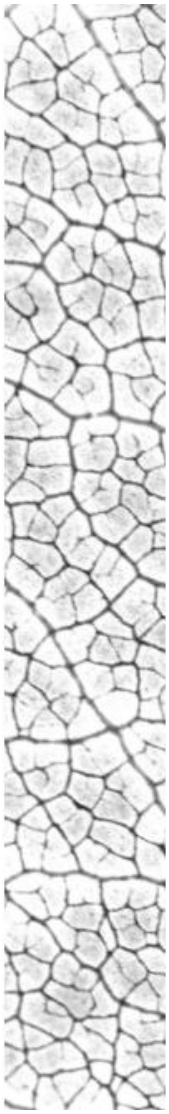

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Eugenia caryophyllus toothpaste reduces periodontal pathogens in saliva of Indonesian subjects

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Abstract
Eugenol, an essential oil extracted from Eugenia caryophyllus, or clove, is used as an antiseptic and anesthetic agent in dentistry and is known for its antibacterial properties. The aim of this study was to analyze the efficacy of Eugenia caryophyllus in toothpaste against the periodontal pathogens Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans in patients' saliva. Patients (n = 10, age 19 to 21) brushed their teeth using toothpaste containing Eugenia caryophyllus twice a day for 120 sec at a time, for a period of 2 weeks. Saliva was collected at baseline and after two weeks. The bacterial DNA in the saliva was extracted using a DNA extraction kit, and the numbers of Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans were calculated by quantitative polymerase chain reaction using 16S ribosomal RNA gene-specific primers for each bacterium. The difference in the numbers of the periodontal pathogens at baseline and after two weeks of using the toothpaste was analyzed using a t-paired test. A significant reduction in the numbers of A. actynomycetemcomitans and P. gingivalis was observed after two weeks ($p < 0.05$). Eugenia caryophyllus may be useful as an ingredient in toothpaste for periodontal disease prevention. Further studies are needed to prove its mechanism against oral pathogens.

Keywords: Aggregatibacter actinomycetemcomitans, antibacterial, Eugenia caryophyllus, eugenol, Porphyromonas gingivalis, toothpaste

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INTRODUCTION

The most common oral and dental issues in Indonesia are inflammation of tooth surrounding tissue and halitosis. The inflammation is caused by bacteria that consume food debris accumulated on the tooth surface, especially on the cervical tooth area, and break it down into sugars and acids that irritate the tooth-surrounding tissue (Mattulada, 2015. Dhillon, Govila, & Verma, 2014). Furthermore, inflammation lowers the gingival margins and deforms the periodontal pockets, leading to alveolar bone resorption, opening the cementum layer, and eventually causing tooth loss. Pathological conditions such as periodontitis can be caused by focal infections, affecting many vital systems, such as the cardiovascular and renal systems (Gough, 2010).

An oral focal infection beginning with gingivitis, periodontitis, chronic periodontitis, or chronic periapical abscess can develop into subacute bacterial endocarditis, carditis atheroma, or glomerulonephritis (Wisniewska-Spychala, et al. 2012).Systemic changes such as pregnancy or medical treatment can alter oral flora numbers and proportions, as these conditions change the salivary flow, composition and viability of components such as immunoglobulin and cytokine in the saliva (Brandtzaeg, 2013).

Gingival inflammation starts with accumulated and calcified plaque known as calculus, which is difficult to remove. The anaerobic Gram-negative bacterial in the calculus grow and develop in gingival inflammation. Some strains of Spirochaetae can also be found (Loesche, 1996). Some bacteria found in gingivitis are Aggregatibacter actinomycetemcomitans, Bacteroides forsythus, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Treponema denticola, and Eikenella corrodens; (Carrouel, et al. 2016).

A study showed the relation between periodontal pathogen *P. gingivalis* and atherosclerosis. *Porphyromonas gingivalis* found in carotid atheroma in coronary arteries invades and proliferates coronary artery endothelial cells and the heart and, along with *Streptococcus sanguinis*, induces platelet aggregation and thrombus formation. (Kurita-Ochiai, & Yamamoto,

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Table 1. The primers sequence used in this study

Primers	*Sequence (5'-3')*
A. actinomycetemcomitans Forward	CTT ACC TAC TCT TGA CAT CCG AA
A. actinomycetemcomitans Reverse	ATG CAG CAC CTG TCT CAA AGC
P. gingivalis Forward	TGC AAC TTG CCT TAC AGA GGG
P. gingivalis Reverse	ACT CGT ATC GCC CGT TAT TC

2014).Other bacteria involved in this disruption are *Streptococcus mutans*, *Staphylococcus aureus*, *Aggregatibacter actinomycetemcomitans*, and *Lactobacillus* (Armingohar, Jørgensen, & Kristoffersen, 2014).

Treatments for gingival inflammation marked by gingival bleeding and halitosis include toothpaste and mouthwash usage (Birang, et al. 2013). Toothpaste is one of the most common materials used for oral and dental cleaning. It is sometimes combined with the use of mouthwash but cannot be substituted with the latter. Toothpaste can remove debris on teeth, has a desensitizing effect, reduces tooth hypersensitivity caused by abrasion, and contains antiseptic agents (Pandey, et al. 2017).

Eugenol, an essential oil extracted from *Eugenia caryophyllus*, is commonly used in dentistry in the form of zinc oxide eugenol (ZOE) as a temporary tooth filling or cementation material. Zinc oxide eugenol is also used in deep cavities because of its healing effect on tooth pulp (Pavithra, 2014). Eugenol was recommended as a desensitizing agent by Aishwarya (2014), who found that clove oil dominates the main peripheral mechanism, and eugenol in clove oil can suppress the pain receptors in teeth (Aishwarya, Harini, & Karthikeyan, 2014). Eugenol is also known as an antibacterial agent (Towaha, 2014).The aim of this study was to investigate the efficacy of Eugenol in toothpaste against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in patients' saliva.

MATERIALS AND METHODS

Research Design

This preliminary clinical experimental study aimed to analyze the efficacy of eugenol in toothpaste against the periodontal pathogens *Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans*. Patients (*n* = 10, age 19 to 21) were patients in Dental Hospital, Faculty of Dentistry, Trisakti University. Randomly selected according to the inclusion criteria such as patients with minimum tooth decay (DMFT < 1) and periodontal index scores (PBI <1). Subjects were brushed their teeth using eugenol toothpaste with 1 cm toothpaste containing Eugenol (Antiplaque, Triple-Ace, Indonesia) twice a day–in the morning and in the evening—for 120 sec at a time, for a period of 2 weeks. Saliva was collected at baseline and after two weeks. The study was approved by Faculty of Dentistry, Trisakti University Ethics Committee with approval number 019/S1/KEPK/FKG/ 9/2017.

Saliva Sampling

All subjects were informed of the procedure and signed informed consent forms. Saliva samples were collected by spitting into 15-mL macro-centrifuged tubes using sterile funnels until 2 mL was collected in each tube. The samples were placed in a cooler box and later stored in a laboratory refrigerator at −20°C until the next procedure.

DNA Extraction

The saliva samples were centrifuged at 4,500 g for 15 min. They were then added to 1 mL of phosphate-buffered saline and inserted into macro-centrifuged tubes with pellets for washing. They were subsequently transferred to 1.5-mL micro-centrifuged tubes, homogenized using a vortexer and re-centrifuged at 10,000 g for 10 min. The supernatant was removed, and natant was added in 100 µL of double-distilled water (ddH_2O) and inserted into 1.5-mL micro-centrifuged tubes, which were closed with Sherlock tube closures. The tube contents were then incubated in a water bath at 100°C for 20 min using floating boats. The tubes were subsequently placed in ice for 10 min. The samples were then homogenized using a vortexer and centrifuged at 10,000 g for 2 min. The supernatants were taken, and transferred to new 1.5-mL micro-centrifuged tubes. Subsequently, DNA concentration was calculated using biodrop.

Quantitative Polymerase Chain Reaction (qPCR)

The quantification using qPCR with SYBR Green fluorescence (Applied Biosystems, USA) was applied. The number of DNA target were identified using 16s rRNA gene specific primers for *P. gingivalis* [15] and *A. actinomycetemcomitans*[16]. A PCR mix was made by mixing 5 µL of SYBR Green, 1 µL of forward and reverse primers, 3 µL of DNA sample, and nuclease free water (NFW) in a 1.5-mL micro-centrifuged tube. Standard bacterial dilutions (for each bacterium) were added in the PCR tube. The PCR tube was then filled with the PCR mix and closed, and the sample was centrifuged at 1,500 rpm for 1 min. The PCR tube was then inserted into the PCR machine. Amplifications were done with the following temperature profiles. Initial template denaturation step at 95 °C for 10 minutes (1 cycle), followed by 40 cycles of 94 °C for 15 seconds and annealing at 57-62 °C for 1 min, 95 °C for 15 seconds. The RT-PCR was performed in triplicate. [17] The PCR results were saved and analyzed.

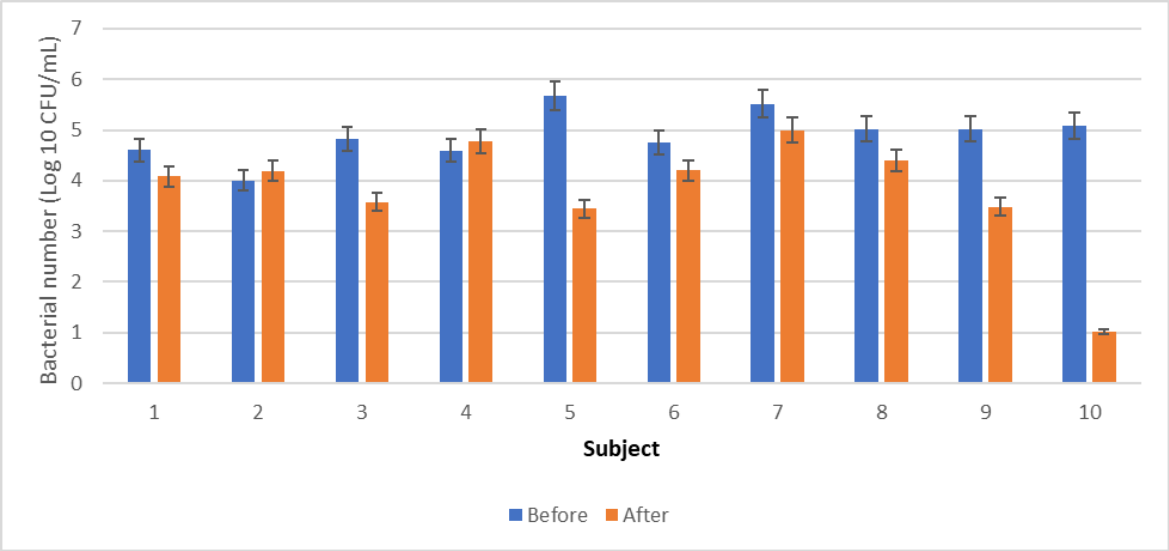

Fig. 1. The numbers of *Aggregatibacter actinomycetemcomitans* in saliva at baseline and two weeks after treatment with *Eugenia caryophyllus* toothpaste. The *Aggregatibacter actinomycetemcomitans* number were significantly reduced after tooth brushing using eugenol toothpaste. ($p < 0.05$)

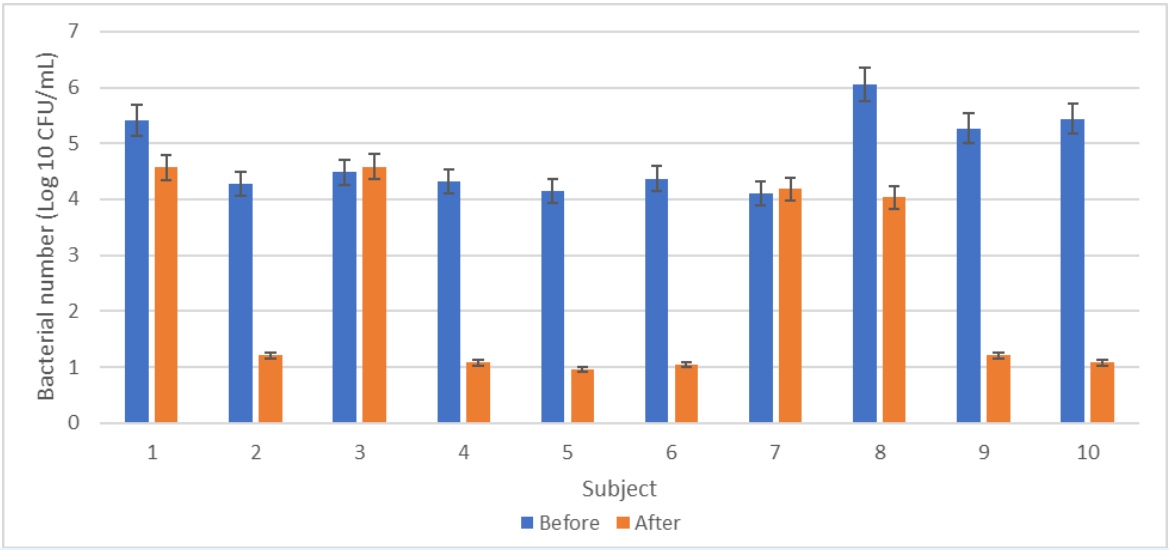

Fig. 2. The numbers of *Porphyromonas gingivalis* in saliva at baseline and two weeks after treatment with *Eugenia caryophyllus* toothpaste. The average number of *Porphyromonas gingivalis* were significantly reduced after tooth brushing using eugenol toothpaste compare to baseline. ($p < 0.05$)

Statistical Analysis

A Shapiro–Wilk test was used to analyze the data normality. The difference in the numbers of periodontal pathogens at baseline and after two weeks of using the toothpaste was analyzed using a *t*-paired test. A *p*-value less than 0.05 was considered statistically significant. The statistical analysis was performed using SPSS Statistics 20 for Windows (IBM, Armonk, NY, USA).

RESULTS

A significant reduction in the numbers of both *A. actynomycetemcomitans* and *P. gingivalis* was observed after two weeks of brushing using *Eugenia caryophyllus* toothpaste. The *A. actynomycetemcomitans* number decreased from 4.91 ± 0.26 log CFU/mL at baseline to 3.81 ± 0.22 log CFU/mL after the treatment ($p < 0.05$) [**Fig. 1**]. The *P. gingivalis* number decreased from 4.79 ± 0.23 log CFU/mL at baseline to 2.39 ± 0.12 log CFU/mL after the treatment ($p < 0.05$) [**Fig. 2**]. Different subjects will have a different bacterial number in their oral cavity even though the inclusion criteria have been fulfilled in this study. It may be the reason there's some different effect of this treatment to the bacterial number of each subject. Habit and oral hygiene condition as well as eating patterns of each subject might affects the reduction number of bacteria.

DISCUSSION

Eugenol can be found in some plants, such as basil, nutmeg, cinnamon, and lemon but is mostly extracted from clove (Pavithra, 2014).It can be extracted from its buds, leaves, or stems (Aishwarya, Harini, & Karthikeyan, 2014).The term “eugenol” is derived from the scientific name of clove itself, which is Eugenia aromaticum or Eugenia caryophyllata (Pavithra, 2014).Eugenia caryophyllus contains eugenol in high concentrations (60–90%) (Aishwarya, Harini, & Karthikeyan, 2014).

In medicine, eugenol, is used as an antiseptic and anesthetic agent, it can relieve pain if applied on the skin or a wound area. In dentistry, eugenol is believed to relieve pain when applied in a tooth cavity, and it is used as a restorative material, as a barrier applied on the gingiva before denture insertion, and as an analgesic agent to relieve pulpitis pain or tooth hypersensitivity (Pavithra, 2014. Tammannavar, et al. 2013). Eugenol is a clove oil component that plays an important role in oral and dental cleansing, as it is used to relieve irritation and as a local anesthetic and flavoring agent (Pavithra, 2014)..Eugenol in *E. caryophyllus* (clove oil) is sometimes used as deodorizing agent in perfume and cosmetics, and also a toothpaste ingredient (Putri, 2019).

Eugenol is commonly combined with zinc oxide and used as a pulp capping material, for endodontic treatment, or for temporary fillings (Cummins, 2009). Eugenol in the form of zinc oxide eugenol (ZOE) temporary filling diffuses the tooth pulp through dentin and easily heals the tooth pulp (Pavithra, 2014). In low concentrations, eugenol has local anesthetic and anti-inflammatory effects on the tooth pulp. Aishwarya (2014) found that clove oil dominates the main peripheral mechanism, and eugenol in clove oil can suppress pain receptors in the tooth. Other than pulp capping and endodontic material, temporary filling, and toothpaste ingredient, eugenol is also used as a flavoring material in cosmetic and food products (Putri, 2019).

In this study, six of ten subjects showed reduction of *Porphyromonas gingivalis* population to more than 60%, while only one subject showed similar pattern against *A. actinomycetemcomitans*. Eighty percent of subjects showed a significant reduction in the amount of *P. gingivalis* or *A. actinomycetemcomitans*, although greater reduction were shown in the amount of *P. gingivalis* ($p < 0.05$). This result showed that *A. actinomycetemcomitans* was not greatly affected by the use of eugenol toothpaste for two weeks. Hence, it can be predicted that using eugenol toothpaste can reduced accumulation of bacteria in plaque or calculus, which lead to prevention of gingival inflammation, periodontal disease and other diseases caused by these bacteria (Xu, et al. 2013. Tjandrawinata, Widyarman, & Liliany, 2019).

Eugenol is considered safe as a food ingredient (Kamatou, Vermaak, & Viljoen, 2012). According to the World Health Organization, the acceptable clove daily intake for humans is 2.5 mg/kg of body weight. According to the US Food and Drug Administration, eugenol is non-carcinogenic, non-mutagenic, and non toxic (Mammen, et al. 2018).However, although it can be used for many treatments, as a material to relieve tooth pain, it can be harmful, especially in incorrect doses, as it can cause pharyngitis, nausea, breathing difficulty, seizures, bleeding disorder, and in extreme cases, renal and liver failure. For these reasons, its usage is limited (Aishwarya, Harini, & Karthikeyan, 2014).

CONCLUSION

Eugenia caryophyllus toothpaste reduced the numbers of *A. actinomycetemcomitans* and *P. gingivalis* in patients’ saliva. Therefore, it may be useful as a toothpaste ingredient for periodontal disease prevention. However, further studies are needed to prove its mechanism against oral pathogens.

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