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The effect of ethanol extract of soursop leaf (*Annona muricata* L.) on adhesion of *Streptococcus mutans* ATCC 35668 to hydroxyapatite discs

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ABSTRACT

The demineralization of dental hard tissues can be caused by dental plaque. Dental plaque contains various components, especially bacteria attached to the extracellular matrix. *Streptococcus mutans* (*S. mutans*) has extracellular glucan as adhesin that is important in the attachment mechanism of tooth surface. The natural substance can be used for preventing plaque formation by inhibiting the attachment of *S. mutans*. Soursop plant has been used in treating various diseases. The leaves of the soursop (*Annona muricata* L.) are used as a material to inhibit potential attachment of bacteria *S. mutans*. Common surfaces that is used in adhesion testing is hydroxyapatite (HA). The aim of this study was to evaluate the effect of ethanol extract of soursop leaf (EESL) on the adhesion of *S. mutans* ATCC 35668 to HA discs. Soursop leaves were extracted by the maceration method using 70% ethanol. The experiment was carried out by analyzing the inhibition adhesion of *S. mutans* ATCC 35668 on HA discs after incubation with different concentrations of soursop leaf extract. The concentrations of extract tested were: 150; 125; 100; 75; and 50 mg/ml. *Chlorhexidine* 0.2% was used as a positive control while DMSO 5% was used a negative one. Data were evaluated by One Way Anova. This study statistically showed significant differences of *S. mutans* colony count between groups ($p<0.05$).The results of a post hoc Dunnett T3 test showed that the 2 highest concentrations of extract (125 and 150 mg/ml) reduced *S. mutans* adhesion on HA discs.The obtained results showed that ethanol extract of soursop leave inhibits the adherence of *S. mutans* to the HA disc.

Keywords: adhesion; ethanol extract of soursop leaf *(Annona muricata* L.); hydroxyapatite discs; *Streptococcus mutans*

INTRODUCTION

Dental caries is a disease that destroys the tooth structure which is indicated with demineralization of dental hard tissue. This is view of the accumulation of plaque on the tooth surface.[1] Dental plaque is a bacterial deposit, and its product attaches to the tooth surface.[2] *Streptococcus mutans* (*S. mutans*) plays an important role in the formation of dental caries.[3] It has *surface protein antigen peptide* (SpaP), Ag I/II *family proteins*, that will bind to receptors on the pellicle on the tooth surface. The adhesion of *S. mutans* to the tooth surface is also influenced by the enzyme glucosyltransferase (GTF) which is capable to convert sucrose into glucan. Glucan plays an important role in facilitating bacteria adherence on the tooth surface which is lead to the increasing accumulation of plaque and initiate caries on the tooth surface.[4]

The prevention of *S. mutans* attachment is an effort in reducing the incidence of caries. One approach to reducing the incidence of caries is to develop the therapeutic agents with antimicrobial and antiadherent properties to prevent the bacterial adhesion on the tooth surface.[5] One of the medicinal plants used in several regions in Indonesia is soursop *(Annona muricata* L.)[6] in which its leaves are the part of the soursop plants which is widely used.[7] In our previous studies, it showed that the ethanol extract of soursop leaf (EESL) contain secondary metabolites including saponins, terpenoids, steroids, flavonoids, tannins and alkaloids. The EESL inhibit the growth of *S. mutans* ATCC 35668 in a dose-dependent manner.[8] The inhibition of the adherence of *S.mutans* to the surface of the tooth is necessary for the prevention of plaque formation. Therefore, in this study, we investigated the inhibitory effect the EESL on the adhesion of *S. mutans* to HA discs

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which have been used as one of the experimental model systems. HA discs are the bacterial adhesion objects to simulated tooth enamel structure. Tooth enamel is composed of 99% inorganic compounds of HA.[9]

MATERIALS AND METHODS

This was a laboratory study. This study has been approved by The Research Ethics Team of Faculty of Dentistry Universitas Gadjah Mada with the number: 00247/KKEP/FKG-UGM/EC/2015.

Soursop leaves were obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B_2P_2TOOT) Tawangmangu, Ministry of Health, Indonesia. The leaves have been identified in B_2P_2TOOT and extracted by the maceration method in Laboratorium Penelitian dan Pengujian Terpadu (LPPT) UGM. A reference stock of *S. mutans* (ATCC® 35668) was used as a standardized suspension. Mc Farland standard solution ($1.5x10^8$ CFU/ml) was used as the bacterial suspension turbidity standard.

HA discs were prepared as described by Guan.[10] HA discs (ϕ 10 mm X 2 mm) were made by pressing HA particles by Universal Testing Machine with 4 kgf pressure (Pharmacy Technology Laboratory of Faculty of Pharmacy UGM). HA discs were then sintered (120 min, 1300°C) through the heating of 5 °C/minute in air atmosphere to obtain a solid HA discs (Ceramics and Composite Technology Laboratory of Chemical Engineering Department of Faculty of Engineering UGM). HA discs were sterilized by autoclave (121 °C, 20 min).

Whole saliva was collected from a healthy volunteer (dental conditions free from cavities and good oral hygiene) based on methods described previously.[11] Fresh saliva samples were collected without stimulation and obtained in the morning without stimulation. The samples were dispersed using vortex mixer for 60 min and centrifuged at 20,000 rcf for 60 min at 4 °C. The supernatant was filtered through two low-protein-binding filters (pore sizes of 0.45 µm and 0.22 µm).

The assay of *S. mutans* adhesion on HA discs. was done according to Lee, et.al.[12] *S. mutans* was cultured on BHI for 24 h at 37°C. A total of 14 discs HA were coated with saliva for 1 h at the room temperature. The saliva-coated HA discs (S-HA disc) were washed 3 times with potassium phosphate buffer (KPB) pH 7.0 and 2 HA discs were added to every tube. Everytube was filled with 0.5 ml EESL at different concentration (150; 125; 100; 75; 50 mg/ml) and 0.5 ml of a suspension of bacteria *S. mutans* ATCC 35668 ($1.5x10^7$ CFU/ml). Positive control tube contained 0.5 ml *Chlorhexidine* 0.2% and 0.5 ml of a suspension of bacteria *S. mutans* ($1.5x10^7$ CFU/ml). Negative control tube contained 0.5 ml 5% DMSO and 0.5 ml of a suspension of bacteria *S. mutans* ($1.5x10^7$ CFU/ml). Based on a method as described by Anggraeni[13] tubes were incubated for 24 hours at 37°C. HA discs were washed with KPB and transferred into a tube containing KPB (pH 7.0). *S. mutans* adsorbed onto the HA discs was dispersed using vortex for 60 seconds, diluted and spread on trypticase soy-sucrose-bacitracin agars (TYS20B) plates. After incubation for 24 h at 37°C number of bacterial colonies were counted on each TYS20B plate. Five replicates were made for each concentration of test extracts, and the number of Colony Forming Unit (CFU) was calculated.

RESULTS

The bacterial adherence assay was performed to determine whether EESL inhibits *S. mutans* adherence to HA discs. As shown in Figure 1, the adhesion of *S mutans* to HA disc decreased following the increasing concentrations of EESL. ESSL inhibited the adherence of *S. mutans* completely at concentration 150 mg/ml. One Way Anova test showed a significant value of 0.000 ($p < 0.05$) showing asignificant difference between groups. This showed that EESL has a significant effect on a number of colonies of *S. mutans* attaching on HA discs. The post hoc Dunnet T3 test was conducted to determine the differences among groups Table 1.

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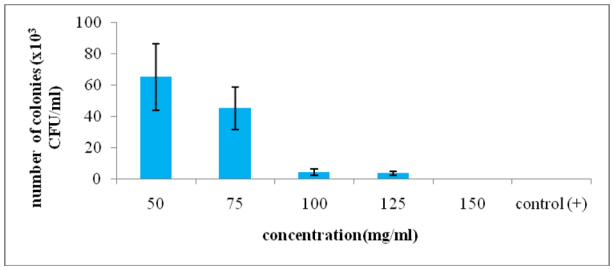

Figure 1. Effect of EESL on *S. mutans* adherence to saliva-coated HA discs. The CFU of *S. mutans* to S-HA discs by various concentrations of EESL was determined.

Table 1. Post hoc analysis of *S. mutans* adherence to saliva-coated HA discs among groups

Among groups		p
50 mg/ml	75 mg/ml	0.765
	100 mg/ml	0.028*
	125 mg/ml	0.027*
	150 mg/ml	0.022*
	Negative control	0.010*
	Positive control	0.022*
75 mg/ml	100 mg/ml	0.022*
	125 mg/ml	0.021*
	150 mg/ml	0.016*
	Negative control	0.009*
	Positive control	0.016*
100 mg/ml	125 mg/ml	1,000
	150 mg/ml	0.071
	Negative control	0.007*
	Positive control	0.071
125 mg/ml	150 mg/ml	0.019*
	Negative control	0.007*
	Positive control	0.019*
150 mg/ml	Negative control	0.006*
	Positive control	-
Negative control	Positive control	0.006*

*p< 0.05 was statistically significant

Several concentrations of the extract were tested regarding their effect on adhesion of *S. mutans* on HA discs (Table 1). The results for 2 highest concentrations of extract that reduced *S.mutans* adhesion on HA discs. The results of a post hoc Dunnett T3 test showed that the group treated with 125 mg/ml significantly decreased in colony counts compared with the negative control and lower concentrations. The group treated with 150 mg/ml EESL showed no difference from positive control indicating that the concentration was effective in reducing the bacterial adherence to HA discs.

DISCUSSION

In the present study, the adhesion of *S. mutans* was evaluated using saliva-coated HA discs (S-HA disc). HA is common materials to analyze bacterial adherence.[14] Saliva was used in this study for containing a glycoprotein that plays an important role in the bacterial adhesion in plaque formation.[15] BHI was used as growth medium without any sucrose to allow the adhesion of *S. mutans* in the form of non-specific attachment followed by the specific attachment of interaction independent on sucrose through adhesion of *S. mutans* binding to salivary glycoprotein attaching to HA discs.[16]

One-way analysis of variance (ANOVA), test on the effect of various concentrations of EESL on the adherence of *S. mutans* on the HA discs showed that EESL could significantly decrease the number of colonies. The group treated with 125 mg/ml showed a significant decrease in colony counts compared with the negative control and lower concentrations. The group treated with 150 mg/ml EESL showed no difference from positive control indicating that the concentration was effective in reducing bacterial adherence to HA discs. This might be the active compound in EESL reducing the bacterial colonies adhering to the HA discs.

The early phases of adhesion of *S. mutans* bacteria on the surface of the HA discs occur through non-specific attachment. One of the non-specific attachments is the hydrophobicity interaction between the cell and the adherence surface. A decrease in the hydrophobicity between the cell and the surface prevents bacterial adherence leading to a decrease bacteria attached to a surface.[17,18] The ability of the extract to prevent the adherence of *S. mutans* could be related to the effect of saponins and flavonoids components. Saponins are known to have a hydrophobic and hydrophilic action. Hydrophobicity of saponin allows binding to hydrophobic end of the bacterial cell membrane while the hydrophilic end is a free end and will bring a complex detergent-protein resulting in bacterial lysis.[19] Flavonoids through their antibacterial action is to form a

complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, and covalent bond formation.[20] In the next phase, adhesin-specific attachment of *S. mutans* that bind to glycoproteins of saliva adhering to HA discs. This specific attachment is thought to be inhibited by EESL through tannins and flavonoids. According to Agnol[21] tannins works by binding bacterial adhesin protein that binds to receptors on the surface of the host, Bennick[22] stated that the weakening bond with the bacterial cell surface protein on pellicle (host) would result in decreased bacterial adhesions. According to Kumar and Pandey[20] flavonoids can inactivate the bacterial adhesion and a disturbance in the bacterial adhesion will lead to a decrease in bacterial adhesion. In conclusion, this study showed the anti-adhesive properties of EESL inhibit adherence of S. *mutans* to the HA disc.

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