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Anti-Apthous Stomatitis Screening of Fresh Fruit Juice

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Abstract

Aim: This study was purposed to determine out the fruit juice with the highest anti-aphtous stomatitis activity of ten selected fruit juices (avocados, grapes, dragon fruit, guava, lime, kiwi, lemon, passion fruit, strawberries, and tomatoes) against *Staphylococcus aureus* and *Streptococcus mutans*.

Methods: The anti-aphtous stomatitis activity of the juices was determined based on the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of each juice. The values of the MIC were determined using the microdilution method. The MIC results then were inoculated onto the surface of agar media and the growth colonies were observed to determine the MBC values of each juice. Comparison of the efficacy between the selected potent juice as anti-aphtous stomatitis were assayed using the agar diffusion method.

Results: Phytochemical screening results showed that all of the tested fruit juices generally contained flavonoids, saponins and tannins which have been known as an antibacterial agent. From the MIC and MBC result, the juice of lemon and lime showed the lowest value of ≤1.5625% w/v. Based on the supported data on the diameter zone of inhibition, the lemon juice strongly inhibited against both tested bacteria, than that of the lime juice.

Conclusion: The results of this study provide promising scientific information for the potential use of lemon juice for aphtous stomatitis treatment.

Keywords: aphtous stomatitis, juice, fruit, *Staphylococcus aureus, Streptococcus mutans*

INTRODUCTION

Aphthous stomatitis, also known as canker sores or recurrent aphthous stomatitis (RAS) is the most common chronic oral cavity disease. This illness usually affecting 5-25% of the population [1,2]. The symptoms of aphthous stomatitis appear as small ulcers in the mouth, usually inside the lips, on the tongue, or on the cheeks [3]. The disease can be affected because of many factors as follows: genetic factors, local trauma, food allergens, endocrine alterations (menstrual cycle), certain chemical products, stress and anxiety, smoking cessation, and microbial agents [1, 4-6]. Aphthous stomatitis will generally heal within 4 days, but the presence of a microorganism infection that causes aphthous stomatitis can aggravate the condition.

Staphylococcus aureus and *Streptococcus mutans* are normal microflora that are present in the mouth. From patients with atrophic denture stomatitis, *S. aureus* was isolated in a higher percentage (84% of patients) than *S. mutans* (16% of the patients) [7]. The surface adhesin (P1) of *S. mutans* can bind to salivary agglutinin on the tooth surface and bind to extracellular matrix proteins such as collagen and fibronectin. Therefore, after brushing the tooth, *Streptococcus* can enter the bloodstream and causes inflammation that lead to plaque accumulation in the arteries and can develop atherosclerosis. This condition related to heart attack, cardiovascular disease, endocarditis and stroke [8].

Aphthous ulcers can spread and causing systemic diseases, thus it is important to decide the suitable therapy. There are topical and systemic medications used to treat this infection. The topical medication availabe as topical corticosteroids (triamcinolone, dexamethasone, clobetasol or fluocinonide) and antimicrobial mouthwash. The purposed of topical medication is primary to reduce pain and to improve time of healing, but not improve remission or recurrence rates. Meanwhile the systemic medications alternatively used if topical therapy is not effective [9]. However, the use of chemical drugs is relatively expensive and has side effects. Recently, a lot of natural products have been studied as an antimicrobial agent [10]. The natural product has been popularly used in dentistry because of its low cost and antimicrobial potency [11]. Many consumable fruits could be the safest natural medicine against the pathogenic bacteria in the oral cavity. Therefore, the use of fruit that has a chemical content that acts as an antibacterial can be given in the form of juice.

Fruits juices are nutritious, has great taste and provide health benefits because of its content, such as polyphenolic compounds, flavonoids, terpenoids, steroids, alkaloids, saccharides, vitamin C and minerals [12-14]. These compounds can be effective against bacterial infections. It has been traced in the literature, there are many fruits that contain chemical ingredients that empirically used as traditional herbal medicine for aphthous stomatitis and scientifically prove can act as antibacterial agents, as follows: avocados, guava, red grapes, dragon fruit, lemon, tomatoes, kiwi, lemons, passion fruit, and strawberries [15-22]. Based on those data research, it is necessary to conduct a study to determine the antibacterial potential of those ten fruit juices against *S. aureus* and *S. mutans* bacteria as aphthous stomatitis causative bacteria.

MATERIALS AND METHODS

Plant Materials

Ten fruits of avocados, grapes, dragon fruit, guava, lime, kiwi, lemon, passion fruit, strawberries, and tomatoes were local fruits collected from supermarkets in Bandung, West Java, Indonesia. All the samples were obtained in fresh condition and directly prepared for further processing. Each fruit was identified in Plant Taxonomy Laboratory of Biology Major, Faculty of Mathematics and Natural Science Padjadjaran University.

Chemical materials

The chemicals used consist of ethanol (CV. Agung Menara Abadi), aquadest (Chemistry dept. UNPAD), potassium permanganate (Christa), hydrochloric acid (Merck), ether (Merck, acetic acid (Merck), amyl alcohol (Merck), sulfuric acid (Merck), chloroform (Merck), methanol (Merck), dragendorff reagents (Merck), mayer reagents (Merck), iron (III) chloride (Merck), metal powder (CV. Agung Menara Abadi, 1% gelatin solution (CV. Medilabs), ammonia solution (Merck), Liebermann Burchard reagents (Merck), vanillin (merck) and sodium hydroxide (PT. Brataco). The growth medium used in this study were fresh blood of goats (Faculty of Medicine UNPAD), Blood Agar Base (Pronadisa), Mueller Hilton Agar (Oxoid) and Mueller Hilton Broth (Oxoid).

Bacterial Strain

The test bacteria used in this research were *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* ATCC 12392 that obtained from the Faculty of Medicine, Padjadjaran University, Jatinangor, West Java, Indonesia.

Juice Preparation

The juice was prepared from single fruit. About 100 g of fresh fruit was cleaned using tap water and finally washed by sterile aquadest. Each fruit was cut into pieces and soaked using potassium permanganate for 15 min. After that the fruit was pressed using a juicer that has been previously cleaned with 70% alcohol then rinsed with sterile distilled water. The resulting juice was then centrifuged for 30 min at 4000 rpm in order to separate the fruit pulp and its supernatan. The fruit juice (supernatan) obtained was then taken and pasteurized at 65ºC for 30 min.

Phytochemical Screening

Phytochemical screenings of each fresh fruit juice were carried out to determine the secondary metabolite content, such as flavonoids, tannins, polyphenolates, triterpenoids, and saponins. The screening method was performed according to standard methods [23].

Preparation of Bacterial Suspension

The turbidity of the bacterial suspension for MIC assayed must be equivalent to a concentration of 10^6 CFU/ml. This concentration can be obtained by diluting the 0.5 Mc Farland standard solutions, which indicated that the suspension contain of 1 x 10^8 CFU/ mL. The Mc Farland solution contains about 1.175% $BaCl_2$ solution and 1% H_2SO_4 solution. The preparation of 0.5 Mc Farland solution was done by taking 0.05 mL of $BaCl_2$ 1.175% solution and mixed with 9.95 mL of 1% H_2SO_4 solution. After that the mixing solution was shaked until homogeneous. The bacteria colonies which had been cultured in MHA agar medium for 18-24 h at 37 °C were taken by Ose, then suspended in physiological NaCl sterile. The turbidity of the solution was measured using distilled water as the blank at 625 nm. To achieve 0.5 Mc Farland solution absorbance, so the value of the bacterial suspension must be in the range of 0.08 to 0.13. Bacterial turbidity was measured with McFarland 0.5 standard solutions. Then, the 0.5 Mc Farland standard solutions were diluted to achieve 10^6 CFU/ml.

MIC and MBC Determination

The MIC value of each fresh fruit juice was determined by microdilution plate assay. Juice dilution was applied in a range of 1,5625% -50% w/v with serially twofold dilution concentration, using sterile aquadest as the solvent. In each well, 100 µl of sterile MHB was poured to all well in microdilution plate, then a 100 µl of each fresh fruit juice in a concentration of 100% was diluted to the second well to achieve the concentration of 50% w/v. So as the next well, until the lowest concentration. After that, a volume of 100 µl bacterium cell with a concentration of 10^6 CFU/ml was inoculated to each well, thus the final inoculum in each well was $5x10^4$CFU / 200µl. Each test plate contained an uninoculated control (100 µl juice + 100 µl media) and negative control (200 µl medium). The samples were conducted in triplicate and incubated for 24 h at 37°C. The absence of bacterial growth at the lowest concentration was interpreted as MIC value. The MBC value was determined by inoculating a volume of 10 µl MIC results to the surface of MHA agar media. Then the tested media were incubated for 24 h at 37°C. MBC value was defined as the lowest concentration without colony growth on the media.

Determination of the Most Potent Juice

Based on the MIC result, there were two fruit juices (lime and lemon fruit juice) that exhibiting the lowest concentration of bacterial inhibition at the same concentration range. Therefore, it needs a furthermore evaluation to determine the most potent juice as an anti-aphthous stomatitis. The selection was conducted using the agar diffusion method at the same test concentration (50% w/v). A 100% w/v of each fruit juice was diluted into 50% % w/v using sterile aquadest as the solvent. The bacterial suspension turbidity of this method was different to that of MIC determination method. The bacterial suspension of each test bacteria was made the same as the turbidity of 0.5 Mc Farland standard solutions. The sterile petri dish containing 20 mL MHA (40-45^{0}C) was inoculated with 20 µL bacterial suspensions. The media was homogenized and allowed to solidify. The petri dish was homogenized slowly, then allowed to solidify. The medium then perforated to make holes for storing the 50 µl of sample and incubated at 37 °C for 24 h. After the incubation period, the diameters of inhibition zone were measured using a caliper.

RESULTS AND DISCUSSION

Yield of Juice Processing

Before processing, fruits were washed and soaked using 0.1% potassium permanganate in order to decontaminate the fruits. Fruit or vegetables decontamination using 0.1% potassium permanganate was found to be more effective than washing using sterile water [24]. Food safety is important because fresh fruit may resulted food borne infections. Fruits can get contaminated during the cultivation process, storage and transport [25,26]. Therefore, disinfection is an important step to sanitize the fresh fruits before used.

The fresh fruit juices were obtained by juice processing using a juicer. The juice processing did not employed using a blender. They both can produce nutritious and delicious fruit juice, but they gave different end result. A juicer can separate the juice from the fruit fiber. Therefore, it will be easy to discard the fiber and leave the fruit juice in the digest form to drink. Meanwhile the blender processes the whole fruit, thus it is difficult to separate the juice from the remaining fruit fiber. Therefore, the end result of fruit processing using blender will produce a smoothie juice which needs time to digest.

A total volume juice yielded from the juice processing were various in rendemen, can bee seen in table 1. The amount of fruit juice produced from each fruit was different, this was because the difference in its water content. The juice yield value is related to the secondary metabolites content number [27].

Table 1: Juice Processing Yield

Fruits (100 g)	Water content (%)	Yield (ml)
avocados	73	2
grapes	86	15
dragon fruit	89.4	7
guava	86	2
lime	92	12
Kiwi	90	10
Lemon	92	13
passion fruit	69	6
strawberries	90.6	9
tomatoes	94	8

Phytochemical Screening Results

The phytochemical screening of ten fruit juices was purposed to investigate the phytochemicals that may play an important role in anti-aphtous stomatitis activity against *S. aureus* and *S. mutans*. In this study, the phytochemical screening of the juices revealed the presence of flavonoids, tannins, polyphenolates, triterpenoids, and saponins. These secondary metabolites abundant of each fruit juice were varied, can be seen in table 2. The differences of phytochemical content may be due to differences in fruit type and geographical location. However, it can be concluded that ten fruit juices generally contain flavonoids, saponins, and tannins.

MIC and MBC Result

The inhibition of each fruit juice performed significantly different antibacterial activity against both tested bacteria, performed on table 3-4 and figure 1. In addition, the bactericidal activity (MBC) was detectable at an equal concentration as MIC value for both strains.

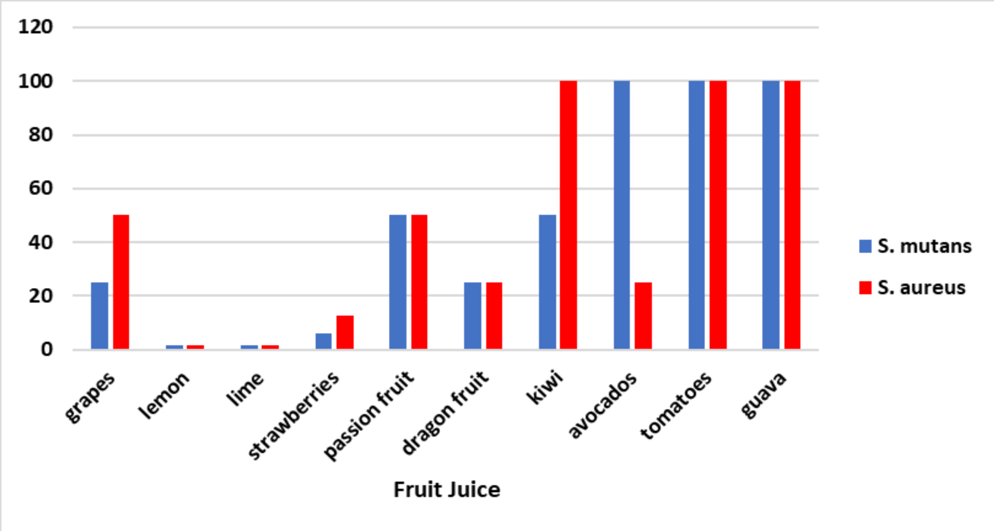

Figure 1: Comparison of the lowest inhibitory concentration of each juice against *S. mutans* and *S. aureus*

Table 2: Phytochemical Screening of Fruit Juice

Fruit	Poly phenolates	Flavonoid	Saponin	Tanin	Triterpenoid
avocados	+	+	+	+	+
grapes	+	+	-	+	-
dragon fruit	-	+	+	-	-
guava	+	+	+	+	+
lime	-	+	+	+	+
Kiwi	-	+	+	+	-
Lemon	-	+	+	+	+
passion fruit	-	+	+	+	+
strawberries	+	+	-	+	-
tomatoes	+	+	+	-	+

Notes: (+) presence; (-) absence

Table 3: MIC and MBC Result of Fruit Juices Against *S. aureus*

Concentration (%)	Fruit Juice									
	1	2	3	4	5	6	7	8	9	10
50	-	-	-	-	-	-	+	-	+	+
25	+	-	-	-	+	-	+	-	+	+
12.5	+	-	-	-	+	+	+	+	+	+
6.125	+	-	-	+	+	+	+	+	+	+
3.125	+	-	-	+	+	+	+	+	+	+
1.5625	+	-	-	+	+	+	+	+	+	+
Kontrol (+)	+	+	+	+	+	+	+	+	+	+
Kontrol (-)	-	-	-	-	-	-	-	-	-	-

Notes: (1) grapes; (2) lemon; (3) lime; (4) strawberries; (5) passion fruit; (6) dragon fruit; (7) Kiwi; (8) avocados; (9) tomatoes; (10) guava; (-) no colonies; (+) colonies present

Table 4: MIC and MBC Result of Fruit Juices Against *S. mutans*

Concentration (%)	Fruit Juice									
	1	2	3	4	5	6	7	8	9	10
50	-	-	-	-	-	-	-	+	+	+
25	-	-	-	-	+	-	+	+	+	+
12.5	+	-	-	-	+	+	+	+	+	+
6.125	+	-	-	-	+	+	+	+	+	+
3.125	+	-	-	+	+	+	+	+	+	+
1.5625	+	-	-	+	+	+	+	+	+	+
Kontrol (+)	+	+	+	+	+	+	+	+	+	+
Kontrol (-)	-	-	-	-	-	-	-	-	-	-

Notes: (1) grapes; (2) lemon; (3) lime; (4) strawberries; (5) passion fruit; (6) dragon fruit; (7) Kiwi; (8) avocados; (9) tomatoes; (10) guava; (-) no colonies; (+) colonies present

The antibacterial property of lemon and lime juice were found to be maximum against *S. mutans* and *S. aureus*. The antibacterial activity was observed from the lowest concentration of inhibition at > 1.5625 %w/v among others fruits juice. While juice extract of tomatoes and guava juice did not show any inhibitory effect against both bacteria. Both bacteria gave the same bacterial response to lime, lemon, passion and dragon fruit juice, can be seen from the resulted of the same MIC value. In contrast, the difference antibacterial activities were shown in the avocados juice result, which only inhibited *S. aureus* at 25 %w/v but not against *S. mutans*. The same result also given by kiwi juice, that only inhibited *S. mutans* at 50 %w/v but not to *S. aureus*. The grapes juice also produced different antibacterial activity, that was more potent against *S. mutans* than that of *S. aureus*. The hypothesis for these different bacterial responses against fruit juice is may be due to different physical interaction between fruit juice as a drug molecule and its specific target on certain bacteria. This different interaction will affect the alterations to the affected bacterium at the molecular, biochemical and ultrastructural levels. Therefore, these different gave various inhibition responses of juice against different bacteria.

MIC and MBC values of the fruit juice can inform that the fruit juice contains of antibacterial compound and can be applied for determining the minimal dose used in pharmaceutical dosage form. The data of phytochemical screening result supported the capability of each fruit juice in giving MIC and MBC value. In common, each fruit juice revealed the presence of flavonoids, saponins and tannins. The content of polyphenols was owned by several juices as follows: avocados, grapes, guava, strawberries, and tomatoes. This compound has an important role as an antibacterial agent by coagulating the bacterial protein, thus causing the bacterial lysis.But in this study, such fruits that contain polyphenols did not show the significant inhibition against both tested bacteria. This fact, in line with the information literature which stated that ripe fruits display lower phenol contents than the immature fruits and in this study, the fruit using for juice processing was in ripe stages [28, 29]. Interestingly, all the fruit juice had flavonoid that can damage the cell membran of bacteria by discharging intracellular compounds [30]. However, but differences in the levels of flavonoids in each fruit juice produced differences in the antibacterial strength. All fruit juice contains of saponin, except grapes and strawberries. Saponin also support the other metabolites to lysis the bacteria using a different mechanism of action. The saponins interact with lipopolysaccharide of bacterial cell wall and reduce the surface stress, lead to the bacterial cell wall degradation [31]. Another secondary metabolites that were also important was tannins, which owned by all the fruit juice, except dragon fruit and tomatoes. Tannins can disrupt the bacterial cell membrane and caused cell wall shrinkage, which lead to bacterial lysis [32]. Meanwhile, approximately half of the total fruit juice was detected possess terpenoids, which can disrupt the cell membrane of bacteria by the lipophilic compounds [33]. Those antibacterial compounds of each fruit juice worked integratedly in contributing inhibition affect for the treatment of aphtous stomatitis infection.

Comparison of Antibacterial Inhibition Effect

From the MIC and MBC results, the lime and lemon juice exhibited the same inhibitory effect at the lowest concentration of >1.5625 %w/v. Generally, both citrus juices showed the broadest antibacterial activity. The inhibition comparison of both citrus juices was summarized in table 5 and figure 2. These juices displayed antimicrobial active against both bacteria because the acid concentrations in lime and lemon are higher than in any other fruit juices [34]. The presence of many antibacterial substances such as: citric acid, linalool, limonene, linalyl acetate, cymen and turpinol, may contribute to the strong antibacterial activity of citrus fruit juices [35]. After the follow up study using the agar diffusion method, the lemon juice showed higher inhibitory activity against both bacteria, compared with lime juice. However, the lemon juice provide better inhibition to *S. mutans* than that of *S. aureus*. Based on the Clinical and Laboratory Standards Institute (CLSI), there are three categories of antibacterial strength according to the its inhibition diameter, as follows: strong antibacterial (≥ 20 mm); moderate antibacterial (15-19 mm); and weak antibacterial (≤ 14mm) [36]. Based on CLSI categories, the lemon juice characterized as a moderate antibacterial agent against *S. mutans*, but had a weak antibacterial properties against *S. aureus.*

Table 5: Comparison of Antibacterial Inhibition Effect Between Lime and Lemon Juice

Bacteria	Diameter of Inhibition Zone (mm)	
	Lemon Juice	Lime Juice
Staphylococcus aureus	12.2±0.001	11.3±0.003
Streptococcus mutans	16.0±0.000	13.7±0.002

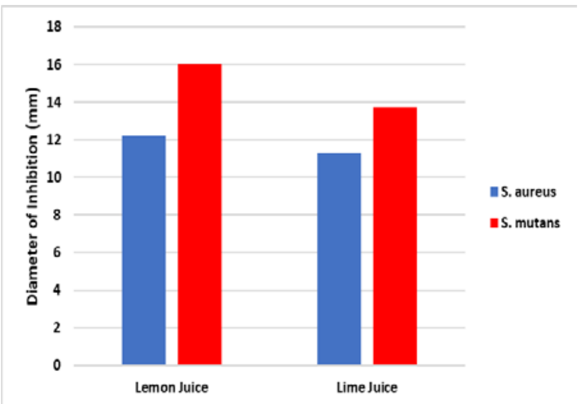

Figure 2: Comparison of Antibacterial Inhibition Effect Between Lime and Lemon Juice

Conclusion

The results of this study clearly exhibited a significant anti-aphtous stomatitis properties of lemon fruit juice against *Staphylococcus aureus* and *Streptococcus mutans*. Lemon juice showed highest anti-aphtous stomatitis against both tested bacteria, followed by lime, strawberries, dragon fruit, grapes, passion fruit, avocados, and kiwi juice. Tomatoes and guava juice showed no anti-aphtous stomatitis infection.

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