Antiseptic Effect of Betel Quid Extract on Lip Mucosal Wound of male Wistar (Rattus novergicus) Rats

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Abstract

Antiseptic is commonly used in dentistry practices, but some antiseptics have side effects such as allergy. Therefore, natural substances are developed as substitute, i.e betel quid which consisted of betel leaf, areca nut, gambier, and mineral slaked lime.

The aim of this study was to determine antiseptic effect of betel quid extract on lip mucosal wound of male Wistar (Rattus norvegicus) rats.

This study was true experimental in vivo. Thirty rats were divided into five groups; three groups were treated with betel quid extract ointments at concentration of 5%, 10% and 20%, one group was positive control (hyaluronic acid) and last group was negative control (placebo). One-mm diameter of wound was made on lower lip mucosa of rats with cylinder diamond bur. All wounds were swabbed before and after treatment, and the number of bacterial colonies grown in agar plates were counted.

The results showed that there was significant decrease of bacterial colony numbers for respective groups, except for negative control ($p < 0.05$). Ointment containing 20% betel quid extract had antiseptic effect similar to 0.2% hyaluronic acid.

It can be concluded that betel quid extract had antiseptic effect on lip mucosal wound.

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Introduction

Antiseptic is chemical compound used to destroy the structure or to inhibit the growth of microorganisms on living tissue such as skin, mucosa and open wounds.[1] In dentistry practice, antiseptic is widely used before the implementation of teeth extraction procedures, biopsies, scaling and other procedures that have potential to injure soft tissue in oral mucosa.[2] The antiseptic addition has objective to provide aseptic condition in injured mucosa in order to prevent bacterial entrance into circulation and to control infection that can inhibit wound healing process.[1,3]

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One example of antiseptic substance that frequently used on oral mucosal wound is *hyaluronic acid gel* at concentration of 0.2 %.[4] However, some studies showed that the use of topical medications of *hyaluronic acid* had side effect such as allergic reaction.[5] Therefore, natural topical medications that has a role as antibacterial substance is required to help wound healing process. One of natural substances which contain antibacterial substance is betel quid.

Chewing betel quid is prevalent in some part of Asian countries, such as Indonesia, Malaysia, India and Taiwan.[6,7] Traditionally, most people believed that betel quid had several medical effects such as anti-inflammation, antiseptic, anti ulcer, antimicrobial and antivirus.[8] Betel quid, commonly chewed in Indonesia, is a mixture of betel leaf (*Piper betle* Linn), areca nut (*Areca catechu*), gambier (*Uncaria gambir* Roxb.) and mineral slaked lime (calcium hydroxide). Each components of this mixture contain

antibacterial subtances as follows: 1.3% and 4.5% of tanin and essential oil for betel leaf, 0.6% of tanin for areca nut, as well as 2-4% of quersetin and 2.7-33 % catechin for gambier.[9-11] Mineral slaked lime/betel lime (calcium hydroxide) has good antibacterial characteristics through division mechanism of calcium and hydroxyl ions into enzymatic reaction on bacteria that will inhibit DNA replication process.[12,13]

Hoque et al. had reported that ethanol extract from betel leaf at the dose of 10 g/ml was capable to inhibit the growth of microorganisms on food contaminated by pathogens such as *Vibrio cholerae*, *Escherichia coli*, *Shigella dysenteriae* and *Staphylococcus aureus*.[14] Rahman et al. stated that ethanol extract from areca nut seed at the dose of 20 mg/l had ability to inhibit the growth of microorganisms of *Bacillus subtilis* and *Staphylococcus aureus.*[15] Katu et al. conducted antibacterial test on gambier extract and showed that ethanol extract from gambier at concentration of 1 % was effective in inhibiting the growth of bacteria of *Enterococcus faecalis*.[16] Mohammadi et al. exhibited the antimicrobial activity of calcium hydroxide in root canal bacteria.[17]

Antibacterial and antiseptic effect of each constituents in betel quid has been done in *in-vitro* study. Study about the effect of the mixture of all components consisted in betel quid in *in vivo* would be important to be done. The objective of this study was to determine the antiseptic effect of betel quid extract on lower lip mucosal wound of male Wistar *(Rattus norvegicus)* rats.

Materials and methods

This study was true experimental *in vivo* with pretest-posttest only control group design. The study was conducted at Animal House of Medical Faculty of Sriwijaya University Palembang to do the research and at Microbiology Laboratory of Medical Faculty of Sriwijaya University Palembang to determine the number of bacterial colonies. It had been approved by Research Ethical Commission of Mohammad Hoesin General Hospital (RSMH) Palembang and Medical Faculty of Sriwijaya University with ethical certificate No. 390/kepkrsmhfkunsri/2017.

Wistar (*Rattus norvegicus* L.) rats were obtained from Pharmacy School of Bandung Institute of Technology (SF-ITB), with certificate no. 524.3/3691-Dispangtan/2017. Inclusion criterias were male white rats of Wistar (*Rattus norvegicus* L.) strain, 8-12 weeks old, body weight of 150-200 grams and in healthy condition without defect. Rats that had been used in the previous study were excluded. Betel quid components, including betel leaf (*Piper betle* L.), areca nut (*Areca catechu* L.), gambier (*Uncaria gambir* Roxb.) and mineral slaked lime (calcium hydroxide) were collected from Babatoman Village, Sekayu Subdistrict, Musi Banyuasin District, South Sumatra Province, Indonesia. All the constituents and material were identified and authenticated by Faculty of Agriculture, Sriwijaya University, Indonesia.

Preparation of Animal

Thirty male Wistar rats were divided into 5 groups, Group A was treated with 5% betel quid extract ointment, Group B was treated with 10% betel quid extract ointment, Group C was treated with 5% betel quid extract ointment, Group D was positive control, treated with 0.2% hyaluronic acid ointment (purchased from Ricefarma Pharm.Co, Surabaya, Indonesia), and Group E was negative control, treated with placebo oinment.

Samples were acclimated for 8 days at room temperature of 20-25^{o}C.[18] During acclimatization process, rats were fed with standard pellet diet and water *ad libitum*.

Preparation of betel quid extract

Betel quid was extracted by using soxhletation method. Betel quid components consisting of 8 g of betel leaf, 3.5 g of areca nut, 2.5 g of gambier and 2 g of betel lime were mixed. Soxhlet system was filled with 96% ethanol solvent at ratio of 1:4 and heated at temperature of 50° C for five hours. The crude extract was vaporized to dryness by using rotary evaporator (IKA RV10, Staufen, Germany) for 2 days.

Preparation of oitment

Preparations of betel quid extract ointment for respective treatment groups was 5 g and the dose given to exprimental rats was 50 mg. The basic materials for ointment were lipid based material consisted of 15% *adeps lanae*

and 85% *vaselin album*. Each concentrations of 5%, 10% and 20% was made as following formula :

$$M1.\ V1=M2.V2$$

The hot mortar and pestle was taken out from oven (Cole-Palmer Ltd, UK) and adeps lane was poured into mortar and stirred by using pestle until melt. Subsequently, vaseline album was poured into mortar and was stirred by using pestle at constant velocity until homogenous and form ointment base. Betel quid extract was added according to formulation of each groups and was stirred until homogenous. Ointment extract was flattened on surface of glass base for subsequent homogenity test. An ointment was considered homogenous if particles mixture was evenly distributed. Homogenous ointment preparations extract was put into ointment pot and was labeled according to treatments.

Induction of mucosal wound

Mucosal wound induction on lower lip of rats was done by using 1-mm diameter of cylinder diamond bur (Microdont, USA). Prior to wound induction, animals were anesthetized with 0.2 mL ketamine by i.m. injection. The lower lip of rats was withdrawn by using tweezer (Fisher brand™, Thermofisher Co, UK) and was swabbed with aquadest-wetted sterile cotton. Mucosal wound was made by using cylinder diamond bur at depth of about 1 mm in accordance to bur diameter used in this process. Blood was cleaned with aquadest-wetted sterile cotton and dried.

Topical wound application

Before giving treatment, each wound was swabbed by using sterile cotton swab with back-and-forth motion for two seconds. Pre-test speciments were placed into sterile transport container and sent to the microbiology laboratory. The treatments were conducted according to respective groups by evenly rub cotton buds on injury part, left it for one minute and followed by conducting re-swab as the previous stage. Post-test speciments were placed into sterile transport container and sent to microbiology laboratory.

Bacterial Culture

The speciments were put into 10 mL test tube containing 1 mL of NaCl, homogenized and covered with sterile cotton. The suspension was diluted in ratio 1:10, and then the tube was rotated for 30 seconds. One mL homogenate suspension was taken from the tube by using micropipette and then put into agar media, flattened by using sterile loop and labeled according to treatments of each groups. All agar plates were incubated within incubator (ThermoFisher Co, UK) at temperature of 37^0C for 24 hours. The number of bacterial colony was counted.

Antiseptic assay

The number of bacterial colonies growth in agar plates was counted by using automatic colony counter camera (Colonizer, Sunil Laxman Lab, India). Plate was put on previously prepared pad. A light transmission was used to help lighting. Plate containing bacterial colonies was photographed and was awaited for about 30 seconds. The results were shown on screen display which was connected to camera. Data were recorded and analyzed.

Statistical Analysis

The analysis was performed with Statistical Package for Social Sciences version 22 (IBM® inc.pvt ltd, US) and Microsoft Excel (Microsoft inc®, Redmond). Data were tested with Levene's test to know the homogenity of samples and normality test of Shapiro Wilk to know the distribution of samples. P value of > 0.05 was considered that data were homogen and normal. Paired t-test was used to compare the changes between "before and after" study. One-way Anova was used for significance of difference in all groups. Post-hoc Tukey's test was continued to evaluate the effect of antiseptic among the groups and to compare the effect with positive control group. Statistically significant differences were indicated by p value of <0.05.

Results

The number of bacterial colonies on mucosal wound was evaluated. Normality test using Saphiro Wilk and homogenity test using

Levene's test showed p value of >0.05. It meant that all data were normally distributed and homogen. All groups showed the reduction of bacterial colonies, except group E which treated with pacebo oinment. Table 1 described that betel quid extracts in all concentrations were effective in decreasing bacterial colonies significantly (p value<0.05) in which hyaluronic acid showed the best results followed by betel quid extract of 20%, 10% and 5% concentration.

One-way Anova test was performed, and the results was p=0.00. It meant that there was a significant different difference in reducing the number of bacterial colonies between and within groups after treatment. Analysis was continued to Post-hoc Tukey's test (Tabel 2).

Group	Means ± SD		P value
	Before	After	
5% betel quid extract	115.83 ± 9.99	101.67 ± 8.94	0.00*
10% betel quid extract	119.67 ± 16.11	78.00 ± 15.53	0.00*
20% betel quid extract	121.00 ± 14.34	62.50 ± 13.94	0.00*
Positive control (hyaluronic acid)	125.17 ± 11.00	49.33 ± 5.99	0.00*
Negative control (placebo)	120.17 ± 5.57	152.33 ± 12.47	0.00*

Table 1. The number of bacterial colonies before and after treatment. * Significant level, Paired t test, p<0.05.

Groups/P value	5% betel quid extract	10% betel quid extract	20% betel quid extract	Positive control	Negative control
5% betel quid extract		0.02*	0.00*	0.00*	0.00*
10% betel quid extract	0.02*		0.19	0.00*	0.00*
20% betel quid extract	0.00*	0.19		0.33	0.00*
Positive control	0.00*	0.00*	0.33		0.00*
Negative control	0.00*	0.00*	0.00*	0.00*	

Table 2. The effect of antiseptic of betel quid extract among groups.
*Significant level, Post-hoc Tukey's test, p<0.05.

It could be seen in tabel 2 that all concentration of betel quid extract had significanly difference with negative control in reducing bacterial colonies. It signified that betel quid extracts had antiseptic effect on mucosal wound. Betel quid extract at 20% concentration had no different effect in decreasing the number of bacterial colonies compare to positive control. It was implied that 20% betel quid extract had similar antiseptic effect to 0.2% hyaluronic acid.

Discussion

The existence of antiseptic effect from betel quid extract is due to active ingredients available on betel quid components such as betel leaf, areca nut seed, gambier and mineral slaked lime or betel lime. Betel leaf contains eugenol, essential oil, cavicol, allipyrocatecol and cavibetol compounds that have capability as antiseptic compound.[19] Amalia *et al* found that *Piper betle* infusion was potential as antiseptic due to its phenolic derivates.[20] Eugenol has capability to destroy permeability of bacterial membrane cells resulting in disturbance of bacterial activity and subsequently the death of bacterial cells.[21,22]

Areca nut seed has antimicrobial effect on several bacteria found in mouth such as *Streptococcus mutans, Streptococcus salivarius, Candida albicans* and *Fusobacterium nucleatum*.[23,24] The compound in areca nut seed that is estimated to have antibacterial effect is proantocyanidin, a condensed tannin which belongs to the class of flavonoid.[24,25] This condensed tannin has ability in inhibiting bacteria found in oral cavity such as *Enterococcus faecalis, Phorphyromonas gingivalis* and *Fusobacterium nucleatum,* fungus such as *Candida albicans,* and virus.[26,27] Tomiyama et al revealed that condensed tannin had strong antibacterial actions againts bacteria included in biofilms.[28]

The antiseptic effect of gambier product is due to its chemical compounds, such as catechin and tannin. Dewi et al reported that gambier extract had capability to decrease the number of bacterial colonies on teeth which were soaked within artificial saliva.[29] Gambier extract takes part in inhibiting gram positive bacteria and less effective in inhibiting gram negative bacteria such as *Escherichia coli*.[30] This is due to the fact that cell wall structure of gram negative bacteria is more complex, because it has three layers consisting of lipoprotein at external layer, polysaccharides at central layer that has a role as barrier for the entrance of antibacterial bioactive compound and peptidoglycan having high lipid content at internal layer. On the other hand, cell wall structure of gram positive bacteria is simpler and consisting of one layer with low lipid content so that catechin can effectively kill gram positive bacteria such as *Streptococcus mutans.*[31,32] Catechin can inhibit biofilm formation of some bacteria, such as *Streptococcus mutans* and *Lactobacillus*. Catechin binds with protein, damages the cytoplasmic membran, leaks cytoplasmic membran, induces cell lysis and death.[33] Catechin inactivates glucosyltransferase enzymes, blocks the extracellular polysaccharide (EPS) synthesis, reduces the initial formation and accumulation of biofilm, and eliminates the growth of bacteria.[34,35] Catechin also keeps wound sterile and stimulates the healing process on ulcer.[35,36,37] Tannin has bacteriostatic and bactericidal againts gram positive bacteria, because tannin generates the complexation with the substrate of bacteria. Tannin binds with metal ion involved in the bacteria metabolism, forms chelat and leads toxicity of cells.[38]

Calcium hydroxide within betel quid has capability to change the environment into alkaline condition. This alkaline condition can affect the environment so that bacteria can not proliferate and produce neutral condition which stimulate the formation of hard tissue due to the decomposition of calcium hydroxide into ions of Ca^{2+} and OH^-.[39] Highly alkaline pH was also effective in eliminating resistant bacteria, such as *Enterococcus faecalis.*[40] The antibacterial mechanisms from calcium hydroxide is directly related to the increase of pH due to the release of ion OH^-. High decomposition of hydroxyde ions kill bacterial cells through damaging of cytoplasm membrane, protein denaturation and DNA damage.[41] Dixit et al reported that calcium hydroxide can eliminate bacteria in contaminated area in patients with periapical lesion.[42]

Conclusion

In conclusion, the study shows that betel quid extract has antiseptic effect on lower lip mucosal wound of male Wistar rats. 20% betel quid extract has equal effect compared to 0.2% hyaluronic acid and it has dose-dependent effect.

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