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Antibacterial Activity and Mechanism of Action of Methanol Extract from Kasturi Mango Fruit (*Mangifera casturi*) on Caries-Causing Bacterium *Streptococcus mutans*

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Abstract

One of the problems frequently found in the oral cavity is dental caries caused by *Streptococcus mutans*. Thus far, dental caries is treated using antibiotics. However, the bacterium is known to be resistant to many antibiotics; hence, another alternative is needed. An alternative option is found in the Kasturi mango (*Mangifera casturi*). This study aims to identify the bioactive compounds of Kasturi mango and find out the mechanism of its action in inhibiting the growth of *Streptococcus mutans*. Kasturi mangoes were macerated using 96% methanol, then the phytochemical compounds were identified qualitatively. Antibacterial activity testing was carried out using the agar diffusion method, and bioactive compounds were identified using GCMS. The results showed that the methanol extract of Kasturi mango contains alkaloids, flavonoids, phenolics, terpenoids, and saponins. In 1 gr/mL of the methanol extract of Kasturi mango fruit has an inhibitory activity against the growth of *Streptococcus mutans* with a zone of inhibition of ± 10 mm and MIC (minimum inhibitory concentration) value of 25% extract. The inhibitory action is suspected to be through a mechanism where holes in the bacterial cell membrane are made. This can be seen from the results of SEM (scanning electron microscope) images showing that cell leakage or lysis has occurred. This research also, for the first time, revealed the types of bioactive compounds from the methanol extracts of Kasturi mango (*Mangifera casturi*) consisting of 18 compounds with the most abundance is 5-Hydroxymethylfurfural compounds, Octadecenoic acid, n-Hexadecanoic acid, Phenyl 4-methyl-1-piperidine carboxylate, and Methyl linolenate.

1. Introduction

The Kasturi mango plant (*Mangifera casturi*) is an endemic plant from the island of Borneo. According to the Decree of the Minister of Home Affairs No.48 of 1989, the Kasturi mango plant is determined as the floral identity of the South Kalimantan Province. Kasturi mangoes are round to elliptical with a weight of 60-84 g, 4.5 - 5.5 cm long, and 3.5-3.9 cm wide. The flesh is yellow or orange with fibers; the texture of the fruit flesh is rather rough, has a sweet, slightly sour fruit taste and unique aroma.

Dental caries is the main problem that is most often found in the oral cavity, wherein Indonesia alone dental caries have a prevalence of 90.05%, meaning that it can attack all levels of society from various age groups and economies [1]. Dental caries is caused by the interaction of multiple factors, such as host factors (teeth and saliva), food, and microorganisms. The microorganisms that cause caries are bacteria of the types Streptococcus and Lactobacillus. However, *Streptococcus mutans (S. mutans)* is the primary bacterium that causes dental caries formation. The prevention and treatment of dental caries to date have not been limited to traditional methods such as regular dental check-ups, brushing teeth with fluoride toothpaste, and low-sugar foods. The use of some natural ingredients as problem-controlling agents in the oral

cavity has been reported previously by Sogandi and Nilasari [2], who revealed that the noni fruit extract could inhibit the growth of *S. mutans* bacteria that cause dental caries.

Extracts from Kasturi mangoes have also been reported to have inhibitory activity against some bacteria, such as the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli.* Kasturi mangoes are said to contain terpenoid compounds that have antioxidant activity along with triterpenes and phenolic compounds that act as anti-inflammatory [3]. Kasturi mango is also a plant with the highest total flavonoid content compared to other medicinal plants in South Kalimantan, such as kelakai, gerunggang, and pasak bumi [4]. This study aims to determine the inhibitory activity of Kasturi mango extract and determine the types of bioactive compounds that act as inhibitors of the growth of *S. mutans* bacteria that cause dental caries.

2. Research Methodology

2.1. Sample Extraction

Kasturi mango *(Mangifera casturi)* was obtained from Banjar District, South Kalimantan, and observation was carried out at LIPI Cibinong, Bogor. The process of drying the Kasturi mango was done using an oven at 50°C for seven days. The dry herb obtained was sorted and selected for components that have no bad characteristics or fungus-free. The clean herb was dried and chopped into small pieces, then crushed to form a 40 mesh powder [3]. As much as 1 kg of the Kasturi mango which had been formed into powder was macerated using methanol solvent in a ratio of 1: 5. The herb was shaken every 2 hours, left for 24 hours, and then the filtrate was concentrated.

2.2. Secondary Metabolites Screening

2.2.1. Alkaloid Identification

1 mL of sample was added with 5 mL of 10% hydrochloric acid. Then, ammonium hydroxide and chloroform were added, and the mixture was shaken. The chloroform layer at the bottom was taken and evaporated to dry. The residue was added with 2 mL 2% HCl, then added with Mayer and Bouchardad reagents. The addition of Mayer reagent will give a white precipitate, and the addition of Bouchardad reagent will provide a brown precipitate, which combined indicate that the sample contains alkaloids [5].

2.2.2. Flavonoid Identification

As much as 1 mL of sample was added with a small piece of magnesium plate, 1 mL of concentrated HCL solution, and 5 mL of amyl alcohol solution. It was shaken vigorously and allowed to separate. The appearance of red color rising to the top shows that the sample contains flavonoids [5].

2.2.3. Steroid and Triterpenoid

A total of 30 mg of extract was added with five drops of glacial acetic acid and two drops of concentrated sulfuric acid. Extracts contain steroids if they form blue or green coloration, whereas if it contains triterpenoids, the formation of red or purple coloration would occur [5].

2.2.4. Saponin Identification

The solution from the experimental results of the flavonoid group identification was shaken vertically for 10 seconds, then left for 10 minutes. The formation of foams and its long remaining appearance show the presence of saponins in the sample [5].

2.2.5. Tannin and Phenolic Identification

The sample was added with three drops of $FeCl_3$ solution, and the color changes that occurred were observed. A positive tannin is indicated by the formation of a greenish-blue color, whereas the establishment of a green, purple, or blue to black color indicates the presence of phenolics [5].

2.2.6. Making of Mc. Farland Standard Solution

Prior to the testing of antibacterial activity, the *S. mutans* test bacterium was standardized using the 0.5 Mc Farland standard solution to obtain bacterial cell concentration of ± 1.5 x 10^8 CFU/mL. The 0.5 Mc Farland standard solution was made by mixing 1% $BaCl_2$ solution with 1% H_2SO_4. The turbidity of the Mc Farland 0.5 standard solution was compared with the results of the suspension of S. mutans bacteria using a 0.9% NaCl solution.

2.3. Antibacterial Activity Testing

2.3.1. Media Preparation

All tools and materials are sterilized using an autoclave at 121°C for 15 minutes. The agar media that have been sterilized were cooled to a temperature of 45-50°C and then added with 5% goat blood. The media was poured into a disk and was left until it solidified.

2.3.2. Test Bacteria Regeneration

Planting of *S. mutans* bacteria in the blood agar media was done by dipping a sterile inoculation loop into the bacterial suspension first, then etching it onto the surface of the media to form four quadrants and incubating it at 37°C for 24 hours. A single colony was taken using the ose, grown in a liquid media, and used as test bacteria.

2.3.3. Measurement of Antibacterial Activity

The measurement of antibacterial activity was carried out by modification of the method of Sogandi and Nilasari [2]. With the *S. mutans* bacteria that had been matured, the day before was taken as much as 3 mL to be diluted with 5 mL of 0.9% NaCl solution. 3 mL of the suspension was taken and mixed with a blood agar medium as much as 17 mL, poured into a petri dish, and was allowed to stand for 15 minutes. Then, paper disks containing a sample of methanol extract of the Kasturi mango fruit, a negative control (distilled water), and positive control were placed on the surface of blood agar media. Petri dishes were incubated at 37°C for 24 hours, and the clear zone formed was measured using a caliper [2].

2.4. Minimum Inhibitory Concentration Test

The MIC test was carried out using the agar diffusion method. The extract concentrations used in this test were 10, 15, 20, 25, and 30%. Clear zone measurements were made after the bacteria were incubated for 24 hours at 37°C. The lowest concentration of the sample, which still gives inhibitory zones to the media, is determined as the MIC value.

2.5. GCMS Analysis

The bioactive compounds from the methanol extract of the Kasturi mango fruit were analyzed using GCMS (Shimadzu GCMS-QP 2010 Ultra) to determine the types of compounds present in the extract. The stationary phase used was Rxi-1MS (100% dimethyl polysiloxane) with a column length of 30 m and a diameter of 0.25 mm. The conditions set were ultra-high purity helium carrier gas pressurized at 37.1 kPa, injection volume of 5 µL, 250°C injector temperature, 230°C ion source temperature, 230°C interface temperature, and split mode of 10. The column was programmed from 70°C then increased to 230°C with a rate of increase of 10°C/minute and 3 minutes hold time. The final column temperature was 270°C with a rate of increase of 5°C/minute and was held for 3 minutes.

2.6. Cell Damage Analysis

Analysis of changes in the cell morphology was performed to determine changes in the cell structure after being treated with extracts. The treatment procedure was done by adding 250 µL of *S. mutans* bacteria that had been cultured overnight in a nutrient broth liquid medium grown at 37°C for 5 hours. The culture was then centrifuged (6000 g, 4°C for 3 minutes), and the cells were resuspended with 1000 µL PBS buffer containing MIC 1 and MIC 2. The PBS buffer without the addition of extract was used as control. The cell suspension was then incubated at 37°C for 30 minutes and was shaken during the duration. The suspension was then centrifuged again. Cells were then fixed using 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer. The fixation results are then analyzed using the JCM Scan Electron Microscope (SEM) series.

3. Results and Discussion

The study began with the acquirement of the Kasturi mango sample from the peat area in Banjar District, South Kalimantan Province. The determination of specimen was carried out at the LIPI Cibinong biological research center, Bogor, to ensure the species to be used was, in fact, a Kasturi mango species. Based on the results of the determination, it is known that the plant used in this study is *Mangifera casturi*.

3.1. Extract Characteristics

3.1.1. Extract Yield

The Kasturi mangoes were extracted using the maceration method. Maceration was done by soaking 200 grams of the herb powder into a methanol solvent; this immersion process would cause the methanol to penetrate the cell wall and into the cell cavity. This is caused by the difference in concentration between the inner and outer parts of the cell, making the entire contents of the cell pushed out. The event recurs until there is a balance of concentration between the solution outside the cell and inside the cell [6]. The choice of methanol as the solvent solution is because, in the antibacterial test, water is very influential on the sensitivity of the antibacterial test in which water is a suitable growth medium for microorganisms. The 96% methanol solvent, which has a water content of only 4%, can reduce the contamination of the extract. Furthermore, methanol, as a solvent, is more selective, able to attract more polar substances, has good absorption, volatile, and is a difficult medium for mold and yeast to grow [7].

The viscous extract obtained from the maceration of 200 grams of Kasturi mango herb powder was 66.2 grams with a yield of 33.1%, determined by dividing the yield weight (viscous extract) by the initial weight of the powdered mango herb.

3.1.2. Phytochemical Screening

The phytochemical compound screening was carried out qualitatively to provide preliminary information regarding the compounds contained in the methanol extract of the Kasturi mango. The methanol extract of the Kasturi mango contains several secondary metabolites, as shown in Table 1 below:

3.2. Antibacterial Activity

The disk diffusion method was used to measure the antibacterial activity of the Kasturi mango fruit (*Mangifera casturi*) methanol extract against the *S. mutans* test bacteria. The inhibitory activity is determined by the amount of inhibition zone formed within the disk with ampicillin as a positive control and sterile distilled water as a negative control. *S. mutans* bacteria were regenerated one day before being used as test bacteria to obtain bacteria in the log phase as a starter [8].

Table 1. Examination of secondary metabolites of methanol extract of Kasturi mango

Test method	Observed compound	Test Result
Qualitative	Alkaloid	+
	Saponin	+
	Tannin	+
	Phenolic	+
	Flavonoid	+
	Terpenoid	+
	Steroid	-

The results show that the inhibitory activity of the methanol extract of the Kasturi mango with a concentration of 500 mg/mL could inhibit the growth of *S. mutans* bacteria, as indicated by the presence of a clear zone of ± 10 mm in diameter around the paper disc. The presence of this clear zone is thought to be due to the presence of hostile activity originating from the secondary metabolites of the Kasturi mango such as flavonoids, tannins, saponins, phenolics, and alkaloids [9]. The sensitivity of bacteria to a chemical compound

can be seen from the size of the clear zone formed. The bigger the clear zone, the more sensitive the bacteria are to the chemical compounds being tested. In this study, the *S. mutans* bacteria have a low sensitivity to the Kasturi mango extract used as a test material. This is in contrast to the ampicillin antibiotic used as a positive control; ampicillin showed potent activity against *S. mutans* bacteria [10].

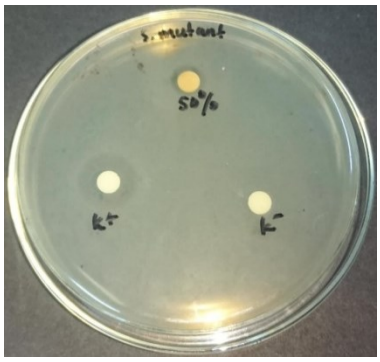

Figure 1. Antibacterial activity of methanol extract of Kasturi mango fruit extract with a concentration of 50%, ampicillin positive control (K^+) and negative control (K^-)

The results of this study support the results of a study conducted by Rosyidah *et al.* [9] in which Kasturi mango plant whose bark contains terpenoids and saponins also have inhibitory activity against Gram-negative and positive bacteria. To be able to kill bacteria, a compound must be able to penetrate the cell through the bacterial cell wall. There is a difference between Gram-negative and positive bacterial cell walls. In general, Gram-negative bacteria contain relatively more lipids than Gram-positive bacteria, such as the *S. mutans* bacteria. The mechanism of action of ampicillin antibiotics as a positive control in this study is by inhibiting the activity of the enzyme transpeptidase needed for the irreversible synthesis of bacterial cell walls. Specifically, ampicillin can inhibit the last three stages in the stage of the formation of bacterial cell walls that can cause the bacterial cells to begin to perish [11].

Some types of mango plants are also reported to have inhibitory activity against bacterial growth, including mango leaf extract obtained from Pakistan, which has activity against antibiotic-resistant bacteria *Salmonella typhi* [12]. It is also known from the research of Van Quang *et al.* [13] that mango leaf extract *(Mangifera indica)* originating from Vietnam contains active compounds in the form of mangiferin, which can inhibit the growth of S. *mutans* bacteria. The methanol extract of mango bark originating from Malaysia is also known to have better inhibitory activity against the growth of *S. mutans* ATCC 2517 bacteria compared to the extracts of eucalyptus twigs [14].

3.3. Determination of MIC Value

Based on the MIC value testing performed at concentrations of 10, 15, 20, 25, and 30%, it is found that at a concentration of 25%, inhibitory activity exists, shown by the clear visible zone around the disc paper indicating the absence of growth of *S. mutans* bacteria at that concentration. The results of this MIC test support the research on the activity of the Kasturi mango bark against *Staphylococcus aureus* bacteria, demonstrating a MIC value of 25% extract concentration [14]. Nevertheless, methanol extract of *Syzygium aromaticum* is also known to have better MIC value 20% against the growth of *S. mutans* bacteria [15].

3.4. GCMS Analysis

The identification of the types of secondary metabolite compounds contained in the methanol extract of the Kasturi mango fruit was carried out by GCMS analysis. The identification results show that 18 compounds are present, with the most abundant of the compounds are 5-Hydroxymethylfurfural (15.44%); 9-Octadecenoic acid (13.46%); n-Hexadecanoic acid (12.19%); 4-Methylpiperidine-1-carboxylic acid, phenyl ester (9.47%); and Methyl linolenate (8.46%).

Table 2. GCMS results of methanol extract of Kasturi mango

RT	Compound	Content (%)	Similarity (%)
7.313	5-Hydroxymethylfurfural	15.44	94
19.629	9-Octadecenoic acid	13.46	93
17.104	n-Hexadecanoic acid	12.19	92
5.479	Phenyl 4-methyl-1-piperidinecarboxylate	9.47	90
19.014	Methyl linolenate	8.46	94

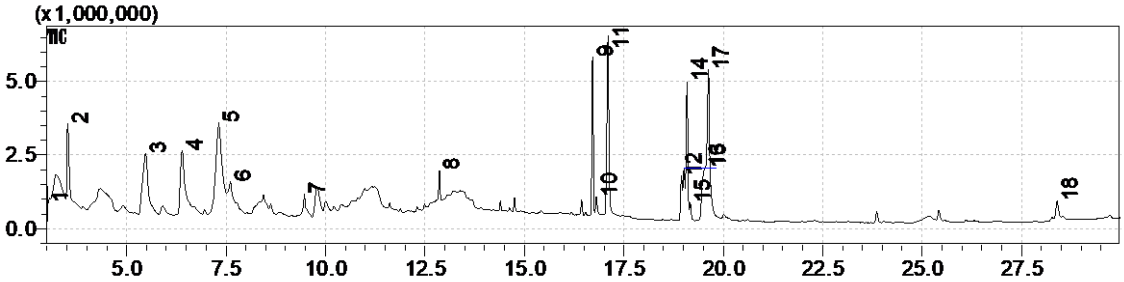

Figure 2. GCMS spectrogram results of methanol extract of Kasturi mango

The 5-Hydroxymethylfurfural (5-HMF) compound is an organic compound present in the methanol extract of Kasturi mango with the most abundant content (15.44%). The 5-HMF compound is usually obtained from the dehydration process of some glucose and has the molecular formula $C_6H_6O_3$. The 5-Hydroxymethylfurfural (5-HMF) molecule consists of a furan ring containing an aldehyde group and an alcohol

group. The 5-HMF has the IUPAC name 5-(hydroxymethyl) -2-furaldehyde. The following is an image of the 5-HMF compound structure:

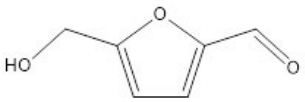

Figure 3. 5-*Hidroxymethylfurfural* (5-HMF) compound

The 5-Hydroxymethylfurfural (5-HMF) compound is commonly found in honey and has been reported to have inhibitory activity against bacterial growth of both Gram-negative and Gram-positive bacteria. HMF can inhibit the bacteria *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Methicillin-Resistant Staphylococcus aureus* (MRSA) [16]. HMF compounds are usually used as an indicator of the quality of honey. 5-Hydroxymethylfurfural is also known to have benefits as an antioxidant, hypo-allergenic, anti-inflammatory, and antibiotic agent [17].

The 9-octadecanoic acid is a long-chain fatty acid compound with the molecular formula $C_{18}H_{34}O_2$. This compound is found in neem oil *(Azadirachta indica)* and has the ability to actively inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella sp* [18].

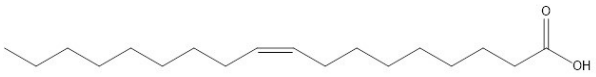

Figure 4. 9-octadecanoic acid compound

The n-hexadecanoic acid compound is a fat derivative that has the formula $C_{16}H_{32}O_2$ that possesses an inhibitory mechanism against bacteria by absorbing their nutrients, inhibiting the entry of water, and inhibiting the work of enzymes in the Gram-positive bacteria [18]. The n-hexadecanoic acid compound is also present in the ethyl acetate fraction of licorice extract (*Glycyrrhiza glabra L*). As described by Sogandi *et al.* [19], licorice extract has inhibitory activity against *Bacillus cereus* bacteria classified as strong. Aside from being an antibacterial, n-hexadecanoic acid compounds are also reported to have antioxidant activity [20].

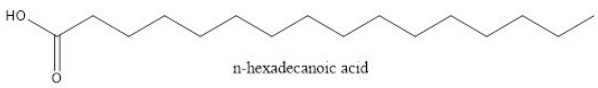

Figure 5. N-hexadecanoic acid compound

Phenyl 4-methyl-1-piperidine carboxylate compound has the molecular formula $C_{13}H_{17}NO_2$, and the Methyl linolenate compound has the molecular formula $C_{19}H_{32}O_2$. These compounds are found in ethanol extracts of water cabbage plants (*Pistia stratiotes L*) possessing the antifungal activity and antibacterial activities against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeroginosa* [21, 22].

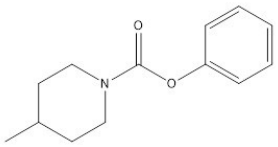

Figure 6. Phenyl 4-methyl-1-piperidine carboxylate structure

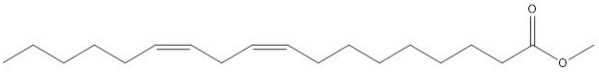

Figure 7. Methyl linolenate compound structure

3.5. Mechanism of Action

The results of this study also show that the methanol extract of Kasturi mango has the ability to interfere with the membrane integrity of the *S. mutans* bacteria. It is believed to be sensitive to the Kasturi mango extract since the bacterial cells experience lysis (Figure 5). The cause behind the cell lysis is possibly due to the phenolic compounds contained in the Kasturi mango extract known to be able to react with phospholipids from bacterial cell membranes resulting in changes in cell membrane permeability [23]. Damage to the cell membrane results in the intracellular components such as amino acids, nucleic acids, and proteins to come out of the cell, meaning that the bacterial cell will die.

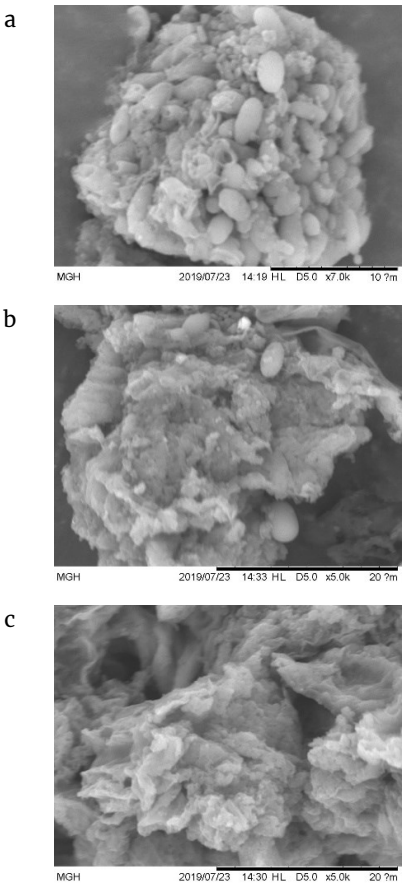

Figure 8. Changes in the cell morphology caused by methanol extracts of Kasturi mango shown through SEM. (a): without treatment, intact round cell shape, (b): with 25% concentration treatment, some cells appear to be torn and experience lysis, (c): with 50% concentration treatment, the cell appears to be broken and experience necrosis

The application of extract on the test bacteria appears to cause severe cell damage. Where the administration of extract with a concentration of 25% caused cells to begin to experience shrinkage and undergo lysis possible because the peptidoglycan in the test bacterial cells cannot withstand the pressure exerted by the Kasturi mango extract, resulting in cell lysis and the cytoplasm to sput out [24]. When treated with 50% extract concentration, the cell is severely damaged, and necrosis occurred. The entire contents of the cell were discharged, and the cell no longer appears to form a rounded cell.

4. Conclusion

This research has proven that the methanol extract of Kasturi mango (*Mangifera casturi)* has the potential to prevent the formation of dental caries caused by *Streptococcus mutans* bacteria. GCMS analysis results revealed that the most abundant content of the extract was 5-Hydroxymethylfurfural compound, Octadecenoic acid, n-Hexadecanoic acid, Phenyl 4-methyl-1-piperidine carboxylate, and Methyl linolenate. The results of SEM micrographs show that the extract can lyse the target cell. These results provide a theoretical foundation for the application of the use of Kasturi mangoes as a natural antibacterial.

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