ranged from 2.0 to 1000  $\mu$ g/ml, respectively. Columns 11 and 12 served as growth and media controls, respectively. The plates were then incubated at 37°C for 24 hrs and were visually read for the absence or presence of microbial growth. The MIC was considered as the lowest concentration of the sample which completely prevented the visible growth [11].

## RESULTS AND DISCUSSION

#### Plant determination result

Plant determination was performed in Laboratory of Taxonomy, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. Whole parts of dried plants were used, and the results showed that sample plant are identic with *Phyllanthus emblica* L.

#### Extraction result

Since the active compound of Malacca fruit acting as an antimicrobial agent has not been discovered yet, the safest and simplest extraction method that can be used is maceration. After 4 days of extraction process using 10 l of 70% ethanol as solvent, from 1119.19 g dried plant, we can obtained 225.20 g ethanolic extract as shown in Table 1. The characteristic of the extract was dark brown viscous liquid with distinctive odor.

#### Fractionation

Fractionation method was performed using liquid-liquid fractionation technique. The result of fractionation is shown in Table 2. The solvents used were water and ethyl acetate, and from Table 2, it can be seen that from 255.20 g Malacca fruit ethanolic extract, we obtained two fractions, which is water as much as 9.18 g (36.69% yield) and ethyl acetate fraction amounted about 9.05 (36.17% yield).

The phytochemical compounds detected are known to have medicinal importance. For example, alkaloids have been reported as powerful poison, and many alkaloids derived from medicinal plants show biological activities such as anti-inflammatory, antimalarial, antimicrobial, cytotoxicity, antispasmodic, and pharmacological effects (Table 3).

Table 1: Extraction result

Dried sample weight	Extract weight	Yield
1119.19 g	252.20 g	22.80%

Table 2: Fractionation result

Fraction	Weight (g)	Yield (% w/w)
Water	9.18	36.69
Ethyl acetate	9.05	36.17
N-hexane	NA	NA

Table 3: Phytochemical screening

Phytochemicals	Ethanollic extract	Ethyl acetate fraction
Alkaloids	+	+
Flavonoids	+	+
Poliphenols	+	+
Tannins	+	+
Quinones	+	+
Monoterpenes and sesquiterpenes	+	+
Saponins	+	+

+: Present, -: Not present

Table 4: Phytochemical screening result

Fraction concentration (% w/v)	<i>B. cereus</i> FNCC0057	<i>S. dysenteriae</i> ATCC13313
6,0000	-	-
3,0000	-	-
1,5000	-	-
0,7500	-	-
0,3750	-	-
0,1875	+	-
0,0937	+	+
0,0468	+	+
0,0234	+	+

+: Bacteria growth, -: No bacteria growth, *B. cereus*: *Bacillus cereus*, *S. dysenteriae*: *Shigella dysenteriae*

#### Antibacterial activity test

Preliminary antibacterial activity test was performed by doing agar diffusion method. This method used extract and fraction with various concentrations. Antibacterial activity of the extract was shown by the clear zone at about the hole in the test medium. The clear zone indicated that Malacca fruit ethanolic extract and ethyl acetate fraction could inhibit bacterial growth.

The activity of plant extracts was considered as significant if MIC values were below 100 µg/ml, moderate when 100 < MIC ≤ 625 µg/ml, or weak when MIC > 625 µg/ml. On the other hand, the activity of phytochemicals was significant if MIC < 10 µg/ml, moderate if 1 < MIC ≤ 1 µg/ml, and low or negligible when MIC > 100 µg/ml. MIC test only performed for ethyl acetate fraction. Therefore, the activity recorded

for the ethyl acetate fraction could be considered to be moderate, as result shown in Table 4.

#### CONCLUSION

This is the first study reported about antibacterial activity of Malacca fruit ethanol extract and fraction against pathogenic *S. dysenteriae* and *B. cereus*. The results of this study provided a scientific basis for the traditional use of Malacca fruit against bacterial-administered diarrhea. The results of the suggested that the extracts of the studied plants can be used as potential leads to discover new drugs to control some enteric bacterial infections.

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