Inhibition of Streptococcus Mutans Growth Induced by the Extract of Citrus Aurantifolia Peel

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Abstract

The aim of this study was conducted to assess the in vitro antimicrobial potential and also determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Citrus aurantifolia peel extracts.

This study will analyze the antibacterial activity of C. aurantifolia peel. This test uses the disc diffusion and microdilution method. The extracts prepared from peel of C. aurantifolia were screened for in vitro antimicrobial activity against Streptococcus mutans (ATCC 25175).

Results showed that the inhibition zone was obtained at a concentration of 50% in methanol extract, hexane, ethyl acetate, and water fractions were 22.1; 18.1; 28.7 and 20.8 mm, respectively. MIC and MBC values of hexane fractions were 0.19 and 0.39%. MIC and MBC values of ethyl acetate fractions were 0.78 and 1.56%. This data reinforce the statement that the hexane fraction is the most active fraction.

This study shows that the extract of C. aurantifolia peel contain compounds with therapeutic potential has been verified. It has an effect as an inhibiting agent the formation of S. mutans.

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Introduction

Character of Streptococcus mutans is a major factor in formation of cariogenic biofilms. It is a gram-positive facultative anaerobic bacteria and as an obligate biofilm organism is the principal etiological agent of dental caries and has the ability to metabolize carbohydrates.[1–3] These bacteria can adhere then form tenacious biofilms on tooth surfaces.[2,4,5]

Acidogenic and aciduric character of S. mutans and ability to synthesize glucan extracellular, is a major factor in formation of cariogenic biofilms.[6–9] Rapidly reduce the pH in dental biofilms due to sucrose fermentation by oral bacteria can result in a shift in the balance of resident plaque microflora to become more cariogenic.[2,6,9–12] Sucrose serves as a substrate for the synthesis of polysaccharides, especially extracellular polysaccharides (EPS) in dental biofilm.[2,10–12]

The bacterial adhesion mechanism is mediated by several ways of which the synthesis of extracellular polysaccharides (glucans and fructans) is main in dental biofilm formation.[2,11,12] These polysaccharides are synthesized by extracellular enzymes glucosyltransferase (GTF) and fructosyltransferase (FTF).[2,4,8,13–15] S. mutans produces three glucosyltransferases, coded by gtfB, gtfC, and gtfD, whose cooperative action is essential for sucrose dependent cellular adhesion.[4,5,7,8,12,14,16] This cellular adhesion plays an important role in the formation of dental plaque and also the initiation of dental caries.[4,5,14,17]

The popularity of herbal medicines is increasing day by day and is currently in

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demand. WHO recommends and encourages the use of traditional herbs or remedies in the healthcare sector because huge amount of raw material is easily available. Plants are very complex in nature and their therapeutic activity varies according to species, harvesting processes, and geographical location. Improper authentication of herbs, pesticide residue, adulterations by microorganism; has made standardization of herbal drug of primary importance.[18,19]

One of the important medicinal and food plant cultivated in many parts of the world is Citrus aurantifolia. Citrus aurantifolia is a polyembryonic plant cultivated in many countries all over the world and grows in hot tropical or subtropical regions.[20] These health benefits of C. aurantifolia are associated with its high amounts of phytochemical and bioactive compounds such as flavonoids, limonoids, carotenoids, phenols, minerals and vitamins.[20,21]

Plants of the genus Citrus are primarily valued for their edible fruit, but also have traditional medicinal value.[20] The peel of Citrus has been used in traditional Asian medicine for centuries.[22,23] Lime peel contains antibacterial active compounds with the highest concentration compared to other parts.[24]

The peel of Citrus is a rich source of flavanones (polymethoxylated flavones) which are very rare in other plants. The most prevalent flavanones are hesperetin and naringenin, both of which are found in the fruit peel largely as their glycosides, hesperidin and naringin, respectively. Tangeretin and nobiletin are two polymethoxylated flavones that are commonly found in lime peels. These compounds play important physiological, ecological roles, and also of commercial interest because they have a multitude of applications in the food and pharmaceutical industries.[23] Citrus aurantifolia peel contains antibacterial active compounds with the highest concentration compared to other parts. Various activities of C. aurantifolia include leaves, seeds, and fleshs have been studied, including the extract of the peels, but no one has examined it to the fraction level.[20,25,26]

These citrus residues, which are generally discarded as waste in the environment, can act as potential nutraceutical resources. Due to their low cost and easy availability such wastes are capable of offering significant low-cost nutritional dietary supplements. The utilization of these bioactive rich citrus residues can provide an inexpensive, efficient, and environment friendly platform for the production of novel nutraceuticals and improvement of older ones.[4] Finally, there should be a translation from pre-clinical screening of the plant's fruit peels to the isolation of active compounds and the actual development of useful drugs from the plant. It was therefore of interest to develop the methods through the suppression of cariogenic biofilm formation in the oral cavity for improving oral health care and decreasing teeth decay. This study was conducted to find out the effect of extract of C. aurantifolia peel as an inhibiting agent the formation of Streptococcus mutans(ATCC 25175), and evaluate the MIC and MBC.[4,20]

Materials and methods

This type of research used in this study was a laboratory experimental design to compare the antibacterial activity of extract of lime peel (Citrus aurantifolia) and chlorhexidine against S. mutans. The characteristic of the study was a factorial design where there are two or more groups as a control variable or a comparison of the variables studied. The experiments were done in duplicate.[2,27]

Materials used in this research is the extract of lime peel, S. mutans ATCC 25175, BHI media, 2% sucrose, 2% chlorhexidine gluconate, methanol, n-hexane, ethyl acetate, sterile aquadest.

Collection of Citrus aurantifolia samples

Citrus aurantifolia peel samples were collected from the lime plantation of Sukawana Village in the Kertajati District, Majalengka, West Java, Indonesia.

Plant materials used in the study

Research begins with identification plants to ensure that plants which used true lime plant. Citrus aurantifolia were collected based on the information received from herbalists and their consorts on the basis of their effectiveness against microbial diseases. Plant part used were peels and collected from their natural environment. The plants were stored at room temperature.

Extraction and antibacterial activity test of Citrus aurantifolia peel

Collected C. aurantifolia were washed thoroughly under water, squeezed, and disposed

of water and seeds. The peel was extracted in 50 litre methanol under continuous shaking for 24 h. The extract was filtered through a 22 µm paper filter. The filtrate was evaporated to dryness using a rotatory evaporator at 40°C. The residues in the form of powder materials were preserved in sterile glass bottles at room temperate until further use.

The next step was prepared S. mutans as the agent test inhibitors. This test uses disc diffusion and microdilution method.

a. Sensitivity bacteria determined by disc diffusion method. Bacteria prepared by making a deep suspension medium BHI and used 0.5 McFarland standard. Samples were prepared in the form of solution at concentrations: 50, 20, 10, and 2% in medium BHI broth + 2% sucrose. As a solvent control used aquadestand positive controls were used 2% chlorhexidine gluconate. Paper discs (6 mm) were impregnated with 20 µL of each sample. Discs loaded with samples were placed onto the surface of the agar and then it incubated for 48 hours at the incubator temperature 37°C. The diameter of clear zone around the disk was observed. Inhibition zone around the disk was measured by using a caliper to determine the major inhibitory zone.

b.Determining minimal inhibitory concentration (MIC) value used micro dilution method. The bacterial cells were pre-cultured in brain heart infusion (BHI) broth at 37°C for 48 hours under anaerobic conditions. They were incubated in the presence of sample (extract) with the concentrations obtained by serial two-fold dilution. Solution test is placed on the microplate round bottom 96wells, with a total volume of 100 µl per well using a micropipet. The test grain contains an extract of lime peel with the addition of a 5 µl bacterial suspension, while the blanks containing the solution blank extract of lime peel without added bacterial suspension. Microplate round bottom 96well then incubated for 48 hours at the incubator temperature 37°C. Microplate round bottom 96wells removed from the incubator. Test results in form optical density (OD) are read with microplate reader Benchmark with wavelength 620nm, in the Chemistry laboratory, Faculty of Chemistry Universitas Padjadjaran (Unpad), Jatinangor.

Results

Results of the phytochemical of fraction of Citrus aurantifolia peel can be seen in Table 1.

	Secondary Metabolit	Methods (Reagents)	Result			
			MeOH	EA	Hex	H_2O
1	Phenolic	$FeCl_3$ 5%	+	+	+	+
2	Flavonoid	a. HCl + Mg	+	+	-	+
		b. H_2SO_4 2N	+	+	-	+
		c. NaOH10%	+	+	-	+
3	Steroid	Lieberman-Burchard	+	+	+	+
4	Triterpenoid		+	+	+	-
5	Saponin	HCl + H_2O	+	+	-	+
6	Tanin	$FeCl_3$ 1%	+	+	-	-
7	Alkaloid	Dragendorf	+	+	+	+

Table 1. Results of the phytochemical of fraction of *Citrus aurantifolia.*

Note: - MeOH : methanol fraction
- EA : ethyl acetate fraction
- Hex : n-hexane fraction
- H_2O : H_2O fraction

The inhibitory zone value of methanol extract, hexane, ethyl acetate, and water fractions at a concentration of 2, 10, 20, 50% can be seen in Table 2.

	Sample (fraction of lime)	The inhibitory zone (mm) at a concentration (%)			
		50	20	10	2
1	methanol	22.1	13.6	0	0
2	n-hexane	18.1	13.6	12.5	10.1
3	ethyl acetate	28.7	21.0	13.0	0
4	H_2O	20.8	0	0	0
5	chlorhexidine gluconate 2%				26.5

Table 2. Testing of inhibitory zones at a concentration of 2, 10, 20, and 50%.

Result of the testing of minimum inhibitory and minimum bactericidal concentration values of lime peel extract and fraction to *S. mutans* can be seen in Table 3.

	Sample (fraction of lime)	MIC (%)	MBC (%)
1	methanol	1.56	3.12
2	n-hexane	0.19	0.39
3	ethyl acetate	0.78	1.56
4	H_2O	6.25	12.5
5	chlorhexidine gluconate 2%	0.0019	0.0039

Table 3. Testing of minimum inhibitory and minimum bactericidal concentration values of lime peel extract and fraction to *S. mutans.*

Discussion

Results of plant identification in Laboratory of Plant Taxonomy, Department of Biology, Faculty of Science, Unpad, Jatinangor, Indonesia, stated that plants used in this study is a true lime plant (*Citrus aurantifolia*). In this study, lime fruit as much as 41.98 kg was cut into pieces and separated the water produced 27.4 kg of lime peel and flesh. The separated lime is then macerated with methanol to produce 506.7 g of methanol extract. Furthermore, lime methanol extract was fractionated with n-hexane, ethyl acetate, and water solvent to produce n-hexane fraction (2 g), ethyl acetate fraction (50 g) and water fraction (450 g). Results of the phytochemical of fraction of lime peel can be seen in Table 1. Majority of secondary metabolite from results of fraction of lime peel is phenolic, steroid, and alkaloid. This secondary metabolite is active in methanol fraction, ethylacetate fraction, n-hexane fraction, and water fraction of lime. The extracts as mentioned above, were subjected to various qualitative phytochemical tests for the identification of chemical constituents present in the plant material.

This study reports for the first time the phytochemical of fraction of *C. aurantifolia* cultivated in the Kertajati District, Majalengka, West Java, Indonesia. The phytochemical variability *C. aurantifolia* could be attributed to the chemical–physical properties, the composition of the soil, and the influence of other factors such as solar exposure and geographical coordinates. This variability reflects the biological properties of *C. aurantifolia* extracts found.[28]

Lime is an essential ingredient in the preparation of most herbal concoctions. Lime peel extract has antibacterial activity for inhibited bacteria growth which resulted in reduced number of bacteria so the ability of bacterial colonies to communicate with each other becomes inhibited.[20–22]

Results of research show that extract of lime peel able to inhibit *S. mutans*. Result of inhibition test can be seen in Table 2.

The three fractions that have been obtained were tested for antibacterial activity against *S. mutans* ATCC 25175 at a concentration of 50%. The inhibitory zone value of methanol extract, hexane, ethyl acetate, and water fractions were 22.1; 18.1; 28.7; and 20.8 mm, respectively. Based on this data, the n-hexane fraction has the highest activity. Therefore, hexane and ethyl acetate fractions were determined by the MIC and MBC values. From the test results, MIC values and MBC hexane fractions were 0.19 and 0.39% and the MIC and MBC values of ethyl acetate fractions were 0.78 and 1.56%. MIC and MBC data reinforce the statement that the hexane fraction is the most active fraction. (Table 3)

The results of this study show linear relationship between lime peel extract concentration with the inhibition of *S. mutans*, so the higher of concentration of lime peel extract, the greater inhibition of *S. mutans*. Ability lime peel extract in the enable enzyme which react with activity cell membranes, or interfering functionality of genetic material, energy production, and synthesis of structural components, possibly causing disruption glucosyltransferase enzyme activity which *S. mutans* use to synthesize sucrose in a medium glucan. Glucan is a bacteria attachment medium, if the amount is small, resulting in the formation of inhibited biofilms.[3]

The content of compounds in fractions can be determined by phytochemical testing. The test results showed that methanol extract contained all classes of secondary metabolites (phenolics, flavonoids, steroids, triterpenoids, saponins, tannins and alkaloids). The hexane fraction contains phenolics, steroids, triterpenoids, and alkaloids. Ethyl acetate fraction contains the same class as methanol extract except saponins. In the water fraction, almost all contain except tannins and triterpenoids.

Natural herbs such as lime peel are used as single or in combination have been

scientifically proven to be safe and effective medicine without side effects. The major strength of these natural herbs is that their use has not been reported with any side-effects till date.[29,30]

Chlorhexidine, a cationic bisbiguanide with a very broad antimicrobial spectrum, is considered the gold standard antimicrobial agent againts which the efficacy of other antimicrobial, antiplaque agents, and many herbal extracts is assessed.[29–31] Chlorhexidine as with other drugs is not devoid of side effects.[29] The free chlorhexidin molecules enter the cell and coagulate the proteins that cause decrease in activity of vital cell resulting in cell death.[32]

Both chlorhexidine and herbal can be effectively used for plaque control in the prevention of plaque and gingivitis. However, owing to the side effects reported due to the use of chlorhexidine and also biocompatibility and well acceptance of herbal, it can be effectively used as an alternative to chlorhexidine.[29]

Conclusions

Based on the results of this study then it can be concluded that extract of lime peel (*Citrus aurantifolia*) contain compounds with therapeutic potential and has an effect as an inhibiting agent the formation of *Streptococcus mutans* enzym activity. The higher level of lime peel extract, the larger zone of inhibition is formed. However, chlorhexidin with a 2% concentration has a better upward level when compared with lime peel extract (*Citrus aurantifolia*) from low concentrations to high concentrations.

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Declaration of Interest

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