Ethanol extract of soursop leaf inhibits acid production and adhesion of *Streptococcus mutans*

Friska Ani Rahman[1*], Rahsunji Intan[1]

[1]**Dental Hygiene Study Program, Faculty of Dentistry Gadjah Mada University, Indonesia**

ABSTRACT

Introduction: Dental caries and dental plaque are the most popular global oral health problems. The primary step of dental caries is characterized by damage of tooth surfaces affected by acids which are by-products of sugar metabolism by a cariogenic bacteria. One of the cariogenic bacteria is *Streptococcus mutans*. *Annona muricata* is traditionally applied as an herbal remedy for various illnesses and has been recognized in a previous study as an antimicrobial agent. This study was aimed to determine inhibition of ethanol extract of soursop on acid production and adhesion of *Streptococcus mutans*. **Methods:** Soursop leaf extracted by maceration using 70% ethanol solvent. The extracts obtained were tested at various concentrations. To examine the effect of ethanol extract of Soursop on acid production by *S. mutans*, the pH of the culture was determined using a pH meter. Inhibition of adhesion of *S. mutans* to the saliva-coated hydroxyapatite (S-HA) discs was quantified using colony counting on TYS20B agar plates. **Results:** Ethanol extract of soursop showed significant inhibition of acid production at the concentrations of 50, 75, 100, 125 and 150 mg/ml compared to the negative control group. The pH of *S. mutans* cultures in the presence of ethanol extract of Soursop leaf at various concentrations was higher than negative control, but there were no differences in pH value between the various concentrations of ethanol extract of Soursop leaf. The extract clearly inhibited *S. mutans* adhesion to saliva coated hydroxyapatite beads at the concentration 50, 75, 100, 125 and 150 mg/ml compared to the negative control group. Adhesion decreased with increasing concentrations of ethanol extract of Soursop leaf, but there was not significant difference in colony count between the various concentrations of ethanol extract of Soursop leaf. **Conclusions:** The ethanol extract of *Annona muricata* leaf can inhibit acid production and adhesion of *S. mutans*.

Keywords: soursop; *Annona muricata*; *Streptococcus mutans*; acid production; adhesion

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Corresponding author: Friska Ani Rahman, Dental Hygiene Study Program, Faculty of Dentistry Gadjah Mada University, Indonesia. 1, Jalan Denta, Sleman, Special Region of Yogyakarta, 55281, Indonesia. Phone: +62815-7855-7944; Email: friska_ani@ugm.ac.id

INTRODUCTION

Based on the Indonesia Basic Health Research (Riskesdas) in 2013, dental caries is the most common oral cavity infection in Indonesia.[1] The virulent properties of cariogenic bacteria in the oral cavity cannot be separated from the occurrence of caries. One of the cariogenic bacteria that plays an important role in plaque formation and dental caries is *Streptococcus mutans* (*S. mutans*).[2] Pathogenesis of dental caries requires numerous steps, such as attachment of bacteria to the pellicle on the superficial tooth structures caused by acids which produced during dietary sugars metabolism by bacteria.[3]

Streptococcus mutans metabolize carbohydrates and produce by-product in the form of organic acids. Accumulation of metabolic acids induces demineralization of the tooth surface, in which can develop into dental caries.[4] Plant-based therapy is growing in developed and developing countries because of the increasing recognition that they are natural products. Secondary metabolites produced by plants can be used as antimicrobials.[5]

Many studies have revealed that natural remedies can interfere with virulence factors of *S. mutans*.[6,7,8,9] *Annona muricata* leaves are traditionally used to treat headaches, fever, toothache, cough, and asthma.[10.] *Annona muricata* leaf has proven to be able to inhibits the growth of *S. mutans* in previous studies.[11,12,13,14] This study was aimed to determine inhibition of ethanol extract of soursop on acid production and adhesion of *Streptococcus mutans*.

METHODS

Extraction

The protocol experiment was approved by the Institutional Ethical Committee of the Faculty of Dentistry Gadjah Mada University Indonesia. *Annona muricata* leaves were extracted by maceration method, then the dried leaves were chopped into small pieces and extracted with 70% ethanol for 24 h at room temperature. The extracted solutions were filtered and evaporated in a rotary evaporator. The crude extracts were weighed and stored in refrigerator at 4°C. Concentration of 150 mg/ml; 125 mg/ml; 100 mg/ml; 75 mg/ml; 50 mg/ml extracts were dissolved with 5% dimethyl sulfoxide (DMSO). The extracts were stored in tiny containers and labeled accordingly. Chlorhexidine gluconate (0.2%) was used as positive control due to its a broad-spectrum antiseptic that is bactericidal while 5% DMSO as solvent for dissolving the extracts, was used as negative control.

Acid production

Bacterial strain used in this study was *S. mutans* (ATCC 25175). The bacterial suspension turbidity using 0.5 McFarland standard that corresponded to approximately ($1.5x10^7$ CFU/ml). To examine the effect of acid production by *S. mutans*, various concentrations of ethanol extract of *Anona muricata* leaf was added to 7 ml of *S. mutans* suspension in brain heart infusion (BHI) broth containing 1% sucrose. Two ml of the culture was scooped up during cultivation at 37°C and the pH was examined using a pH meter digital as described previously, with slight modification.[15] Three replications were made for each group of experiment extracts.

Adhesion of S. mutans ATCC 25175 on S-HA discs

Hydroxyapatite (HA) discs were prepared as described in previous study.[14] Briefly, HA discs (10X2mm) were made by pressing HA bovine particles. To obtain solid HA then sintered for 120 min until 1300°C with the heating of 5°C/minute. Sterilised HA discs was store at the room temperature. The whole saliva were prepared as described in previous study.[14]

In the morning, unstimulated saliva was obtained from a healthy volunteer. Saliva was vortexed and centrifuged. Millipore filter membranes were used to purify the supernatant. The assay of bacterial adhesion was based on the technique described previously by Rahman et al.[14] that was modified to simulate the effect of food consumption, *S. mutans* were grown at 37°C for 24 h in a tube containing BHI broth with 1% sucrose. The saliva-coated hydroxyapatite discs (S-HA discs) were immersed in bacterial suspension with various concentration of extract.

The positive control using chlorhexidine 0.2%, and the negative control tube containing DMSO. The tubes were incubated for 24 h at 37°C to create the bacterial adherence.

Saliva-coated hydroxyapatite discs were rinsed with KPB (Kalium Phosphate Buffer) and transferred into a new tube that contained KPB (pH 7.0).The adherent *S. mutans* adsorbed onto the S-HA discs were dispersed using a vortex for 60 sec and the supernatant were spread on agar plate. The number of colonies were counted after 24 h of cultivation at 37˚C.

Statistical Analysis

The pH of the culture was measured using a digital pH meter, and the number of bacterial colonies was counted using a digital colony counter. The mean and standard deviation are presented as data. Statistical analyses were conducted using the one-way ANOVA test. Statistical significance was set at $p<0.05$.

RESULTS

Table 1. Effect of various concentrations of *Annona muricata* leaves extract on *S. mutans* acid production

Concentration (mg/ml)	pH (incubation time/hours)				p-value
	0	4	8	24	
DMSO 5%	7.52±0.03	7.52±0.04	7.26±0.09	5.26±0.47[a]	-
50	6.50±0.03	6.52±0.06	6.60±0.06	4.87±0.04*	1.00
75	6.42±0.04	6.49±0.04	6.51±0.07	5.25±0.09*	0.85
100	6.24±0.07	6.46±0.05	6.54±0.06	5.28±0.11*	0.13
125	6.35±0.05	6.23±0.01	6.56±0.01	5.31±0.03*	0.04
150	6.22±0.04	6.24±0.04	6.44±0.07	5.76±0.02*	0.02
Chx 0.2%	7.17±0.03	7.38±0.02	7.33±0.06	7.45±0.02*	0.01

[a]**The mean and standard deviation are presented as a data (pH)**
***Post-hoc test results compared with the negative control group after 24 h incubation**

Annona muricata leaf extract reduced the rate of acid production by S. mutans containing 1% sucrose (Table 1). As a result, there was a noticeable decrease in pH in the negative control group. However, the decrease was substantially repressed in the existence of the extracts (125-150 mg/ml) and significant compared to the negative control group after 24 h incubation ($p<0.05$). The decrease of pH was also inhibited in the positive control group.

The ability of the ethanol extract of *Annona muricata* leaf in inhibits the adhesion of S. mutans to the tooth surface was investigated using an *in-vitro* model by incorporating a hydroxyapatite discs. Adhesion decreased with increasing concentrations of ethanol extract of *Annona muricata* leaf.

Table 2 shows inhibition of adhesion by ethanol extract of *Annona muricata* leaf and manifested significant inhibition at concentrations 50-150 mg/ml compared with the negative control group ($p<0.05$), also, the positive control group indicated an anti adherence activity. However, compared with the previous study[14], the inhibition value in the present study at the concentration of 150mg/ml was not suggested a similar value.

Table 2. Effect of various concentrations of *Annona muricata* leaves extract on *S. mutans* adherence to hydroxyapatite discs

Concentration (mg/ml)	CFU/ml (x10^3)	p-value
DMSO 5%	78,467 ± 14,670[a]	-
50	4,593 ± 1,474	0.00
75	3,480 ± 1,911	0.00
100	2,090 ± 306.43	0.00
125	1,677 ± 94.52	0.00
150	770 ± 60	0.00
Chx 0.2%	0	0.00

[a]**The mean and standard deviation are presented as a data (pH)**
***Post-hoc test results compared with the negative control group**

DISCUSSION

The application of natural products has been published to be one of the strategies for the development of new medicines.[16] *S. mutans* is usually recognized as one of the major roles in the accumulation of dental plaque and dental caries.[2] The main virulence factors of Streptococcus mutans related to cariogenicity are adhesion, acidogenicity, and acid tolerance.

These three properties will be interrelated in changing the ecology of dental plaque. The ecological changes are marked by an increased proportion of Streptococcus mutans and other aciduric and acidogenic species.[17] In the present study, we investigated the effect of ethanol extract of *Annona muricata* leaf on acid production and adhesion of *S. mutans*.

The acid production of the *S. mutans* are associated with dental caries. Demineralization on the tooth surfaces can occur due to the acid produced by the *S. mutans* during dietary sugar metabolism.[16] In the present study, 1% sucrose was added at the culture medium to simulate the food diet.

Furthermore, sucrose among these sugars is investigated the most cariogenic carbohydrate in the human intake as it is a substrate for the unique capability to support the synthesis of polysaccharides (water-soluble or water-insoluble) and acids by oral microorganisms, such as *Streptococcus mutans*.[18] The alteration of pH is applied as an indicator to discover the influence of anticariogenic agents.[15]

As presented in Table 1, the ethanol extract of *Annona muricata* leaf at the concentration of 125 and 150 mg/ml significantly decreased the pH of S. mutans cells after 24 hours compared to the negative control ($p<0.05$). The acidic pH rate reduction could be correlated with the intervention of agents to the bacterial glycolytic enzymes, which indicates specific intervention of the glycolytic activity of the bacterial cells rather than intervention by bacterial growth decrease. Furthermore, the interference of the bacterial cells' acid tolerance ability since the pH assay's final pH value shows acid tolerance.[19]

Adhesion of *S. mutans* to the superficial tooth structures is one of the most important steps for dental plaque formation.[17] On the tooth surface, *S. mutants* will colonize after attachment using sucrose-dependent and sucrose-independent mechanicsm.[20] The inhibitory effect of ethanol extract of Soursop leaf on the adhesion of *S. mutans* to S-HA discs was examined since the interference of the adhesion of *S. mutans* to the tooth surface is critical for the prevention of plaque accumulation. In the present study, the adhesion of *S. mutans* was examined using saliva-coated hydroxyapatite discs (S-HA disc) and BHI with 1% sucrose to simulate a sucrose-dependent mechanism. Ferrazano et al.[16] said hydroxyapatite is a common surface to investigate the adherence of bacteria.

The glucosyltransferase enzyme produced by *S. mutans* plays an important role in the sucrose-dependent mechanism. Glucosyltransferase influences the development of dental plaque and converts sucrose into glucan. This glucan will accommodate the possibility of providing both bacterial adhesions to the tooth surfaces and microorganisms to each other.[20]

The data demonstrated that various concentrations of ethanol extract of *Annona muricata* leaf reduced the adhesion of *S. mutans* on S-HA discs compared with the negative control group. Adhesion decreased with increasing concentrations of ethanol extract of Soursop leaf. At concentrations of 150 mg/ml, the ethanol extract of *Annona muricata* leaf shows the lowest adherence colony of *S. mutans* to S-HA discs and significantly compared with negative control. Ethanol extract of *Annona muricata* leaf inhibits sucrose-dependent attachment by decreasing carbohydrate metabolism and sucrose-dependent production.

The reduction in adhesion was assumed to have a marked inhibitory effect on initial colonization and biofilm accumulation by *S. mutans*. But, this result was not completely inhibited at concentration 150 mg/ml compared with the previous study on independent-sucrose mechanism.[14]

This is possibly due to added 1% sucrose on BHI broth which may enhance cell survival under harsh conditions. However, these data imply that ethanol extract of *Annona muricata* leaf may be a novel substance capable of modulating the activity of dental caries-related factors. Further studies using *in-vivo* caries models are needed to explain

and validate whether ethanol extract of *Annona muricata* leaf could be used as anticariogenic agents.

CONCLUSION

The ethanol extract of *Annona muricata* leaf can inhibit acid production and adhesion of *S. mutans*.

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