

Antimicrobial Activity of Ethyl Acetate Fraction from *Stelechocarpus burahol* Fruit against Oral Bacteria and Total Flavonoids Content

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ABSTRACT

Objective: *Stelechocarpus burahol* is one of the native plants of Indonesia that has been used as an oral deodorant. This fruit contains flavonoids, which flavonoids are known to have antimicrobial activity against oral bacteria. The purpose of this study was to demonstrate the ethyl acetate fraction of *S. burahol* fruit to inhibit growth of oral bacteria and determine its total flavonoid content. **Methods:** Antimicrobial activity test was performed against oral bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) by disc diffusion and microdilution methods. The diffusion assay used Brucella Blood Agar (BBA) plate with paper disks soaked in the fraction of ethyl acetate *S. burahol* with various concentration (1,2,4,8,10,20,40, and 60% w/v), the inhibitory zone diameter of each bacteria compared to standard catechins. The minimum inhibition concentration value (MIC) was using the microdilution method with brucella broth for bacterial growth medium. Total flavonoid content was measured by UV/Visible spectrophotometer with standard quercetin at 440 nm wavelength. **Results:** The results showed that ethyl acetate fraction could inhibit the growth of *P. gingivalis* and *F. nucleatum* with concentration of 1% w/v, and the MIC

was 125 ug/ml. The total flavonoid content is 0.833 extract mg/g Quercetin Equivalent. **Conclusion:** The ethyl acetate fraction of *S. burahol* fruit contained flavonoid that can be used as antimicrobial against oral bacteria.

Key words: Antimicrobial, Total flavonoid content, Oral bacteria, *Stelechocarpus burahol*, Ethyl acetate fraction.

Key message: Ethyl acetate fraction of *S. burahol* fruit with flavonoid content can inhibit the growth of oral bacteria.

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DOI: 10.5530/jyp.2018.2s.19

INTRODUCTION

Traditional medicines especially sourced from natural products have been used to prevent, treat, and maintain human health for a long period of time. Most people living in the developing countries use the medicinal plant for primary healthcare and treat many diseases.¹ Nowadays, the uses of traditional medicinal plants for developed as raw drug, new drug compounds, and pharmaceuticals product to health care have steadily increased worldwide, include oral healthcare product,² but research and experiment about phytochemicals and pharmacological activity in medicine plant that could be useful as antimicrobials for treatment of infectious diseases, especially antimicrobial against oral bacterial very few and limited data.³ One of the medicine plants that have potential for oral care is *Stelechocarpus burahol* (Blume) Hook.f. and Thomson. The fruit belonging to family Annonaceae is a traditional herbal medicine in Indonesia, which is commonly found in Java Island, especially Central of Java. These fruit was reported has antibacterial and anti-inflammatory activity.⁴ The fruits have been empirically used by the princesses of Yogyakarta Palace for removal oral malodor (halitosis).⁵⁻⁶ Halitosis is oral diseases in human, commonly derives from the proteolytic activity of Gram negative anaerobic oral bacteria associated with food metabolism, which the bacteria have a role in degrading the food became amino acids containing sulfide to produce Volatile Sulphur Compounds (VSCs).⁷⁻⁸ The *S. burahol* fruit extracts may inhibit and reduce VSCs causing

halitosis, which it is produced by bacteria *P. gingivalis* and *F. nucleatum*. The antimicrobial test from *S. burahol* extract mouthwash has been reported and the results show the mouthwash did not exhibit antimicrobial activity against *P. gingivalis*.⁵⁻⁶

P. gingivalis and *F. nucleatum* are pathogenic bacteria that grow in the oral cavity associated oral disease. Both of these bacteria play an important role as the cause of periodontal disease. *P. gingivalis* are Gram negative anaerobic known as black pigmented bacteria that usually cause gingivitis. *Fusobacterium nucleatum* belong to the oral microbiota, more commonly found in the oral cavity, in gingival pockets as periodontal disease progresses.⁹⁻¹⁰

Based on phytochemical identification data, it is known that the fraction of ethyl acetate of *S. burahol* contains the components of polyphenols and flavonoids.⁶ The polyphenols have been reported antimicrobial activity against oral bacteria to treat dental and periodontal disease. Polyphenols in foods such as red grape skins and green tea were reported can inhibit the growth of oral bacteria that cause periodontitis.¹¹⁻¹² Apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones, includes a group of flavonoid compounds have been shown to possess potent antimicrobial activity. In addition, some other flavonoids such as quercetin, phlorin, naringin, have activity as an oral antibacterial by inhibiting the growth of Gram negative anaerobic bacteria

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associated with periodontitis such as *Prevotella intermedia*, *P. gingivalis*, *Actinomyces*, *F. nucleatum*, *Solanum morei*.¹³⁻¹⁴

Based on the results of *in vitro* study and still limited information about the antimicrobial properties of *S. burahol* fruit against oral bacteria, we want to investigate the antimicrobial activity of the ethyl acetate fraction against oral bacteria and the total flavonoids assay contained in this fruit.

MATERIALS AND METHODS

Plant material

The ripe fruit of *S. burahol* was collected from smallholder estates in Magelang, Central Java, Indonesia. Harvest time is February-March. The fruit flesh was separated from the pericarp of the fruit and the seeds, the fruit flesh was sliced thin and dried at 40°C. Dried fruits powdered and weighed. The sample was ready for the extraction process.

Preparation of *S. burahol* fraction

Preparation fraction was conducted according partition procedure by Amin *et al* and Mun'im *et al.* procedure.⁵⁻⁶ *Burahol* fruit extracted with conventional method by maceration extraction used ethanol solvent.¹⁵ The ethanol extract was dispersed in warm water and ethanol (30.70 ml) and partitioned successively with n-hexane solvent to obtain liquid hexane fraction. The residue is re-partitioned with an ethyl acetate solvent. The organic layer is concentrated in a rotary vacuum evaporator under reduced pressure at 50°C, to obtain concentrated hexane and ethyl acetate fractions.

The Culture of the Bacterial strains.

Oral bacteria were employed in this study including two species Gram negative anaerobic: *P. gingivalis* ATCC 33277, and *Fusobacterium nucleatum* ATCC 25586. Both were cultured in Brucella Blood Agar (BBA) (Difco) medium with the addition of vitamin K and kanamycin, after incubated at 37°C for 72 h under anaerobic condition.

Antimicrobial Activity Assay

Antimicrobial activity assay using disc diffusion method: Petri plates were prepared with 20 mL of sterilized BBA+vit K, then solidified. Bacterial test colonies were suspended in 0.9% saline and adjusted to 0.5 Mc Farland turbidity standard (equivalent to approximately $1-7 \times 10^6$ CFU/ml). 100 µl of each bacterial solution was swabbed onto the plates, and allowed to stand for 15 min. The sterile paper disk blank (6 mm diameter disc) was impregnated with the various concentration of sample (1, 2, 4, 8, 10, 20, 40, 60 mg/ml per disc), sterile distilled water as a negative control, and catechin as the positive control. The loaded discs were placed on the surface of the agar plate. The plates were incubated for 72 h at 37°C in anaerobic condition. The results were evaluated by measuring the diameter of the growth inhibition zones formed around each disc. All experiments were done in triplicate.¹⁶

Minimum Inhibitory Concentration and Minimum Bactericide Concentration.

The minimum inhibitory concentration (MIC) assay using microdilution method,¹⁷ it was performed according to the protocol of Scorzoni, *et al.*¹⁸ with some modification: The burahol fraction was dissolved in water and 2% DMSO. The various concentration sample (1, 2, 4, 8, and 10 µg / ml) were inserted into each well with a total volume of 100 µl per well in the 96-well plates. Each microtiter plate well was inoculated with 5 µL of 0.5 McFarland standard broth bacteria (equivalent to approximately $1-7 \times 10^6$ CFU/ml) was diluted to the ratio of 1:100 in BHI (Merck) broth media. The mixture was incubated anaerobically at 37°C for 24 h. Absorbance reading at 520 nm wavelength using a microtiter plate reader

and cell growth measured post-incubation. DMSO as the negative control, while catechin as the positive control. Minimum inhibitory concentration (MIC) values were evaluated by observing the turbidity and the absorbance reading of the suspension post-incubation. The lowest concentrations of burahol fruit fraction without visible growth with clear suspension were defined as minimum concentrations that inhibited bacterial growth (MIC). To confirm the Minimum Bactericide Concentration (MBC), the suspension samples after incubation were transferred to plate of BBA (conventional plating method) and anaerobically incubated for 3x24 h. The lowest concentration with no visible growth was defined as the MBC. The experiment was performed in triplicates.

Total Flavonoid Content Assay

The total flavonoid content (TFC) was conducted according to using the aluminum chloride colorimetric method with some modification. The fraction was dissolved in 10 ml methanol, 0.5 ml of extract solutions were mixed with 2 mL of distilled water, and added 0.2 ml of 10 % $AlCl_3$ solution, 0.2 mL of 1 M sodium acetate, and volume was made up to 5 ml with distilled water, vortex for 2 min and left at room temperature for 30 min. The mixture solution incubated for 1 h. The absorbance of the reaction mixture was measured at 440 nm with UV/Visible spectrophotometer. The total flavonoid contents were calculated as quercetin from a calibration curve with the solution concentrations of 10 to 60 mg/ml in methanol. The TFC was expressed in mg of quercetin equivalents per Gram of extract. All the measurements were performed in triplicates.¹⁹

RESULTS

Antimicrobial Activity Assay

Antimicrobial activity of ethyl acetate fraction of *S. burahol* fruit to oral bacteria using diffusion method showed that fraction with concentration 1mg/ml can inhibit the growth of *F. nucleatum* bacteria with the diameter of inhibition zone is 8.13 mm, while *P. gingivalis* have diameter of inhibition zone is ≥ 9 mm. The catechin as positive control shows the diameter of inhibition zone is ≥ 10 mm. The results are shown in Table.1.

Minimum Inhibition Concentration and Minimum Bactericidal Concentration Assay

The determination of MIC, and MBC values using microdilution method. The lowest concentration of burahol fruit fraction for inhibiting the growth of *P. gingivalis* and *F. nucleatum* was determined as the MIC. The range of MIC of the fraction recorded was 0.1-1 mg/ml.

The fruit fraction showed MIC of 0.1 mg/ml, whereas catechin as positive control showed MIC of 0.2 mg/ml. DMSO as negative control. The MBC assay cannot be determined by microdilution because the dark/brown color in the test sample makes the turbidity, so the MBC value undetermined, to confirm MBC the suspension samples were transferred to plate of BBA+vit K media. The fruit fraction showed MBC of 1mg/ml. The results is shown in Table 2.

Total Flavonoid Content Assay

The total flavonoid content (TFC) was conducted using the aluminum chloride colorimetric that calculated of quercetin equivalent. The result is shown in Table 3.

DISCUSSION

Natural product has long been known and used as traditional medicine to treat and maintenance of human health. The natural product with antimicrobial activity could become a potential source of new drugs. Today, the antimicrobial activity study of medicinal plant against oral pathogen has been many reported. The Gram negative anaerobic oral

Table 1: Antimicrobial activities of ethyl acetate fractions of *S. burahol* fruit in Disc Diffusion Method.

Antimicrobial	Concentration per disc (mg/ml)	Diameter of inhibition zones (The mean±SD in mm)	
		<i>P.gingivalis</i>	<i>F.nucleaum</i>
<i>S. burahol</i> Fruit	1	8.13±0.25	9.46±0.44
	2	9.17±0.39	10.36±0.26
	4	10.06±0.40	10.83±0.26
	8	10.57±0.36	10.95±0.35
	10	10.66±0.58	11.37±0.33
	20	11.18±0.65	11.78±0.40
	40	11.54±0.53	12.35±0.44
Catechin	60	13.33±0.35	13.53±0.54
	3	10.11±0.23	10.11±0.23

Table 2: MIC and MBC values in microdilution methods.

Antimicrobials	<i>P. gingivalis</i>		<i>F. nucleaum</i>	
	MIC values (mg/ml)	MBC values (mg/ml)	MIC values (mg/ml)	MBC values (mg/ml)
<i>S. burahol</i> fruit	0.1	-	0.1	-
	0.2	-	0.2	-
	0.4	-	0.4	-
	0.8	-	0.8	-
	1	1	1	1
Catechin	0.2	0.4	3	0.4

(-): There is no colony growth, MIC: Minimum Inhibition Concentration, MBC: Minimum Bactericidal Concentration.

Table 3: The Total Flavanoid Content (TFC) From Fraction of *S. burahol* Fruit.

Sample	Concentration (µg/ml)	Absorbance Value	TFC (mg QE/gm)
EAF of <i>S. burahol</i>	5000	0.079±0.0130	0.833±0.013

EAF: Ethyl acetate fraction, the total flavanoid content (TFC) was determined as mean ± SD for triplicates measurements.

pathogen known as causative oral diseases, especially periodontal diseases.⁷⁻⁸ Several medicinal plants belongs to the family of Fabaceae, Ebenaceae, Bombaceae and Annonaceae has been reported for treatment of oral diseases.²⁰ *S. burahol* is one of some Indonesian indigenous plants belonging to family Annonaceae. These fruit have been traditionally used as body deodorant and oral deodorant by the princess of Yogyakarta Palace. As an oral deodorant, the burahol fruit extracts have been studied by *in-vivo* and *in vitro* test, the result of this study prove that the extracts have the ability to reduce volatile sulfur compounds (VSCs) that trigger halitosis.⁴⁻⁶ Halitosis or oral malodor derives from the proteolytic activity of Gram negative anaerobic oral bacteria.⁷ The antimicrobial tests against Gram negative anaerobic bacteria are performed as one step to reduce oral malodor with inhibiting growth oral bacteria. Antimicrobial activity of *S. burahol* of mouthwash extract against *P. gingivalis* (Gram negative anaerobic oral bacteria) showed no antimicrobial activity,⁵ but *in vitro* studies to reduce VSC reported that ethyl acetate fraction could inhibit and reduce VSC from methyl mercaptan produced by Gram negative anaerobic bacteria from physiologic halitosis saliva,⁶ this being an indication that *S. burahol* has potential as an antimicrobial against oral

pathogens. The hexane fraction was not tested in this study because previous research revealed that the hexane fraction is inactive to reduce (VCS).⁵⁻⁶ The ethyl acetate fraction was tested for two anaerobic gram-negative bacteria, such as *P. gingivalis* and *F. nucleatum*. Both of these bacteria belong to oral pathogenic bacteria as the cause of oral disease. The antimicrobial activity of the *S. burahol* fruit fraction was carried out using the method for agar disk diffusion, the advantage of this method was simple, low cost and the use of small sample volumes. The antimicrobial activity of this fraction belongs to moderate activity with inhibition zone diameter at the concentration range of 2 to 40 mg/ml is ≥10 mm. The MIC was defined as the lowest concentration able to inhibit any visible bacteria growth. The results were analyzed visually and spectrophotometrically. The MIC results indicated that the fraction is potentially antimicrobial to oral pathogenic bacteria with the lowest inhibitory concentrations is 0.1 mg/ml of two Gram negative anaerobic oral bacteria tested.

Based on the results of phytochemical tests, it was reported that the fraction of ethyl acetate contains polyphenols and flavonoids. The total flavanoid content of the ethyl acetate fraction is expressed as a total flavanoid value that is equivalent to quercetin. The phenolic compound has potency as antimicrobial property to inhibit oral bacteria, such as allyl pyrocatechol from betel leaves, catechin from green tea and resveratrol from licorice inhibit anaerobic oral bacteria and reduce malodor.¹¹ Catechins include epicatechin, epicatechingallate, and epigallocatechin gallate found in *Camelia sinensis* (Theaceae) are believed to be responsible for the oral antimicrobial activity, prevention of adherence of bacteria to tooth surfaces, inhibition of glucan production and inhibition of amylases.¹² Several polyphenols have been reported as antimicrobial activity against periodontal pathogen. Wine catechins have strong antimicrobial activity against *P. gingivalis* and *P. intermedia*.¹¹ Green tea catechins, used in a slow-release local delivery strip system applied in the periodontal pockets, decrease the pocket depth and the proportion of Gram negative anaerobic black pigment, while the same catechins show an *in vitro* bactericidal effect against *P. gingivalis* and *Prevotella spp.* Several polyphenols inhibit the proteolytic activity of *P. gingivalis*.²¹

Flavonoids are classified under phenolic groups in plants, which have been reported to be effective as antimicrobial property against a wide array of microorganisms with some mechanisms: inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones and chalcones have been shown to possess potent antimicrobial activity. The citrus fruit was shown to inhibit the growth of periodontal pathogens and other common oral microorganisms, such as *Actinobacillus actinomycetemcomitans* and *P. gingivalis* because flavonoid compound in the fruit such as naringin indicates as antimicrobial activity against oral pathogen. Phlorin is a phenolic compound in lemon peel exhibited antimicrobial activity against oral bacteria, such as *P. intermedia*, *S. mutans* and *P. gingivalis*. Quercetin may inhibit oral bacteria, such as *S. mutans*, *S. sobrinus*, *S. sanguis*, *L. acidophilus* that associate cariogenic diseases. It also inhibits oral pathogens *P. gingivalis*, and *A. actinomycetemcomitans* as the main causative agent of periodontal disease.²² The mechanism of antimicrobial activity of quercetin by disrupting the membrane and inactivating the extracellular protein by forming irreversible complexes.¹¹ Several studies have shown that isoflavones, flavones, isoprenylflavones flavanones, naringenin, and quercetin may inhibit cariogenic bacteria such as *Streptococcus spp.*, *Actinobacillus*, and *Lactobacillus* species.

The present study report that the ethyl acetate fraction of *S. burahol* fruit showed antimicrobial activity against oral pathogens. The polyphenolic and flavonoids compounds in this fruit indicate those antimicrobial properties may inhibit the growth of Gram negative anaerobic oral pathogenic

causes of periodontal and other oral diseases. The antimicrobial mechanism is suspected by disrupting of the membrane and inactivate extracellular proteins or by inhibition of nucleic acid synthesis, and energy metabolism.

CONCLUSION

The ethyl acetate fraction of *S. burahol* has antimicrobial property against oral bacteria associated with oral disease, which can inhibit the growth of oral bacteria such as *P. gingivalis* and *F. nucleatum* bacteria, with flavonoids contained in this fruit.

ACKNOWLEDGEMENT

This research was supported by Ministry of Research, Technology, and Higher Education, Republic of Indonesia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

BBA: Brucella Blood Agar; **MIC:** Minimum Inhibition Concentration; **MBC:** Minimum Bactericide Concentration; **VSCs:** Volatile Sulphur Compounds; **ATCC:** American Type Culture Collection.

SUMMARY

Ethyl acetate fraction of *Stelechocarpus burahol* fruit inhibited the growth of anaerobic bacteria causing bad breath such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Total flavonoid content. The total flavonoid content is 0.833 extract mg / g Quercetin Equivalent.

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Article History: Submission Date : 29-11-2017 ; Revised Date : 28-01-2018; Acceptance Date : 12-04-2018.

Cite this article: Amin A, Radji M, Mun'im A, Rahardjo A, Suryadi H. Antimicrobial Activity of Ethyl Acetate Fraction from *Stelechocarpus burahol* Fruit against Oral Bacteria and Total Flavonoids Content. *J Young Pharm.* 2018;10(2)Supps:s97-s100.