

Active compounds isolated from red betel (*Piper crocatum* Ruiz & Pav) leaves active against *Streptococcus mutans* through its inhibition effect on glucosyltransferase activity

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ABSTRACT

Dental caries is a major problem of dental health in Indonesia. *Streptococcus mutans* is a bacteria that can cause caries. In this recent time, the agents used to eliminate the caries is less effective due to *S. mutans* resistance to those agents. Moreover, the agents may produce side effects after 10 years of consumption. Therefore, effort to find alternative agents against dental caries is needed. Red betel (*Piper crocatum* Ruiz & Pav) is a promising natural material to treat dental caries. The *P. crocatum* Ruiz & Pav leaves contain secondary metabolites such as essential oil, flavonoid, alkaloid, and phenolic compounds that active against *S. mutans* involved in caries formation. This study aimed to evaluate the activity of secondary metabolites isolated from red betel leaves against *S. mutans* and its inhibition effect on the glucosyltransferase (GTF) activity. The growth inhibition test of *S. mutans* was carried out with liquid dilution test and diffusion test. Cultures of *S. mutans* were treated with essential oil, alkaloid, flavonoid, phenolic compounds isolated from *P. crocatum* Ruiz & Pav in various concentration and incubated at 37°C in anaerob condition. Povidone iodine was used as positive control and aquadest as negative control. The inhibition test of GTF activity was performed by measuring the levels of formed fructose that was analyzed by Somogyi-nelson method. The data was analyzed using one-way ANOVA with a 95% confidence interval. The results showed that essential oil and alkaloid had lowest MIC (Minimal Inhibitory Concentration) compared with the other active compounds (5000 µg/mL) but higher than povidone iodine as positive control (2500 µg/mL). The essential oil exhibited similar inhibition zone diameter compared with povidone iodine, either at low or high concentration ($p > 0.05$). The essential oil had similar inhibition activity to the glucosyltransferase compared with povidone iodine ($p > 0.05$). In conclusion, the essential oil of red betel leaves had the strongest activity against the *S. mutans* growth through its inhibition effect on GTF activity.

Key words : red betel (*P. crocatum* Ruiz & Pav) – antibacterial - *S. mutans* – essential oil - glucosyltransferase

ABSTRAK

Karies gigi merupakan masalah utama kesehatan gigi. *Streptococcus mutans* adalah bakteri utama penyebab karies gigi. Saat ini, bahan untuk menghilangkan karies gigi sudah kurang efektif karena timbulnya resistensi *S. mutans* terhadap bahan tersebut. Selain itu, bahan tersebut kemungkinan menyebabkan efek samping setelah pemakaian beberapa tahun. Oleh karena itu usaha untuk menemukan senyawa alternatif untuk melawan karies gigi diperlukan. Sirih merah (*Piper crocatum* Ruiz & Pav) adalah bahan alam yang potensial untuk mengobati karies gigi. Daun sirih merah mengandung metabolit sekunder seperti minyak esensial, flavonoid, alkaloid dan senyawa fenol yang kemungkinan aktif terhadap *S. mutans* yang berperan dalam pembentukan karies. Tujuan penelitian ini adalah mengkaji aktivitas metabolit sekunder dari sirih merah terhadap pertumbuhan *S. mutans* dan efek penghambatannya terhadap aktivitas glukosiltransferase (GTF). Uji penghambatan pertumbuhan *S. mutans* dilakukan menggunakan uji difusi cair dan difusi padat. Kultur *S. mutans* ditambahkan minyak esensial, alkaloid, flavonoid, senyawa fenol yang diisolasi dari *P. crocatum* Ruiz & Pav pada berbagai kadar dan diinkubasikan pada suhu 37°C selama 24 atau 48 jam dalam kondisi anaerob. Providon iodine digunakan sebagai kontrol positif dan akuades sebagai kontrol negatif. Uji aktivitas penghambatan glukosiltransferase dilakukan dengan mengukur kadar fruktosa yang terbentuk menggunakan metode Somogyi-Nelson. Data yang diperoleh dianalisa dengan ANAVA satu jalan dengan tingkat kepercayaan 95%. Hasil

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penelitian menunjukkan minyak esensial mempunyai nilai KHM (Kadar Hambat Minimal) paling rendah (5.000 μ /mL) dibandingkan dengan metabolit sedkunder lain tetapi lebih besar dibandingkan dengan povidon iodine sebagai kontrol positif (2.500 μ /mL). Minyak esensial menunjukkan diameter zona penghambatan yang sama dengan povidon iodine baik pada kadar rendah maupun tinggi ($p > 0,05$). Minyak esensial juga menunjukkan aktivitas penghambatan glukosiltransferase yang sama dengan povidon iodine ($p > 0,05$). Dapat disimpulkan bahwa minyak esensial dari sirih merah mempunyai aktivitas paling kuat melawan pertumbuhan *S. mutans* dan aktivitasnya kemungkinan melalui penghambatan aktivitas GTF.

Kata kunci : sirih merah (*P. crocatum* Ruiz & Pav) – antibacteri - *S. mutans* – minyak esensial - glukosiltransferase

INTRODUCTION

Dental caries is a major problem of dental health in Indonesia¹ and its treatment requires high costs.² Dental caries is a local damage of teeth due to bacterial fermentation of carbohydrates from food in the oral cavity. It is characterized by demineralization of tooth enamel that results in caries formation.³ Dental caries can cause a spread of infection to the periapical tissue and causes a sense of pain.⁴ A more severe dental caries can be a source of infection that triggers a systemic disease such as endocarditis.⁵

Streptococcus mutans bacteria is the main cause of dental caries in human.⁶ The attachment of *Streptococcus mutans* to the tooth surface allows the attachment of other microorganisms, which will lead to a dental plaque formation.⁷ The ability of *S. mutans* to synthesize glucans is the major factor of the development of dental caries.⁴

The prevention of dental caries with antibacterial compound is still the main choice. Agents that can inhibit the expression and activity of GTF enzymes, an enzyme involved in exopolysaccharide polymer synthesis in microbes, can be used as a therapeutic choice for dental caries.⁸ In recent time, the existing antibacterial substances are ineffective, and have significant side effects. Therefore, development of antibacterial substances or invention of new drugs for the prevention and treatment of dental caries is important.

One of potential plants to be developed as a dental caries preventive material is red betel (*P. crocatum* Ruiz & Pav.) Red betel is one of the medicinal plants that is empirically known to be effective to cure various diseases.⁹ Ethanol extracts of red betel leaves exhibit antibacterial activity towards *Staphylococcus aureus* and *Escherichia coli*.¹⁰

Phytochemical screening of red betel leaves showed that red betel leaves contain chemical compounds such as polyphenols, flavonoids, tannins, alkaloids, and essential oils.⁹ Polyphenols are toxic to bacteria. Polyphenol compounds have the oxidized group that can inhibit the activity of enzymes in bacteria and deactivate proteins on the cell surface. Catechin, a component of flavonoids was proven to be able to inhibit the growth of *S. mutans in vitro*. Catechin can inhibit the activity of GTF enzymes produced by *S. mutans*.¹¹

This study was conducted to evaluate the activity of secondary metabolites isolated from red betel leaves against *S. mutans* and its inhibition effect on the GTF activity.

MATERIALS AND METHODS

Isolation and identification of the active compounds of red betel leaves

Isolation of essential oil was carried out using a steam distillation. The essential oil was analyzed using TLC with a stationary phase (silica gel GF₂₅₄) plate and mobile phase (toluene-ethyl acetate (93:7, v/v)). The spot was detected using a UV irradiation (254 and 365 nm) and visible light. Vanillin sulfate acid reagent was used to identify the spot.¹² Isolation of flavonoids was conducted using two-phase maceration using methanol-water (9:1, v/v) then methanol-water (1:1, v/v). Flavonoids were identified using TLC with a stationary phase (cellulose) and mobile phase (ethyl acetate-formic acid-acetic acid-water (100:11:11:27, v/v)). The spot was detected using UV 254, UV 365 nm, and visible light. Before the detection, the plate was treated with ammonia.¹² Isolation of alkaloids group was carried out by maceration using 10% acetic acid in ethanol. Identification of alkaloids was carried out using TLC with a stationary phase (silica gel GF₂₅₄) and mobile

phase (methanol-ammonia (200:3, v/v)). The detection of the spots was performed using UV irradiation 254 and 365 nm. Alkaloid detection was conducted using Dragendorff reagent and visible light.¹² Isolation of phenolic compounds was conducted by soxhletation using ethyl acetate as solvent at 60°C. Identification of phenolic compounds was conducted using TLC with a stationary phase (Silica gel GF₂₅₄) and mobile phase (methanol-formic acid (19:1, v/v)). Detection of the spot was performed using UV 254 and 365 nm, and ferric chloride reagent.¹²

Determination of the MIC

Streptococcus mutans culture was grown in 2 mL of BHI media containing 3% TSB and incubated at 37°C in anaerobic condition for 18-24 hours. Cell suspension (200 mL) was added to BHI media and then incubated in anaerobic condition at 37°C for 4 hours. An aliquot (0.9 mL) of BHI media was prepared in sterile glass tube, then of 100 mL of 10⁸ cfu/mL *S. mutans* suspension was added to obtain a 10⁶ cfu/mL bacterial suspension.

The samples were treated in groups. Group I was a negative control. One mL of sterile aquadest was added into the glass tube of samples in group I. Into the samples in group II, 1 mL of povidone iodine was added and the samples acted as positive control. Group III, IV, V, and VI were prepared by adding 1 mL of extract containing essential oils, alkaloids, flavonoids, and phenolic compounds, respectively. The concentration of compounds tested in groups II, III, IV, V, and VI was made in a series of 20000, 10000, 5000, 2500, and 1250 mg/mL. Group VII which contained 1 mL of media and 1 mL of aquadest without the addition of bacteria was used as control media.

All groups were incubated in anaerobic condition at 37°C for 48 hours. After incubation, the growth of bacteria was investigated. The lowest concentration of an extract that inhibits the visible growth of a microorganism after incubation was expressed as the minimum inhibitory concentration (MIC). Bacterial growth was observed with the occurrence of turbidity in the media.

Determination of the inhibition zone

Bacterial suspension (10⁸ cfu/mL, 100 mL) was spreaded on BHIA media with 3% TSB. A well,

with 3 mm of depth and 6 mm of diameter was created in the media, and in each well was poured with 50 mL of test solution. The test solution was the solution that was used in each group. The solution added to group I was sterile distilled water, while the solution added to group II was povidone iodine. The solutions added to group III, IV, V, and VI, respectively, were extract containing essential oils, flavonoids, alkaloids, and phenolic compounds. The concentrations of compounds tested in groups II, III, IV, V, and VI were 20000, 10000, 5000, 2500, and 1250 mg/mL. Bacteria then incubated in anaerobic condition at 37°C for 48 hours. The area around the wells that was clear from bacterial growth was expressed as inhibition zone.

Glucosyltransferase activity test

Streptococcus mutans was inoculated into 20 mL of BHI liquid containing 3% TSB, and shaken at 150 rpm for 48 hours. After cultivation, the bacterial culture was centrifuged at 1500 rpm for 10 minutes at room temperature in order to obtain supernatant containing GTF.

Glucosyltransferase activity assay was conducted by mixing 0.475 mL of supernatant of bacterial culture, 1 mL of 0.25 M sucrose solution, 25 µL of test solution, and 0.5 mL of 0.2 M phosphate buffer up to a total volume of 2 mL in the glass tube. The solution was divided into several groups. Group I was added with aquadest as a negative control. Group II was added with povidone iodine as a positive control. Group III, IV, V, and VI, respectively, were added with extract containing essential oils, flavonoids, alkaloids, and phenolic compounds. Concentrations of the extract that were tested were minimum inhibitory concentration of each group. All tubes were incubated at 37°C for 2 hours. After the incubation, the amount of formed fructose was measured using the Somogyi-Nelson method.

Statistical Analysis

The data were analyzed using one way ANOVA to determine the difference between the groups. When significant differences were observed, analysis was continued using LSD (Least Significant Different) to determine the average difference between groups. The level of significance was set at 5%.

RESULTS

Isolation and identification the active compounds of red betel leaves

Five point one mL essential oils was obtained from 3 kg of fresh red betel leaves. The identification of essential oil was performed by observing the presence of a red-violet spot that indicated the red betel leaves contained the essential oil. The amount of flavonoids obtained from 100 g of dry powder of red betel leaves was 23.16 g of extract. Identification of flavonoid using TLC was performed by observing the presence of yellow spots that indicated red betel leaves contained flavonoids. One hundred g of dry powder of red betel leaves produced 18.31 g of extract contained alkaloids. Identification using TLC provided fluorescence spots, as an evidence that red betel leaves contained alkaloids. From 100 g of dry powder of red betel leaves it can be obtained an amount of 9.89 g of extract containing phenolic compounds. Identification using TLC provided a gray spot indicated the red betel leaves contained phenolic compounds.

Determination of the MIC

Minimal inhibitory concentration value of active compounds contained in red betel leaves is shown in TABLE 1. Essential oil and alkaloids had the lowest MIC value compared with the other active compounds isolated from red betel leaves. However, the MIC values of essential oils and alkaloids were

higher than the MIC of povidone iodine as positive control. MIC values of phenolic compounds was higher than the MIC value of alkaloids and essential oils, but lower when compared with MIC values of flavonoids. The result of the dilution test showed that flavonoids appeared to have the lowest activity against *S. mutans* compared with other active compounds contained in red betel leaves.

TABLE 1. MIC value of the active compound of Red Betel Leaves

Active compound	MIC (µg/mL)
Essential oils	5.000
Flavonoids	>20.000
Alkaloids	5.000
Phenolic compound	20.000
Povidone iodine	2.500

Determination of the inhibition zone

The growth inhibition zone diameter of active compounds of red betel leaves against *S. mutans* is shown in TABLE 2. It can be observed that various concentrations of alkaloids, flavonoids, phenolic compounds, essential oils, and povidone iodine had different growth inhibition zone. The increased concentrations of the active compounds of red betel leaves raised the growth inhibition zone of *S. mutans*. Essential oils had the greatest growth inhibition zone of *S. mutans* compared with the other compounds of red betel leaves.

TABLE 2. Diameter of growth inhibition zone of *S. mutans* after treatment with active compound of red betel leaves

Conc (µg/mL)	Zone Inhibition (mm)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
20000	6.00±0.00	22.46±1.63	23.56±1.49 ^{ab}	12.49±0.68 ^a	16.27±3.59 ^a	12.59±0.99 ^a
10000	6.00±0.00	21.28±2.65	20.34±2.10 ^{ab}	13.77±1.73 ^a	11.58±1.67 ^a	11.56±1.12 ^a
5000	6.00±0.00	16.59±1.85	18.34±4.36 ^{ab}	10.12±2.06 ^a	10.85±0.52 ^a	10.36±1.98 ^a
2500	6.00±0.00	12.50±1.30	18.14±2.06 ^a	10.74±0.72 ^{ab}	10.28±1.11 ^a	8.01±1.21
1250	6.00±0.00	10.92±0.59	12.18±1.51 ^{ab}	7.39±1.43	7.25±2.17	7.39±2.41

Group I: distilled water; Group II: povidone iodine; Group III: essential oils; Group IV: flavonoids; Group V: alkaloids; Group VI: phenolic compounds; ^a: $p < 0.05$ compared with group I; ^b: $p > 0.05$ compared with group II

The post hoc analysis at concentration of 20000 mg/mL showed that the inhibition zone diameter of essential oil (23.56 ± 1.49 mm), flavonoids (12.49 ± 0.68 mm), alkaloids (16.27 ± 3.59 mm), and phenolic compounds (12.59 ± 0.99 mm) was significantly higher compared with the inhibition zone diameter of aquadest (6.00 ± 0.00 mm) as a negative control. Similarly, inhibition zone diameter of povidone iodine (22.46 ± 0.94 mm) as a positive control, was significantly different compared with aquadest as negative control.

On LSD analysis, essential oils had greater diameter of inhibition zone than povidone iodine, but not significantly different ($p = 0.466$), while flavonoids, alkaloids, and phenolic compounds had smaller inhibition zone diameters which was significantly lower compared with povidone iodine ($p = 0.000$, $p = 0.001$, and $p = 0.000$). Compared with flavonoids, alkaloids, and phenolic compounds, essential oils had inhibition zone diameter that significantly higher ($p = 0.000$, $p = 0.000$, and $p = 0.000$).

One way ANOVA showed that at low concentration, the inhibition zone diameters of

povidone iodine, essential oils, flavonoids, alkaloids, and phenolic compounds were larger than the diameter of inhibition zone of aquadest. The inhibition zone diameter of povidone iodine and essential oils was significantly different with aquadest, while in the case of flavonoids, alkaloids, phenolic compounds, the difference was not significant. Compared with povidone iodine, essential oils had larger diameter of inhibition zone, but it was not significantly different. Flavonoids, alkaloids, and phenolic compounds had wider inhibition zone diameter than aquadest, nevertheless it was also not significantly different. Essential oils had the largest inhibition zone diameter compared with the other active compounds. Moreover, flavonoids, alkaloids, and phenolic compounds had no significant differences on inhibition zone diameter.

Glucosyltransferase activity test

Glucosyltransferase activity in this study was represented the amount of fructose as the result of GTF activity within 1 hour. The GTF activity in each group is shown in TABLE 3.

TABLE 3. GTF activity after treatment with active compounds of red betel leaves

Group	GTF activity ($\mu\text{g/mL}$ fructose)			
	Test I	Test II	Test III	Average
Aquadest	62.70	64.87	63.17	63.58 ± 1.15
Povidone Iodine 2500 $\mu\text{g/mL}$	25.12	24.51	27.57	26.73 ± 1.62^a
Essential oil 5000 $\mu\text{g/mL}$	28.39	28.80	27.57	28.25 ± 0.62^{ab}
Flavonoids 20000 $\mu\text{g/mL}$	29.75	32.61	36.15	32.83 ± 3.21^a
Alkaloids 5000 $\mu\text{g/mL}$	45.20	42.61	40.57	42.80 ± 2.32^a
Phenolic compounds 20000 $\mu\text{g/mL}$	44.86	46.49	42.75	44.70 ± 1.88^a

^a: $p < 0.05$ compared with aquadest; ^b: $p > 0.05$ compared with povidone iodine

In Group I, which was treated with aquadest, its GTF activity was the greatest. As a positive control, povidone iodine had the lowest GTF activity. Of the various active compounds of red betel leaves, essential oils showed the greatest inhibition of GTF activity.

The result of one way ANOVA showed that there was a significant different between GTF activity among several test compounds ($p = 0.000$). Post hoc analysis using LSD showed that aquadest as as negative control had significantly greater GTF

activity compared with povidone iodine, essential oils, flavonoids, alkaloids, and phenolic compounds ($p = 0.000$). Essential oils had higher GTF activity compared with povidone iodine, but it was not significantly different. Flavonoids, alkaloids, and phenolic compounds produced higher GTF activity ($p = 0.050$) compared with povidone iodine, and it was significantly different.

Essential oil exhibited the lowest GTF activity compared with other active compounds isolated from red betel leaves, and it was significantly different. Flavonoids had GTF activity that was significantly higher compared with essential oils, and its activity was significantly lower than alkaloids and phenolic compounds. The GTF activity of alkaloids was lower than phenolic compounds but it was not significantly different.

DISCUSSION

Identification of the active compounds of red betel leaves showed that red betel leaves contained essential oils, flavonoids, alkaloids, and phenol, and it was in accordance with previous studies.^{10,13}

The lowest MIC value of the active compounds of red betel leaves was produced by essential oil and alkaloids, but the MIC values of essential oils and alkaloids was higher than the MIC of povidone iodine as the positive control. This value was also much higher than the standards set by CLSI.¹⁴ The MIC values for phenolic compounds were greater than the MIC value of alkaloids and essential oils, but smaller when compared with MIC values of flavonoids. The result of this dilution test showed that flavonoids appeared to have the lowest activity against *S. mutans* compared with other active compounds contained in red betel leaves.

Essential oils which also contain terpenoid class was active against bacteria.¹¹ In the previous study, essential oil proved to be effective against gram-positive bacteria, *Staphylococcus aureus*.¹⁵ Activity of essential oils against bacteria was caused by the lipophilic components in essential oils that were able to disrupt cell membrane function.¹¹

Alkaloids also had strong activity against *S. mutans*, with the fact that MIC values of alkaloids were relatively smaller compared with other active compounds contained in red betel leaves. The strong

activity of alkaloids against bacteria is because of some groups of quaternary alkaloids have planar aromatic group that is capable of reacting with bacterial DNA.¹¹ Berberine, an isoquinolon alkaloid extracted from plants, is able to bind with DNA and RNA of bacteria. Berberine also could inhibit the action of FtsZ, a protein which functions are related to the process of bacterial cell division.¹⁶ With the disruption of the division process, the growth of *S. mutans* will be disrupted.

Phenolic compound also has antibacterial activity, although its action is not as strong as essential oil and alkaloids. The antibacterial activity of phenolic compounds is caused by hydroxyl and carbonyl groups. Phenol compounds can interact with bacterial cell involving the hydrogen bonds. At low concentrations, phenolic compounds will form a complex bond between protein and phenol by a weak bond and immediately causes a dissociation. The process will be followed by the penetration of phenol into the cell and lead to the precipitation and denaturation of bacterial proteins. The high concentrations of phenol can lead to coagulation of protein and lysis of bacterial cell membranes.¹⁵

The measurement of inhibition zone diameter showed that essential oils had the greatest inhibition zone of *S. mutans* than the other active compounds. Determination of the diameter of inhibition zone showed that the effect of essential oils was not significantly different compared with povidone iodine, and at high concentrations the inhibition zone diameter did not much different with the standards set by CLSI.¹⁴

The components of essential oils, such as mentol¹⁷, α -pinen, borneol, and p-cymen have a great potential to inhibit the growth of bacteria.¹⁸ Thymol and karvakrol which are also a component of essential oils are known to have the ability to inhibit the growth of bacteria that cause dental caries, including *S. mutans*.¹⁹

The structure of essential oil consists of isopren, so called as terpen or terpenoid group.²⁰ Terpen or terpenoid group is lipophilic compound that could fight bacteria by disrupting cell membranes using its lipophilic component. Essential oils are stronger against *S. mutans* as gram-positive bacteria. Unlike gram-negative bacteria, *S. mutans* does not have a hydrophilic membrane that can protect cell

membranes from the attack of lipophilic components of essential oils.¹⁷

At the liquid dilution test, alkaloids had the same effect with essential oils, but in the diffusion test, alkaloid had the lowest inhibition zone diameter. The differences were due to the fact that alkaloids did not have the ability to diffuse in the medium of *S. mutans* growth. The ability of an antibacterial material to inhibit bacterial growth depends on the solubility, interaction, and the ability of the test material precipitate in the medium that is used.²¹

The overall data showed that the active ingredients of red betel leaves at high concentrations had a great ability to inhibit the growth of *S. mutans*. In accordance with the previous studies, the ability of a material to inhibit bacterial growth is influenced by the concentration of antibacterial compounds.^{21,22}

Essential oil is an active compounds of red betel leaves that was able to provide the greatest inhibition of GTF activity. Essential oils are also able to provide inhibition to the GTF activity compared with povidone iodine as the positive control. The ability of essential oils in inhibiting GTF activity was possibly due to the ability of essential oils as compounds with low polarity to precipitate GTF which is a protein.

In vitro studies of extracts of *Rosmarinus officinalis* L. showed that methanol extract was able to provide a greater inhibition to GTF activity compared with aqueous extracts.²³ Inhibition of GTF activity is influenced by the polarity of a substance, and a non-polar fraction may provide greater inhibition of GTF activity.²⁴

CONCLUSION

Essential oils isolated from red betel leaves had the highest ability to inhibit the growth of *S. mutans* group compared with other active compounds, and had the same inhibition with *S. mutans* growth produced by povidone iodine. Essential oils isolated from red betel leaves had the greatest ability to inhibit the activity of glucosyltransferase produced by *S. mutans* compared with other active compounds, and had the same inhibition as by povidone iodine.

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