ผลการต้านเชื้อแบคทีเรียของผลิตภัณฑ์ดูแลสุขภาพช่องปากที่ผลิตจากสารธรรมชาติ

นางสาวสุปัญญา นัยวิกุล

จุฬาลงกรณ์มหาวิทยาลัย
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ANTIBACTERIAL EFFECT OF ORAL CARE NATURAL PRODUCTS AGAINST ORAL BACTERIA

Miss Supanya Naivikul

จุฬาลงกรณ์มหาวิทยาลัย
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A Thesis Submitted in Partial Fulfillment of the Requirements
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สุปัญญา นัยวิกุล : ผลการต้านเชื้อแบคทีเรียของผลิตภัณฑ์ดูแลสุขภาพช่องปากที่ผลิตจากสารธรรมชาติ (ANTIBACTERIAL EFFECT OF ORAL CARE NATURAL PRODUCTS AGAINST ORAL BACTERIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ทพญ. ดร.ทิพวรรณ ธราภิวัฒนานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ทพญ. ดร. อัญชลี วัชรักษะ, 74 หน้า.

สารสกัดจากธรรมชาติหลายชนิดมีฤทธิ์ต้านเชื้อก่อโรคฟันผุซึ่งอาจนำมาใช้แทนน้ำยาบ้วนปากคลอร์เฮ็กซิดีนได้ อย่างไรก็ตามยังไม่มีการศึกษาถึงผลของผลิตภัณฑ์ที่มีสารสกัดจากธรรมชาติหลังผ่านกระบวนการทางอุตสาหกรรม การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลการต้านเชื้อ*สเตร็ปโตคอคคัส มิวแทนส์* และ *แลคโตบาซิลลัส คาเซอิ* ในห้องปฏิบัติการของผลิตภัณฑ์สำหรับฉีดพ่นในช่องปากที่มีส่วนผสมของสารสกัดจากน้ำมันหอมระเหย มังคุด พรอพอลิสและใบฝรั่งด้วยวิธีการสัมผัสโดยตรง โดยใช้น้ำยาบ้วนปากคลอร์เฮ็กซิดีนร้อยละ 0.2 เป็นตัวแปรควบคุมเชิงบวก และสารละลายฟอสเฟตบัฟเฟอร์เป็นตัวแปรควบคุมเชิงลบ หลังจากเชื้อทั้งสองชนิดสัมผัสโดยตรงกับสารที่ต้องการทดสอบจึงวัดการเจริญเติบโตของเชื้อโดยใช้การวัดค่าความขุ่นของอาหารเลี้ยงเชื้อและวัดความสามารถในการผลิตกรดของเชื้อโดยการวัดค่าความเป็นกรด-ด่างของอาหารเลี้ยงเชื้อทุกๆ 2 ชั่วโมง จนครบ 10 ชั่วโมง ผลการศึกษาตลอดทั้ง 10 ชั่วโมงของผลิตภัณฑ์สำหรับฉีดพ่นทุกชนิดยกเว้นชนิดที่มีสารสกัดจากใบฝรั่งเป็นส่วนประกอบสามารถยับยั้งการเจริญเติบโตและการผลิตกรดของเชื้อ*สเตร็ปโตคอคคัส มิวแทนส์* และ *แลคโตบาซิลลัส คาเซอิ* เมื่อเปรียบเทียบกับกลุ่มสารละลายฟอสเฟตบัฟเฟอร์อย่างมีนัยสำคัญสถิติ (p=0.001) เมื่อคำนวณในแต่ละช่วงเวลาด้วยสถิติชนิด one-way ANOVA สำหรับผลิตภัณฑ์ที่มีสารสกัดจากใบฝรั่งเป็นส่วนประกอบนั้นสามารถยับยั้งการเจริญเติบโตและการผลิตกรดของเชื้อ *แลคโตบาซิลลัส คาเซอิ* ได้ตลอดการทดลอง แต่สามารถยับยั้งการเจริญเติบโตและการผลิตกรดของเชื้อ *สเตร็ปโตคอคคัส มิวแทนส์* ได้เพียงบางส่วนของการทดลอง จากผลการศึกษาจึงเห็นได้ว่าการนำผลิตภัณฑ์สำหรับฉีดพ่นในช่องปากไปประยุกต์ใช้จริงอาจช่วยป้องกันการเพิ่มจำนวนเชื้อก่อโรคฟันผุในช่องปากได้

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ภาควิชา	ทันตกรรมสำหรับเด็ก	ลายมือชื่อนิสิต .....
สาขาวิชา	ทันตกรรมสำหรับเด็ก	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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SUPANYA NAIVIKUL: ANTIBACTERIAL EFFECT OF ORAL CARE NATURAL PRODUCTS AGAINST ORAL BACTERIA. ADVISOR: ASSOC. PROF. THIPAWAN THARAPIWATTANANON, D.D.S., Ph.D., CO-ADVISOR: ASST. PROF. ANJALEE VACHARAKSA, D.D.S., Ph.D., 74 pp.

Several natural extracts are reported to be an effective antibacterial agent against cariogenic bacteria. These natural extracts can be used as an alternative to replace chlorhexidine mouthrinse. However, the antibacterial activity of the natural extracts in commercial products have never been demonstrated. This study aimed to investigate the antibacterial activity of commercially-available oral spray containing natural extracts; essential oil oral, essential oil with mangosteen extract, propolis extract and guava leaf extract. To determine the antibacterial activity, a direct contact of the oral spray and cariogenic bacteria, including *S. mutans* and *L.casei,* was performed *in vitro*. Chlorhexidine mouthrinse and phosphate buffer saline (PBS) were included as a positive and negative control, respectively. After a direct contact with antibacterial agent, or the oral spray, *S. mutans* and *L.casei* growth was observed in culture medium for up to 10 hr. The optical density measurement at 600 nm (OD_{600}) and pH in cultures were recorded every 2 hr to demonstrate bacterial growth and acid production. The result showed that all oral sprays except guava leaf extract oral spray significantly inhibited growth and acid production of *S. mutans* and *L. casei* for 10 hr (p=0.001) when compared to PBS group analyzed in each time point by one-way ANOVA test. For guava leaf extract oral spray, the growth and acid production of *L. casei* were inhibited throughout the experiment, while the result showed partial growth and acid production inhibition when *S. mutans* exposed to this oral spray.

Department:	Pediatric Dentistry	Student's Signature .....
Field of Study:	Pediatric Dentistry	Advisor's Signature .....
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CHAPTER I
INTRODUCTION

Background and Rationale

Dental caries is a multifactorial disease resulting from environmental changes in the biofilm that support the growth of cariogenic bacteria. Several factors, such as the host factors; saliva, tooth anatomy, immune response, genetic factor; and environmental factors such as high sugar diets, can affect the microbial ecological balance in the dental plaque.(1, 2) The cariogenic bacteria are capable of sugar fermentation which results in soluble and insoluble extracellular polysaccharides and acid production.(3) Therefore, acidic environment becomes persistent when sugar diets are supplied frequently. Acid production during bacterial metabolism of sugars causes the pH in the dental plaque to be lower than the critical pH, which is approximately pH 5.5, for enamel demineralization. When this acidic environment is continued, it promotes the growth of acidogenic and acid-tolerant bacteria and disturb the balance of other microorganisms in the plaque community to an advantage of mutans streptococci and lactobacilli colonization. The increased number of these organisms in plaque, in turn, results in more acidic environment, thereby enhancing further demineralization. (1, 4-6)

The standard care for dental caries is to eliminate bacterial biofilm, by using mechanical methods, such as brushing and interdental flossing, or chemical method such as antibacterial mouthrinse.(7) Chlorhexidine mouthrinse is commonly used, but its unpleasant taste can reduce patient compliance, especially in young children. Moreover, the development of chlorhexidine-resistant microbial strains may possibly occur if long-term use of chlorhexidine is prescribed.(8, 9) Therefore, other

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antibacterial agents including natural extracts are being studied for caries prevention purposes in substitution of chlorhexidine mouthrinse.(10-12)

Several natural products, such as essential oil (13-17), mangosteen(18-21), propolis(22-26) and guava leaf extract (27-31) have been reported to be an effective antibacterial agents. The antiseptic property in essential oils was demonstrated in both *in vitro* and *in situ* studies.(15, 16) In the *in vitro* study, essential oils, i.e. peppermint, tea tree, lavender oil, eugenol oil and thyme oil, have shown an antibacterial effect against common pathogens including *E. faecalis*, *E. coli* and *C. albicans*.(16) In the *in situ* observation, the intraoral device with glass disks was put in 15 subjects, and essential oil mouthwash (Listerine Mentol Listerine Johnson & Johnson, Madrid, Spain) was prescribed for a mouthrinse twice a day in comparison with 0.2% chlorhexidine. After 4 days, biofilms were removed from disks and analyzed by confocal laser scanning microscopy and fluorescence staining. The result showed that essential oil can reduce the bacterial viability and biofilm thickness.(15) For mangosteen extract, an *in vitro* study also show that it can inhibit growth and kill *S. mutans*.(20) After exposure to alpha-mangostin which is the major antibacterial component in mangosteen, acid production ability of *S. mutans* biofilm is decreased. Because the activity of enzymes associated with glucan synthesis, acid production and acid tolerance are significantly inhibited.(18) In addition, guava leaf and propolis extract show proper antibacterial capacity. Guava leaf extract also displayed significant effects against *S. mutans* and *S. mitis* in an *in vitro* study.(29) Propolis extract can produce an inhibitory zone against oral bacteria including *S. mutans*, *E. faecalis* and *L. casei*.(23, 24) Several compounds in propolis inhibit glucosyltransferase activities from *S. mutans*.(25) Moreover, in an *in vivo* study, propolis-extracted mouthrinse

reduce the concentration of *S. mutans* in 81% of volunteer's saliva samples collected after they performed 21 rinses divided into 3 rinses per day for 7 days.(22) Furthermore desalivated rats treated with propolis extract showed statistical difference in either smooth-surface or sulcal caries score when compared to the control group.(26) Taken together, these reports suggest that natural extracts may be used intraorally for a chemical plaque control.

For a long time, natural extracts have been widely used in Thai traditional medicine for treatment and maintenance of healthy condition. Beside toothpaste and mouthwash, oral spray is another product on the market that can be used orally. To use oral spray is feasible and beneficial in young children or disability patients to maintain their oral health. However, most of the studies of natural products for oral care are limited in the *in vitro* study, and the antibacterial activity of the natural extracts after a manufacturing process remains unknown. Therefore, the aim of this study is to investigate the antibacterial activity of oral care natural products against oral microorganisms.

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Research question

Do oral care natural products on the market retain an antibacterial activity against the cariogenic bacteria?

Research objectives

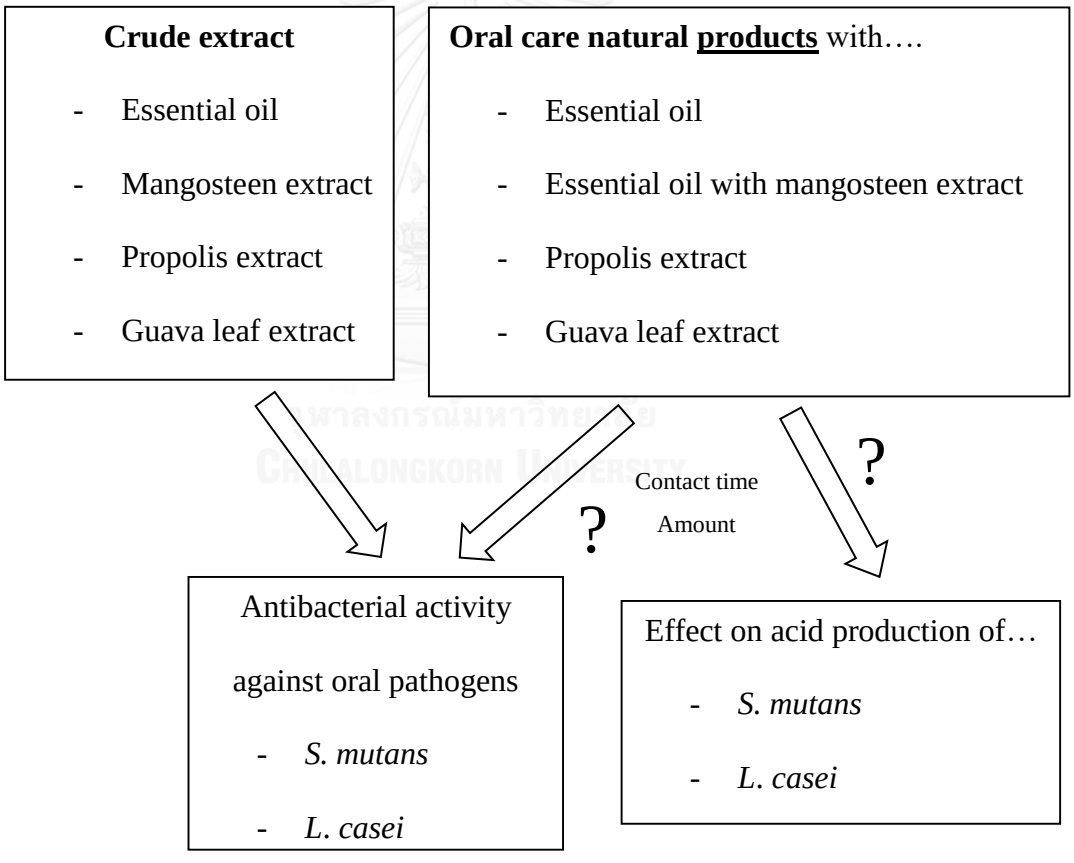
1. To investigate the antibacterial effect of oral care natural products against oral bacteria after a direct contact by evaluation of bacterial growth curve and pH change in the bacterial culture.

2. To investigate the synergistic effect of essential oil with mangosteen extract by evaluation of bacterial growth curve and pH change in the bacterial culture.

Hypothesis

Oral care natural products have antibacterial effect against the cariogenic bacteria, including *S. mutans* and *L. casei*.

Conceptual framework



Research design: Laboratory experimental research

Keywords: Antibacterial activity, natural products, *L. casei*, *S. mutans*

CHAPTER II
REVIEW OF LITERATURE

Dental caries

Dental caries is one of the most common human diseases, which affects the vast majority of individuals.(5) Dental caries is a disease resulting from environmental changes in the biofilm. Several factors, such as the host; saliva, tooth anatomy, immune system, genetic factor; and high sugar diets, can affect the ecological balance and microbial composition of the dental plaque.(1, 2) The causative bacteria can form a cariogenic biofilm by acid production and extracellular polysaccharide synthesis from dietary sugar e.g. sucrose.(3) Therefore, acidic environment becomes persistent when sugar diets are supplied frequently. Acid production during metabolism of sugars causing plaque spends more time below the critical pH for enamel demineralization (approximately pH 5.5). This conditions promote the growth of acidogenic and acid-tolerant bacteria and turn the balance of plaque community in favour of mutans streptococci and lactobacilli. Increased number of mutans streptococci and lactobacilli in plaque results in more acid production at faster rate, thereby enhancing tooth demineralization.(1, 4-6) Therefore caries activity can be assessed by the number of typical colonies (colony-forming units or CFUs); for *Streptococcus mutans*, more than 10^6 CFUs/ml and for Lactobacillus spp., more than 10^5 CFUs/ml is defined as high caries activity.(5) An elevation of mutans streptococci, as the caries risk factor, is one of the criteria for caries risk assessment in young children released by American Academy of Pediatric Dentistry.(32) As dental caries caused by ‘complex’ or ‘multifactorial’ factors, an effective approach should rather be caries control

interventions. Therefore, the first step is dental plaque control followed by elimination of risks, and finally enhance tooth remineralization during follow-up and maintenance visits.(7)

Microorganisms development and metabolic end product

Microorganisms obtain energy for their survival and replication by using different methods. The process is influenced by substrate availability. When carbohydrate is available, the microorganisms are able to convert sugar via the glycolytic pathway to form the high energy compound, ATP, and simultaneously produce the final product, lactic acid. Therefore, pH of the microenvironment surrounding the microorganisms will decrease by 3 minutes, and the physiologic pH can be restored within approximately 20-30 minutes. The low pH may be prolonged if bacterial metabolism continues in the environment that is rich of fermentable carbohydrates.(33)

Streptococcus mutans

Streptococcus is a cocci facultative anaerobic gram-positive bacteria.(33) Survival of microorganisms in the oral environment depends on their ability to adhere to a tooth surface. Only a few specialized organisms are able to adhere to oral surfaces such as *S. mutans* and *S. sanguinis* that can adhere to dental hard tissue since the first teeth appear.(2, 3, 33) Mutans streptococci make soluble and insoluble extracellular polysaccharides (glucan, mutan and fructan) from sucrose that are associated with plaque maturation and cariogenicity.(34) *S. mutans* can ferment a range of sugars very efficiently and generate weak acids including lactic, formic and acetic acid as metabolic

end product. Lactic acid, the strongest acid, produced by *S. mutans* can cause the plaque pH changes to below the critical pH for enamel demineralization. *S. mutans* show the ability to attain the critical pH for enamel demineralization more rapidly than other common plaque bacteria. They are able to grow and survive under the acidic conditions they generate, by the induction of a specific molecular stress response. Moreover, *S. mutans* can synthesize intracellular polysaccharides when there is excess sugar, and these can act as carbohydrate reserves, and be converted to acid during periods when dietary carbohydrates are not available.(34, 35)

There are positive correlations of percent mutans streptococci, including; *S. mutans* and *S. sobrinus*, in saliva and dental plaque with an increased risk of early childhood caries.(5, 36, 37) While *S. oralis*, *S. sanguinis* and *S. gordonii* presented in a significant proportion of the dental plaque from non-carious tooth surfaces. Epidemiological studies have suggested *S. mutans* as a primary cause of early childhood caries in infants, enamel caries in children and young adults, and root surface caries in the elderly.(2, 3, 33, 38)

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Lactobacillus casei

Lactobacillus is a rod, facultative anaerobic gram-positive bacteria which the major metabolic end product of carbohydrate fermentation is lactic acid.(33) They are highly acidogenic and acid-tolerant bacteria. Although lactobacilli are commonly isolated from the oral cavity, they can be rarely detected in dental plaque of non-carious tooth surfaces or from incipient lesions. It is postulated that lactobacilli are the organisms contributed in the progression of the deep enamel lesion and dentine caries.(5, 34, 39) On the other hand, *L. casei* was shown to inhibit the growth of *S. mutans*

and *S. sobrinus*, as well as, periodontal pathogens *P. gingivalis*.(39-41) Therefore, lactobacilli may have a beneficial role during the initiation of disease through interaction between bacterial species.

Chlorhexidine

Chlorhexidine is one of the most widely used antimicrobial agents. It is considered to be the gold standard antiplaque agent.(8, 42) Chlorhexidine is used in addition to a mechanical plaque control by tooth brushing. The cationic chlorhexidine molecule is attracted toward negatively charged bacterial cell surfaces, causing alterations of bacterial cell membrane integrity.(42) Chlorhexidine mouthrinse in 0.2% concentration, rinse 10 ml 1 min twice daily for 7 days, showed an effect in reducing the salivary *S. mutans* in 45 subjects aged 14-15 years.(43) Similar results were obtained in high caries risk patients with fixed orthodontic appliances who used 0.2% chlorhexidine mouthrinse.(44) Thus, the chemical method such as chlorhexidine may be beneficial for patients with mental disability, physical disability, motor function disturbance and muscle coordination disturbance.(42) However, side effects of chlorhexidine include brown discoloration of teeth, restorative materials and dorsum of tongue, oral mucosa erosion and a bitter taste which is difficult to mask.(8) Moreover, the development of chlorhexidine-resistant microbial strains may possibly occur when long term use of chlorhexidine is prescribed.(9)

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Natural products

The use of drugs or dietary supplements derived from plants have increased in recent years. Natural products derived from medicinal plants were reported to be an abundant source of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, many of which have been the basis for the development of new chemicals for pharmaceuticals. They can be useful for the development of alternative or adjunctive anti-caries therapies. With respect to diseases caused by microorganisms, the increasing resistance in many common pathogens to currently used therapeutic agents, such as antibiotics and antiviral agents, has led to renewed interest in the discovery of novel anti-infective compounds.(10-12) Plant-derived medicines have been reported efficacy against oral microbial pathogens. Many of these bioactive agents have been used in combination with fluoride as an alternative prevention approach to replace broad-spectrum antimicrobial agents such as chlorhexidine.(12, 45) The mixture of naturally-occurring agents, 1.0 mM myricetin (Extrasynthese Co., Genay-Sedex, France), and 2.5 mM tt-farnesol with 125 ppm fluoride (Sigma-Aldrich Co., St Louis, MO) in 20% ethanol and 2.5% DMSO effectively disrupted the expression of specific virulence genes, structural organization and accumulation of *S. mutans* biofilm.(46)

Essential oils

Essential oils have been used for various purposes in many countries across the world.(16) The antimicrobial effects of essential oils against *E. faecalis*, *E. coli*, *S. aureus*, *S. pyogenes*, *S. epidermidis*, *P. aeruginosa* and *C. albicans* have been demonstrated.(14, 16) Essential oils for prevention and treatment in dentistry have been developed.(47) Essential oils demonstrated antimicrobial activity against some cariogenic bacteria including *S. mutans* and *L. casei* with MIC values ranging from

31.2-500 mg/ml. Essential oil extracted from *Tetradenia riparia* exhibits a bactericidal effect against *S. mutans* within the first 12 hr with cell contact, and that was similar to the effect by chlorhexidine dihydrochloride.(13)

Quintas et al. studied antiplaque effect of daily essential oils mouthwash (Listerine Mentol Listerine Johnson & Johnson, Madrid, Spain) compared with 0.2% chlorhexidine (Oraldine Perio Johnson & Johnson, Madrid, Spain). The oral biofilm was formed *in situ* on the glass disks in a mandibular occlusal splint made by vinyl sheet. The mandibular occlusal splints were used intraorally for 4 days, except during meals and oral hygiene maintenance. After rinsing cycles with essential oils, 0.2% chlorhexidine, or sterile water, the oral biofilm of glass disks were stained and analyzed by confocal laser scanning microscopy. Essential oils mouthwash showed an effective antiplaque effect *in situ.* Although, 0.2% chlorhexidine is more effective than essential oils to reduce the thickness and bacterial mass of the biofilm, the number of vital cells remaining in biofilm is similar to essential oils treatment.(15) A meta-analysis of 6-months clinical trials supports the benefit of essential oil mouthrinses in combination with mechanical methods for plaque control in gingivitis cases.(17) Furthermore, an *in vitro* study showed an effective intracanal antiseptic solution of essential oils; peppermint, tea tree and thyme oil against common oral pathogens; *S. aureus, E. faecalis, E. coli* and *C. albicans*.(16)

Mangosteen extract

In Southeast Asia, mangosteen has been used in pharmaceuticals because of the antioxidative, antibacterial, antiviral, antifungal, antiallergic and anti-inflammatory properties of xanthones, the active ingredients found in many parts of mangosteen. Mangosteen pericarp contains a variety of xanthones, including α-, β- and γ-mangostins, while α-mangostin demonstrated the most potent antibacterial effect by disruption the development and structural integrity of biofilm, reduction of the acidogenic and aciduric activity of *S. mutans*, and inhibition of enzymes associated with glucan synthesis.(18, 19) α-mangostin at 4,000 μg/ml, or lower, was demonstrated in cytotoxicity test not to be toxic to human gingival fibroblast.(48) Due to non-toxic property and the strong bactericidal activity of mangosteen pericarp extract, it has been suggested to add it into oral spray, oral paste and toothpaste for further development as an anti-plaque agent.(19, 20) Mangosteen pericarp extract has antibacterial activity against the oral pathogenic bacteria including *S. mutans*, *P. gingivalis* and *S. pyogenes*.(18-20) Many *in vitro* studies demonstrated the inhibitory effect of α-mangostin and mangosteen pericarp crude extract on *S. mutans* growth.(19-21) The range of the minimum inhibitory concentration (MIC), 0.625-10 μg/ml, and minimum bactericidal concentration (MBC), 0.625-1000 μg/ml, were demonstrated depending on each study. This variable is caused by chemical components of the extract, which varies depending on the cultivated area of mangosteen, extraction protocol and strain of *S. mutans*.(20, 21) Time-kill assay of mangosteen pericarp extract showed that at 60 min, *S. mutans* treated with mangosteen extract at the concentrations four times higher than MBC, or 2.5 μg/ml, was decreased viable cell count by almost two orders, and

completely killed at 90 min. Furthermore the time-kill kinetics which using the crude extract at 160 µg/ml, the extract completely killed the bacteria within 15 minutes.(20)

Propolis extract

In an *in vitro* study, propolis extract showed antibacterial effect against relevant bacteria in dentistry i.e. *S. mutans, S. salivarius, E. faecalis* and *L. casei*.(23, 24) In the study of Ehsani et al, the antibacterial activities of three different propolis extract (aqueous extract and alcohol extract with 15% and 40% ethanol) on *E. faecalis* were compared using three methods. The first method, material solutions and bacterial suspension were directly contacted and colony count was measured after 24 hr incubation, the hydroalcoholic extract of propolis with 40% alcohol showed significant antibacterial effect against *E. faecalis* similar to chlorhexidine 2% solution used for root canal debridement. On the other hand, in disk diffusion test, chlorhexidine 2% produced significantly higher inhibition zone compared to propolis hydroalcoholic extract with 15% and 40% ethanol. In addition to microdilution test, the MIC results for chlorhexidine, propolis hydroalcoholic extract with 15% and 40% ethanol were 2, 750 and 313 µg/ml respectively. In this study, there was no antibacterial inhibitory effect of propolis aqueous extract.(23) While the study of Jafarzadeh Kashi et al. has found that both propolis extracted with 80% ethanol and propolis extract with water can produce an inhibitory zone against *E. faecalis* and *S. mutans* in agar diffusion method. Propolis extracted with 80% ethanol and water extract of propolis showed a MIC 250 and 500 µg/ml respectively on *S. mutans* and *E. faecalis*.(24) These variations in the antimicrobial activity of propolis may be due to the differences in its chemical component and plant ecology with different climates.(24, 26) Several compound in propolis, i.e. apigenin, kaempferol, pinocembrin and pinobanksin-3-acetate, could

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inhibit streptococcal glucosyltransferase which is the key enzyme in glucans synthesis. Among these compounds, apigenin displayed the most potent inhibition of glucosyltransferase activities. Apigenin inhibited 90.5% to 95% of the activity of all of the glucosyltransferases tested in solution and 30% to 60% on surface-adsorbed enzymes at a concentration as low as 500 µM (135 µg/ml).(25)

In an *in vivo* study(26), the rat model, nineteen days old Wistar rats from CEMIB/UNICAMP (Campinas, Brazil), was infected with *S. sorbrinus* and set in conditions of cariogenic diets and high cariogenic challenge, such as desalivation by ligation of the parotid ducts and removal of sublingual and submandibular gland. The rats treated with ethanolic extract of propolis twice daily for 3 week showed a reduction of infection by *S. sorbrinus*. The rats treated with propolis extract from southern Brazil showed statistical difference in either smooth-surface or sulcal caries score when compared to the control group. However, ethanolic extract of propolis from different cultivated area demonstrated different result. In this study, propolis extract from southern Brazil showed better antibacterial effect than other one from southeastern Brazil. This can be attributed to the qualitative and quantitative differences of bioactive flavonoids between the propolis samples. It is evident that the propolis extract from southern Brazil contains more bioactive flavonoid compounds than the propolis extract from southeastern Brazil.(26) Moreover, Duailibe et al. evaluated the antimicrobial activity of a propolis-extracted mouthrinse on the concentration of *S. mutans* present in the oral cavity of young individuals. Volunteers performed 21 rinses divided into 3 rinses per day for 7 day. Propolis-extracted mouthrinse can reduce the concentration of *S. mutans* in 81% of all saliva samples collected. This can indicate that propolis extract possesses antimicrobial activity against oral pathogen. (22)

Guava leaf extract

Guava has been suggested for oral health promotion to maintain oral hygiene and prevent dental caries.(31) For cytotoxicity test, the 50 µg/ml and 100µg/ml of guava leaf did not exhibit anti-proliferative effects on cultured mouse fibroblast and breast cancer cells.(49) In an *in vitro* study, guava leaf extract has a broad spectrum antibacterial activity. It displays significant effects against several microbes e.g. *S. mutans, S. mitis, S. aureus*, *E. coli* and *C. albican*. The activity is strong against most enteric bacteria both standard strains and clinical isolates.(27-29) For the antibacterial activity against *S. mutans*, ethanol extract of guava leaves has shown optimum results with zone of inhibition 18 mm. While the acetone, methanol and petroleum ether extract of guava leaves has shown moderate activity against *S. mutans*. Phytochemical analysis has revealed that guava contains alkaloids, flavonoids, phenols and tannin which may either individually or in combination be responsible for the antibacterial activity.(28) Guaijavarin, which is the active flavonoid compound in guava, has been found to be bacteriostatic against *S. mutans* with an MIC of 2-4 mg/ml; a crude extract of guava gave a similar result.(30, 31) At sub-MIC level of guaijavarin, there was an effect on the acid production of *S. mutans* of both MTCC 1943 and CLSM 001 strains. Other cariogenic properties also have been disturbed by guaijavarin such as cell-surface hydrophobicity, sucrose-dependent adherence to glass surface and sucrose-induced aggregation of *S. mutans*.(30)

Saraya et al., have reported antibacterial activity against *S. mutans* of guava extract that used in chewable tablets compared with standard antibiotic, kanamycin. The crude extract, equally to the concentration in chewable tablets, demonstrated the bacterial inhibition by agar diffusion method. Time kill curve of *S. mutans* indicated

that 160 mg/ml of guava extract showed bacteriostatic activity after 15 min contact time. Taken together, guava extract is not only suitable to develop as antiplaque agent for dental caries management but also demonstrate low cytotoxicity that can be formulated as oral cavity consumer herbal products such as chewing gums, toothpastes, mouthwashes and dental floss.(31, 49)

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CHAPTER III
MATERIALS AND METHODS

Test solution preparation

Four oral care natural products were selected based on a literature survey:

1. Myherbal mybacin trospray (Greater Pharma Co., Ltd.)
2. Myherbal mybacin trospray with mangosteen extract (Greater Pharma Co., Ltd.)
3. Propolis oral spray (T. man pharma Co., Ltd.)
4. Guava leaf extract oral spray (Abhaibhubejhr brand)

0.2% chlorhexidine mouthwash (Faculty of Dentistry, Chulalongkorn university) was used as positive control and phosphate-buffered saline (PBS) was used as negative control.

Test organism and culture preparation

S. mutans (ATCC 25175) and *L. casei* (IFO 3533) obtained from department of Microbiology, Faculty of Dentistry, Chulalongkorn University. *S. mutans* was cultured on brain-heart infusion (BHI) agar (HIMEDIA®) and *L. casei* was cultured on rogosa agar (Oxoid™) at 37ºC in 5% CO_2 incubator (Forma Steri-Cycle CO_2 Incubator, Thermo Scientific) for 48 hr. A single colony was transferred to BHI broth (HIMEDIA®). Then incubate overnight in the same condition as above, and continuously shaken at 240 rpm (IKA KS 130 basic Shaker, USA) which is according to the standardized protocol of Microbiology laboratory, Faculty of Dentistry, Chulalongkorn University.

Table 1: components, activities and application of 4 natural products

Natural product	**Components**	**Activities**	**Application**
Myherbal mybacin trospray	Menthol, thymol, eucalyptol, chamomile, peppermint oil, anise oil, spearmint oil, pine oil, basil oil, bergamot oil, aloe vera, witch hazel, sage and methyl salicylate	Reduce bad breath and treat oral ulcer	Spray into your mouth 1-2 times after meal or when needed
Myherbal mybacin trospray with mangosteen extract	Mangosteen extract, menthol, thymol, eucalyptol, chamomile, peppermint oil, anise oil, spearmint oil, pine oil, basil oil, bergamot oil, aloe vera, witch hazel, sage and methyl salicylate	Reduce bacterial accumulation, reduce bad breath and treat oral ulcer	Spray into your mouth 1-2 times after meal or when needed

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Natural product	Components	Activities	Application
Propolis oral spray	Menthol, honey, peppermint oil, and spearmint oil	Antibacterial and antiviral causing sore throat	Spray into your mouth or throat 2-3 times or often as you preferred
Guava leaf extract oral spray	Ethyl alcohol, 4-hydroxybenzoic acid, menthol and saccharin sodium	Reduce bad breath	Spray into your mouth

Standard growth curve

Bacterial growth was established using previously described methods with slight modifications.(50, 51) Single colony of *S. mutans* from BHI agar plate or single colony of *L.casei* from rogosa agar plate was inoculated into BHI broth, grown overnight at 37ºC in 5% CO_2 and continuous shaking at 240 rpm. Overnight culture was diluted 6 folds with BHI broth in glass tube. Bacterial cultures which contained bacterial cells with an optical density of 0.1 (0.5 on the McFarland turbidity standard) was incubated in 37ºC in 5% CO_2 and shaken 240 rpm. Bacterial solution was taken 1 ml into cuvette, growth was monitored turbidimetrically every 1 hr by measuring the optical density of the culture at 600 nm wavelength (OD_{600}), using a spectrophotometer

(Thermo Scientific™ GENESYS 20). BHI broth was used as blank. Growth curve was generated from data of 10 hr and the data was used to design the experiment.

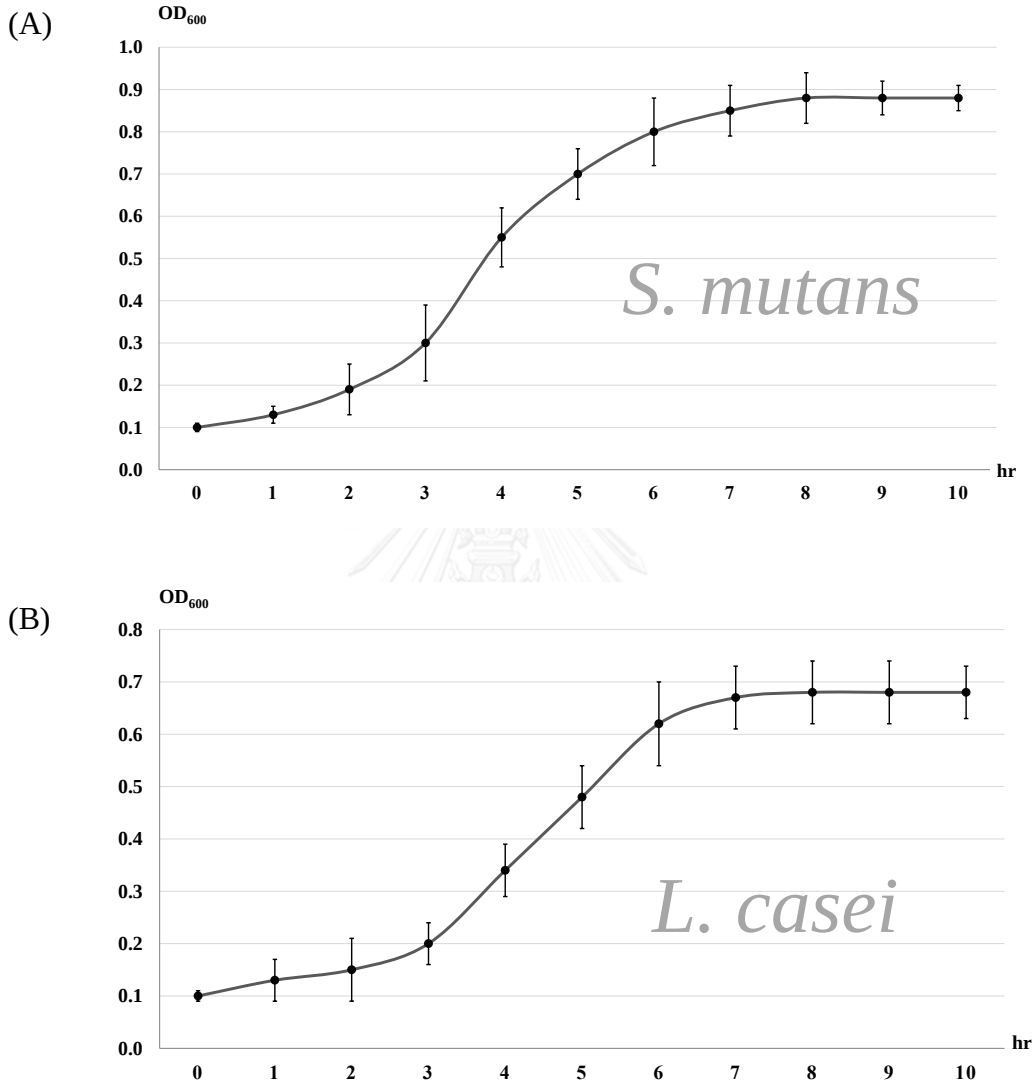

Figure 1. Growth curve of *S. mutans* (A) and *L. casei* (B) cultured in BHI broth. The overnight culture of *S. mutans* or *L. casei* was adjusted to 0.1 OD_{600} at 0 hr, and then incubated in 37ºC for up to 10 hr. The optical density (OD_{600}) of growth media was measured every hour to demonstrate the bacterial growth.

Direct contact test

To assess antibacterial effect of 4 natural products, single colony of *S. mutans* from BHI agar plate or single colony of *L.casei* from rogosa agar plate was inoculated into BHI broth, grown overnight at 37ºC in 5% CO_2 and continuous shaking at 240 rpm. The overnight culture was centrifuged at 5000 rpm for 8 min. BHI broth was discarded from the pellet of bacterial cells, PBS was added instead of BHI broth. Then all solutions was pooled in erlenmeyer flask, the bacterial suspension was adjusted with PBS to optical density of 1.0 at 600 nm wavelength. Prepared bacterial cells in PBS was divided and poured into eppendorf tubes, each tube contained 1 ml of bacterial solution. After that bacterial cells was centrifuged at 8000 rpm for 5 min and PBS was discarded. 100% concentration of each oral care natural product was added to the bacterial cells pellet by 4 different conditions which adapted from Ehsani et al.(23) & Herreara et al.(52)

I) 100 µl of oral care natural product contacted with bacterial cells for 30 sec
II) 100 µl of oral care natural product contacted with bacterial cells for 1 min
III) 300 µl of oral care natural product contacted with bacterial cells for 30 sec
IV) 300 µl of oral care natural product contacted with bacterial cells for 1 min

For the positive control, 100 µl of 0.2% chlorhexidine mouthwash and 30 sec of exposure time was used. After direct contact, 1 ml of PBS was added immediately to dilute the test solution and washed. To obtain only bacterial cells, all solution was centrifuged at 8000 rpm for 5 min and PBS was discarded. Then, the pellet was washed again in 1 ml of PBS. Afterwards, 1 ml of BHI broth was added into pellet of bacterial cells in eppendorf tube and bacterial cells in broth was transferred into 15 ml plastic

test tube containing 9 ml of BHI broth. Bacterial cells in broth was incubated at 37ºC, 5% CO_2 and shaken 240 rpm. Bacterial solution was taken 1 ml into cuvette for optical density measurement (at a wavelength of 600 nm) at 0, 2, 4, 6, 8 and 10 hr. The measurement was performed in triplicates, and means was used for analysis. The optical density was plotted to show exponential growth of microorganisms in each culture.

pH measurement

After optical density measurement, the same samples in cuvettes were used to test effect of oral care natural products on acid productions of *S. mutans* and *L. casei*, which assessed by pH measurement. The pH measurement of the bacterial broth was taken using a pH indicator strip (Panreac, pH range 4.5-10) at 0, 2, 4, 6, 8 and 10 hr. A strip of filter paper was immersed in each bacterial broth until the color change remained stable, and the color on the strip was compared with standard chart provided by the manufacturer to determine the pH value of each sample by only one examiner throughout the study. Before all experiments, the examiner had practiced to use pH strip and the read-out value from the color strip was compared with measurement by a pH meter (Digital Orion pH meter, type 420A). For intra-examiner reproducibility, intra class correlation coefficients (ICCs) were calculated from the collected data. For the reproducibility of test-retest data from the read-out value from the color strip or the pH meter, concordance correlation coefficients (CCCs) were calculated.

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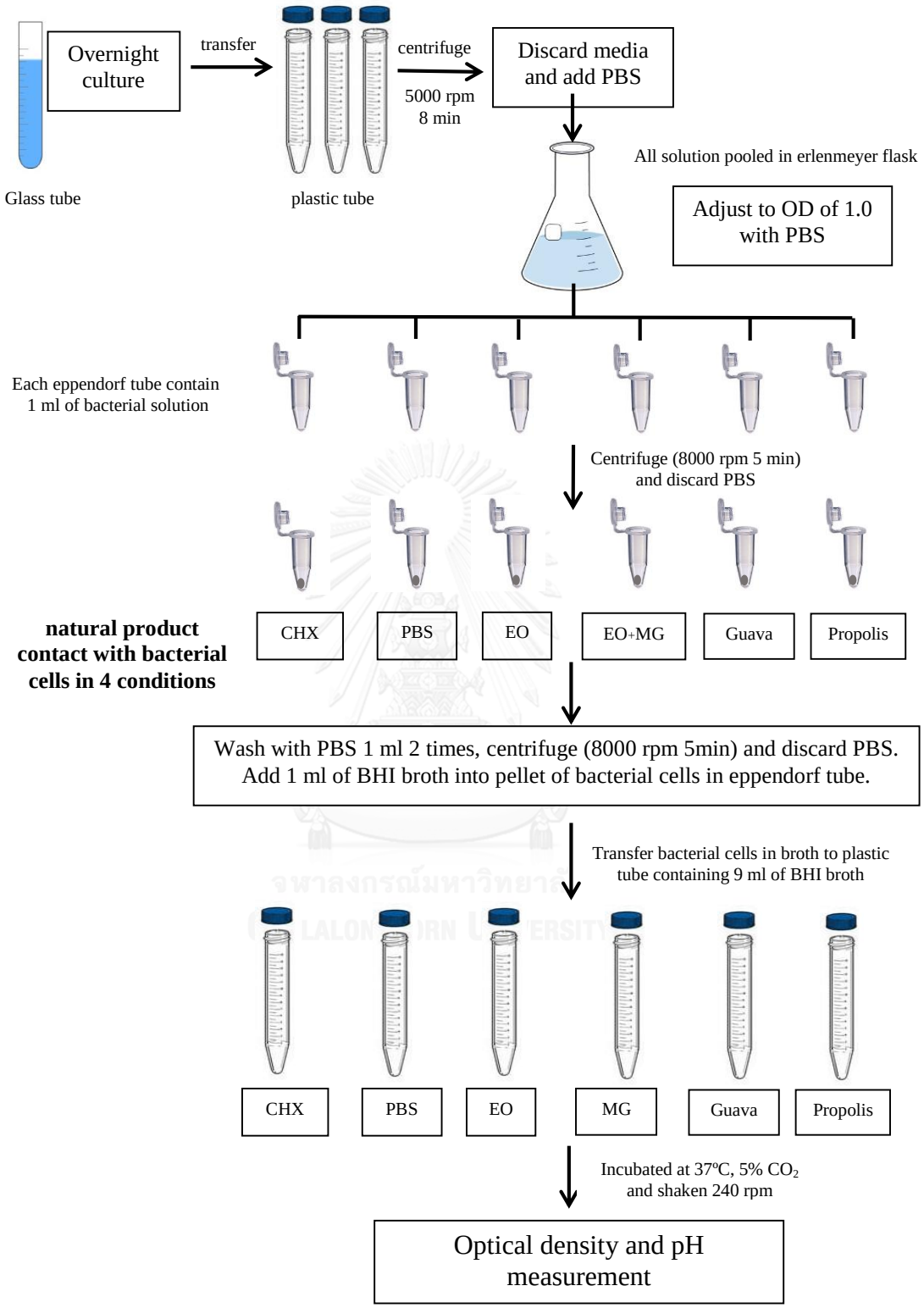


Figure 2 Experimental design of direct contact test; CHX = chlorhexidine (positive control), PBS = phosphate-buffered saline (negative control), EO = essesntial oil, MG = essential oil with mangosteen extract

Data analysis

One-way ANOVA test was chosen for evaluating the significance in each time point of all pairwise comparisons. All statistical analyses was performed using SPSS (version 17.0). The significance level will be set at 0.05

CHAPTER IV
RESULTS

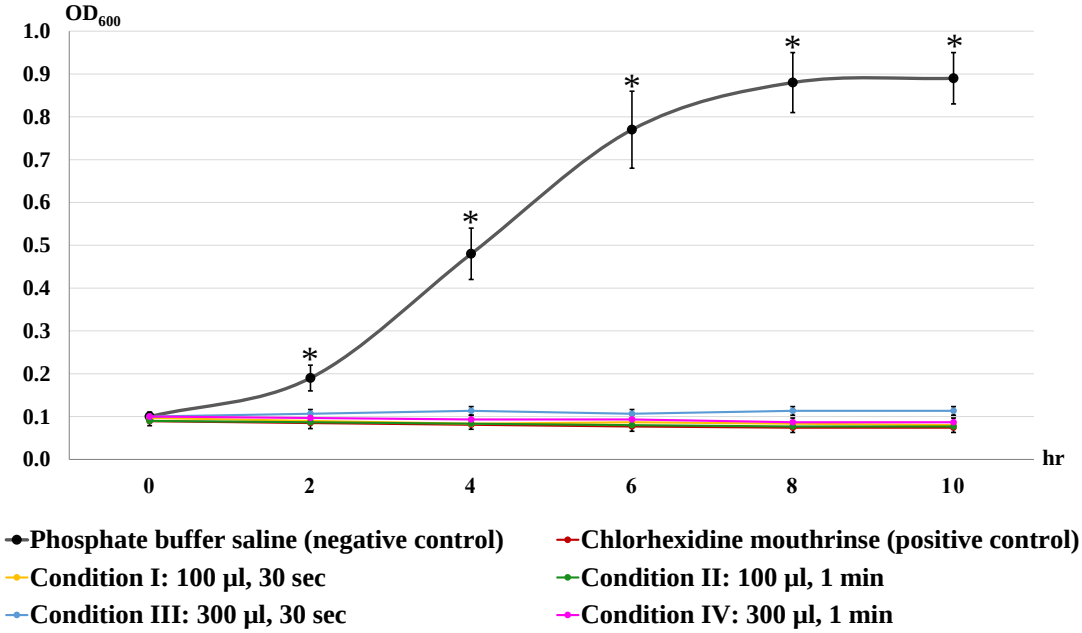
The effects of natural care oral product against *S. mutans*

The antibacterial effect of four oral care natural products against *S. mutans* was demonstrated in 4 different conditions *in vitro*. These condition are varied by the product concentration and contact time to bacterium. Inhibition of *S. mutans* growth after direct contact to the oral care natural product is shown in figure 3-6.

1) Essential oil (Myherbal mybacin trospray, Greater Pharma Co., Ltd.)
2) Essential oil with mangosteen extract (Myherbal mybacin trospray with mangosteen extract, Greater Pharma Co., Ltd.)
3) Propolis extract (oral spray T. man pharma Co., Ltd.)
4) Guava leaf extract (oral spray of Abhaibhubejhr brand)

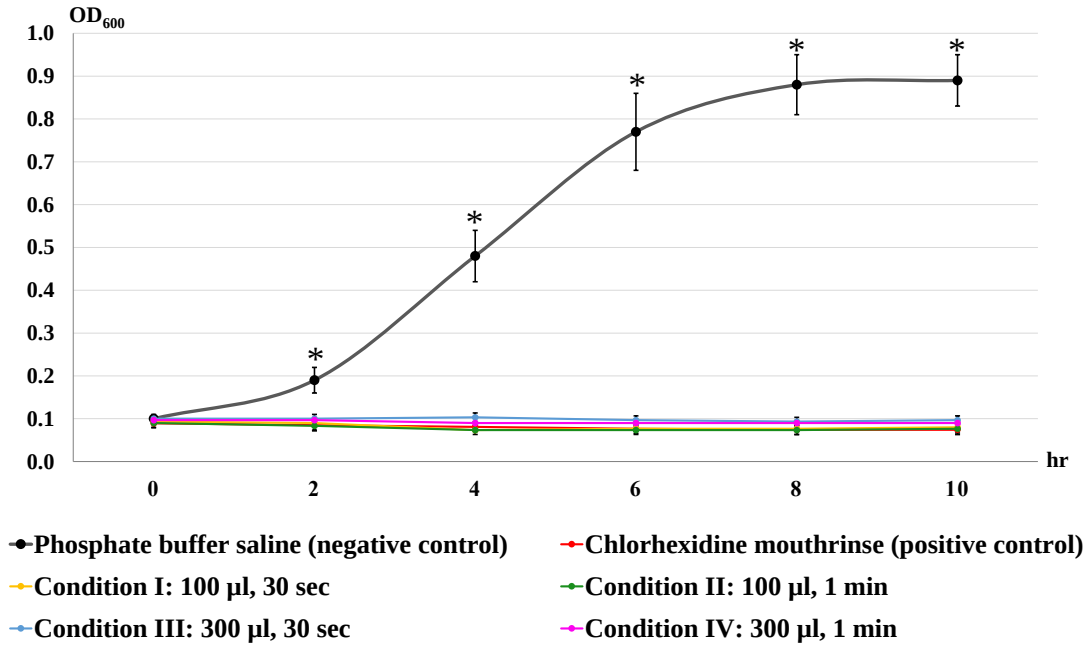
After *S. mutans* was exposed to PBS (negative control) and continued culture in growth medium, bacterial growth reflected by the increase of cell density in growth medium from 0.1±0.01 (mean±SD) OD_{600} or approximately 3 x 10^7 CFUs/mL to 0.88±0.07 (mean±SD) OD_{600} or approximately 2.6 x 10^8 CFUs/mL in 10 hr. By contrast, the optical density of *S. mutans* cultures after a direct contact to essential oil, essential oil with mangosteen extract and propolis extract oral spray, remained at 0.07±0.01, 0.09±0.01 and 0.09±0.01 (mean±SD) OD_{600} throughout 10 hr in culture, respectively. The growth inhibition was similar to the culture contacted with chlorhexidine mouthrinse, 0.07±0.01 (mean±SD) OD_{600}. This result showed that a direct contact to chlorhexidine mouthrinse, essential oil, essential oil with mangosteen extract and

propolis extract oral spray in all conditions significantly inhibited growth of *S. mutans* (p=0.001) (figure 3-5). Data of each time point were compared by one-way ANOVA test and followed by a Tukey post-test for multiple comparisons.



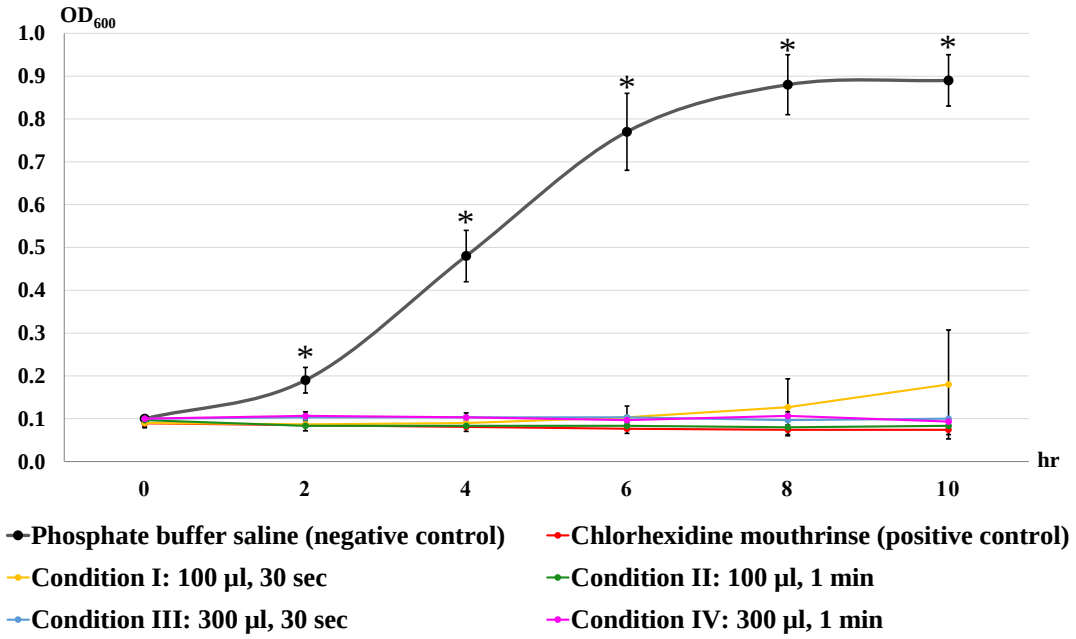
* OD in negative control group is significantly higher than other groups

Figure 3. Growth curve of *S. mutans* culture after treatment with essential oil oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of essential oil oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *S. mutans* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



* OD in negative control group is significantly higher than other groups

Figure 4. Growth curve of *S. mutans* culture after treatment with essential oil with mangosteen extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil with mangosteen extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of essential oil with mangosteen extract oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *S. mutans* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



* OD in negative control group is significantly higher than other groups

Figure 5. Growth curve of *S. mutans* culture after treatment with propolis extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and propolis extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of propolis extract oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *S. mutans* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.

For the treatment with guava leaf extract oral spray, *S. mutans* growth of each condition is shown in figure 6. *S. mutans* treated with 100 µl of spray for either 30 sec or 1 min continued to grow at the same way but in significant slower rate compared with PBS since 2 hr to 8 hr (p=0.01-0.001), data was analyzed in each time point by one-way ANOVA test and followed by a Tukey test. At 10 hr, the cells growth showed 0.83±0.02 and 0.81±0.01 (mean±SD) OD_{600} in condition of 100 µl exposed in 30 sec and 1 min respectively, similar to that achieved by negative control group. This result showed that 100 µl of guava leaf extract oral spray had significant antibacterial effect against *S. mutans* especially at 2 to 8 hr, but not cover to 10 hr. The same as the direct contact to 300 µl of oral spray with guava extract for 30 sec that cells growth was inhibited only 2-8 hr, at 10 hr, *S. mutans* cultures showed 0.76±0.01 (mean±SD) OD_{600} which was no significant difference with PBS (p=0.79). However, when the contact time was longer, 1 min, the growth of *S. mutans* was significantly affected throughout the experiment (p=0.001).

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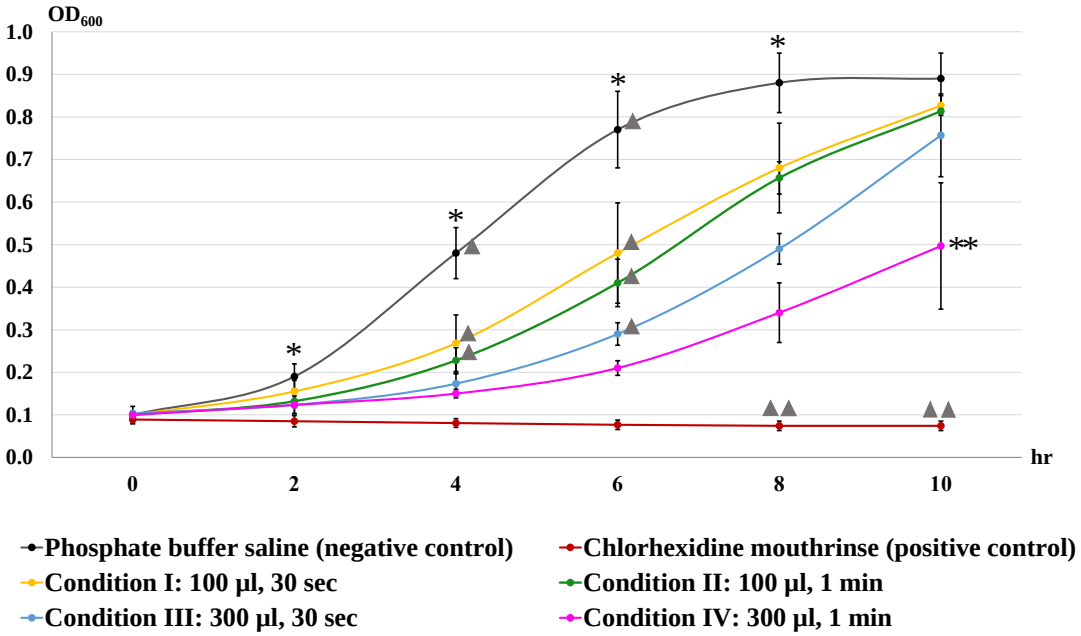
This antibacterial effect did not performed well through the experiment as chlorhexidine mouthrinse. At 2 hr, there was no significant findings in growth inhibition compared to chlorhexidine mouthrinse in all condition of guava leaf extract oral spray. At 4 hr, *S. mutans* which exposed to condition III and IV was inhibited equally to chlorhexidine mouthrinse, however, there were no significant difference in condition I and II. At 6 hr, only *S. mutans* which exposed to condition IV was inhibited growth equally to chlorhexidine mouthrinse. Then lastly at 8–10 hr, no significant difference were shown in all conditions, data was analyzed in each time point by one-way ANOVA and Tukey test.



* OD in negative control group is significantly higher than other groups

** OD in condition IV is significantly lower than negative control and other conditions

▲ OD in conditions that mark with this sign are significantly higher than positive control

▲▲OD in positive control group is significantly lower than other groups

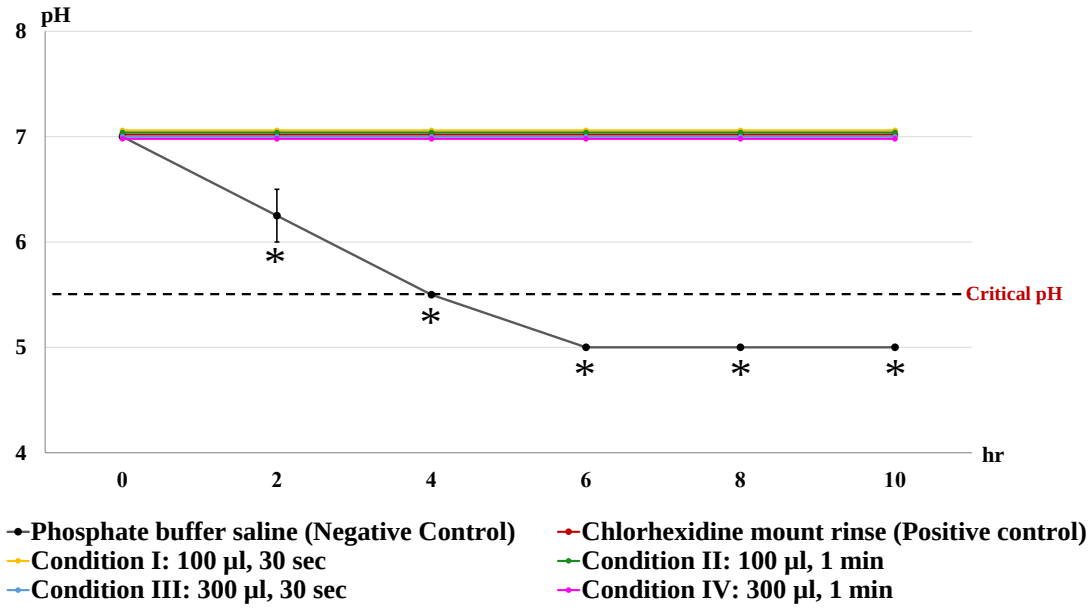
Figure 6. Growth curve of *S. mutans* culture after treatment with guava leaf extract. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and guava leaf extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. There is significant growth inhibition of *S. mutans* which exposed to 100 µl of guava leaf extract oral spray either 30 sec or 1 min (yellow and green respectively) at 2 to 8 hr, but not cover to 10 hr, analyzed data in each time point and condition by one-way ANOVA and Tukey test. The same as the direct contact to 300 µl of oral spray with guava leaf extract for 30 sec that cells growth is also inhibited only 2-8 hr (blue). However, when the contact time is longer, 1 min,

the growth of *S. mutans* is significantly affected throughout the experiment (p=0.001) as shown in pink line.

Culture pH measurement was correspond to the growth curve. pH measured from *S. mutans* cultures in PBS group was reduced from 7 to 5 within 10 hr. On the other hand, pH of *S. mutans* cultures contacted to essential oil, essential oil with mangosteen extract, propolis extract oral spray or chlorhexidine mouthrinse was maintained at pH 7 throughout the experiment (figure 7-10) which significantly higher than pH of PBS group (p=0.001) analyzed by one-way ANOVA test and followed by a Tukey post-test for multiple comparisons. These findings suggested that even if there were different conditions in concentrations and contact times, essential oil, essential oil with mangosteen extract, propolis extract oral spray and chlorhexidine mouthrinse not only affected the growth but also inhibited acid production of *S. mutans*.

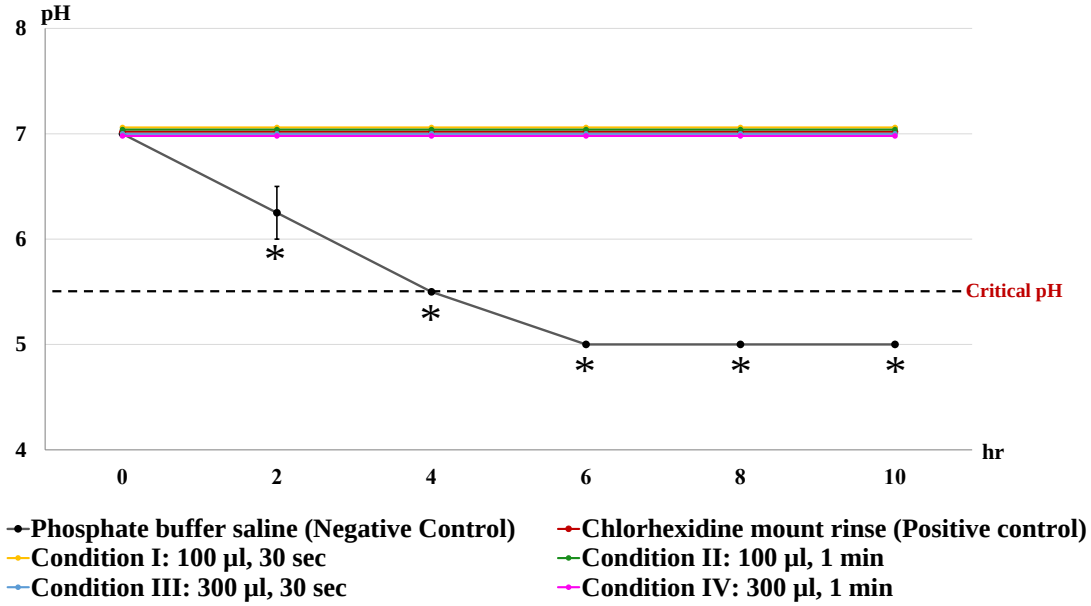
For guava leaf extract oral spray treatment, acid productions of *S. mutans* in all condition was delayed but not comparable to chlorhexidine mouthrinse (p=0.001) from beginning to the end of this experiment as shown in figure 10. Acid production of *S. mutans* exposed to 100 µl of spray was significantly developed slower than PBS at 4-6 hr (p=0.001). However, finally the culture pH were 5 which was equal to PBS group (p=1.00), data was analyzed in each time point by one-way ANOVA test and followed by a Tukey post-test for multiple comparisons. Apart from the previous condition, *S. mutans* treated with 300 µl of guava leaf extract oral spray for either 30 sec or 1 min, acid production was significantly delayed since 4 hr through the end of the experiment (p=0.001), data was analyzed by one-way ANOVA and Tukey test. The pH of both groups were stopped at pH ~5.5 which were not below the critical pH of

demineralization. As the result, the effect on acid production of *S. mutans* after exposed to guava leaf extract oral spray depend on variation of concentration and contact time.



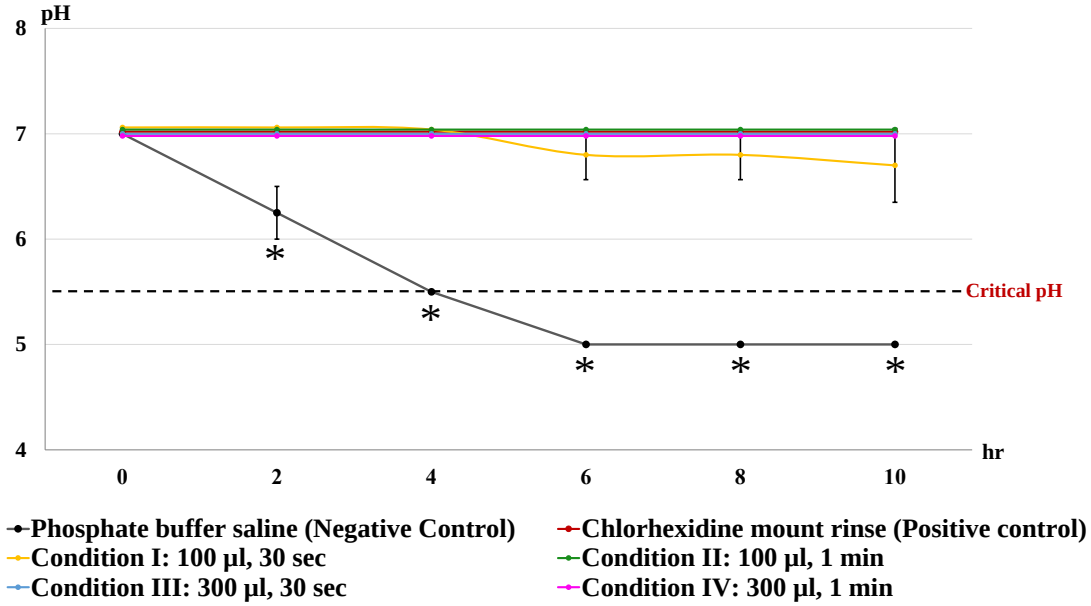
* pH culture in negative control group is significantly lower than other groups

Figure 7. pH of *S. mutans* culture after treatment with essential oil oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *S. mutans* cultures contacted to essential oil oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).



* pH culture in negative control group is significantly lower than other groups

Figure 8. pH of *S. mutans* culture after treatment with essential oil with mangosteen extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil with mangosteen extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *S. mutans* cultures contacted to essential oil with mangosteen extract oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).



* pH culture in negative control group is significantly lower than other groups

Figure 9. pH of *S. mutans* culture after treatment with propolis extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and propolis extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *S. mutans* cultures contacted to propolis extract oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).

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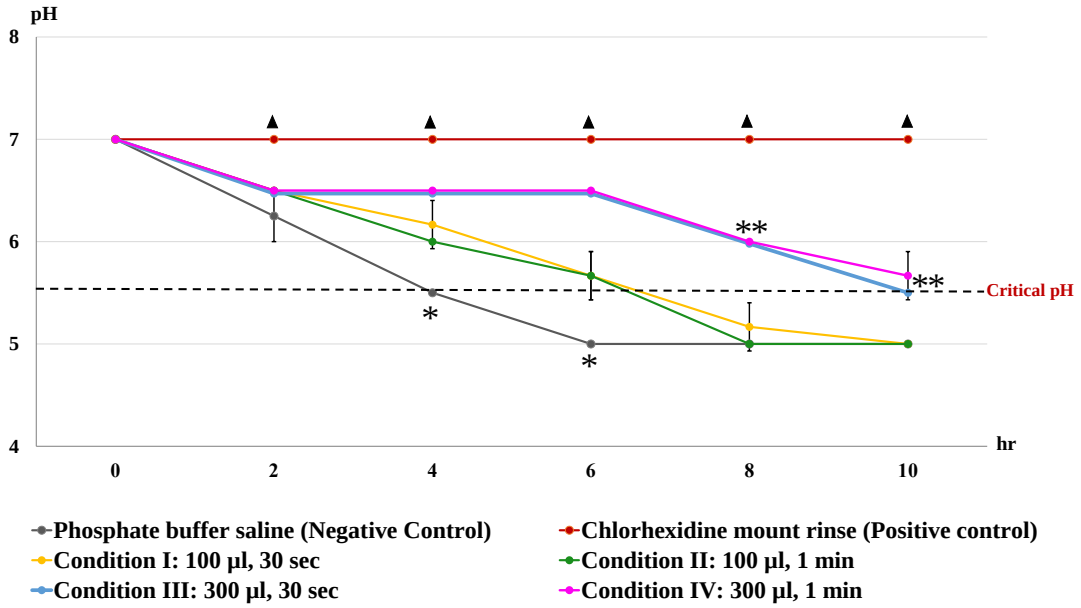


▲ pH culture in positive control group is significantly higher than other groups

* pH culture in negative control group is significantly lower than other groups

** pH culture in condition III and IV are significantly higher than negative control and condition I and II

Figure 10. pH of *S. mutans* culture after treatment with guava leaf extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and guava leaf extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that acid productions of *S. mutans* in all condition was delayed but not comparable to chlorhexidine mouthrinse (p=0.001) throughout the experiment. Acid production of *S. mutans* exposed to 100 µl of spray was significantly developed slower than PBS at 4-6 hr (p=0.001). However, finally the culture pH were 5 which was equal to PBS group (p=1.000) On the other hand, acid production of *S. mutans* treated with 300 µl of guava leaf extract oral spray

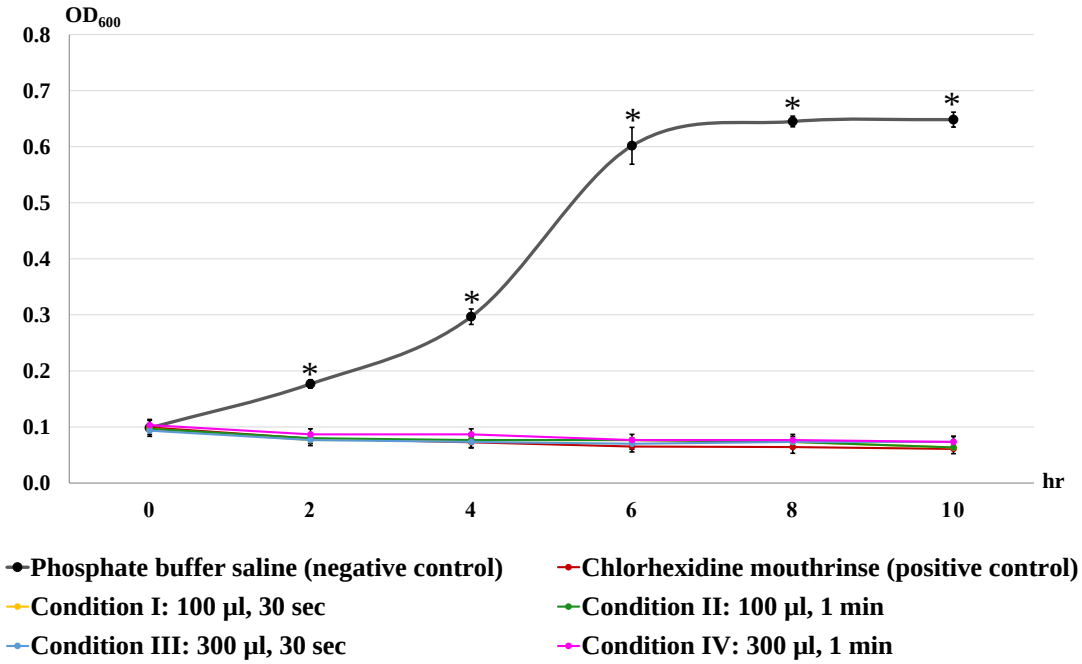
was significantly delayed since 4 hr through the end of the experiment (p=0.001), data was analyzed by one-way ANOVA and Tukey test.

The effects of natural care oral product against *L. casei*

After *L. casei* was exposed to PBS (negative control) and the culture was continued in growth medium, bacterial growth reflected by the increase of cell density in growth medium from 0.1±0.01 (mean±SD) OD_{600} or approximately 2 x 10^5 CFUs/mL to 0.69±0.01 (mean±SD) OD_{600} or approximately 1.4 x 10^6 CFUs/mL in 10 hr. By contrast, the optical density of *L. casei* cultures after a direct contact to essential oil, essential oil with mangosteen extract, propolis extract and guava leaf extract oral spray, remained at 0.07±0.01, 0.08±0.01, 0.1±0.01 and 0.07±0.01 (mean±SD) OD_{600} throughout 10 hr in culture, respectively. The growth inhibition was similar to the culture contacted with chlorhexidine mouthrinse, 0.06±0.01 (mean±SD) OD_{600}. This result showed that a direct contact to chlorhexidine mouthrinse, essential oil, essential oil with mangosteen extract, propolis extract and guava leaf extract oral spray in all conditions significantly inhibited growth of *L. casei* (p=0.001-0.04) (figure 11-14). Data of each time point were compared by one-way ANOVA test and followed by a Tukey post-test for multiple comparisons.

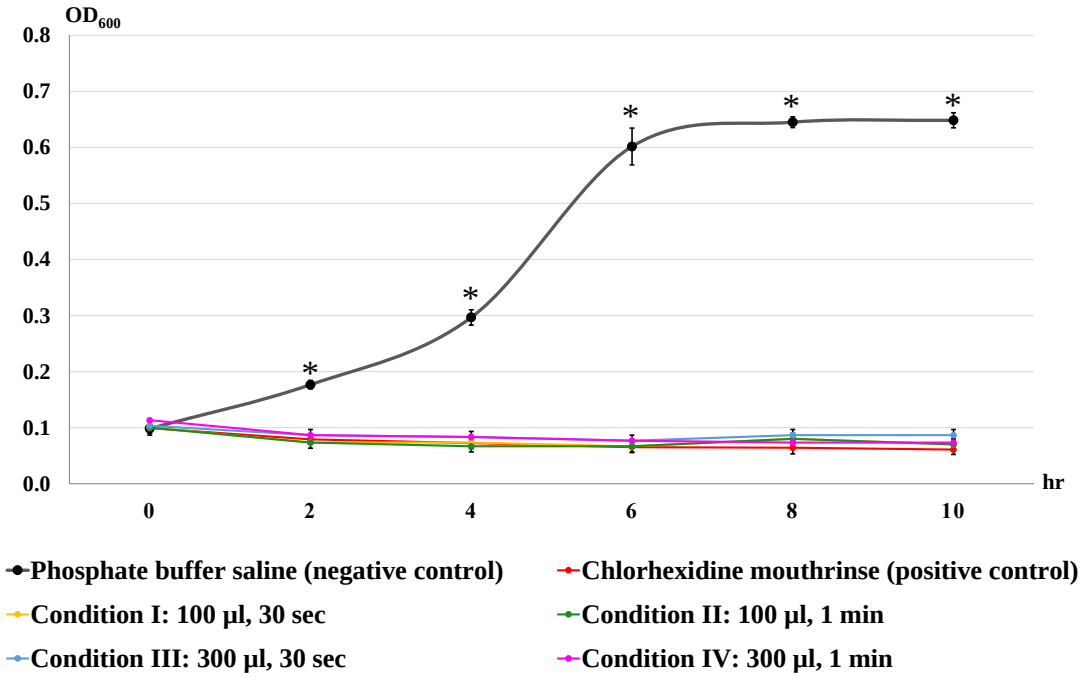
pH measured from *L. casei* cultures in PBS group was reduced from 7 to 5 within 10 hr. On the other hand, pH of *L. casei* cultures contacted to essential oil, essential oil with mangosteen extract, propolis extract, guava leaf extract oral spray or chlorhexidine mouthrinse was maintained at pH 7 throughout the experiment (figure 15-18) which significantly higher than pH culture of PBS group (p=0.001), data was analyzed by one-way ANOVA test and followed by a Tukey post-test for multiple comparisons. These findings suggested that even if there were different conditions in concentrations and contact times, essential oil, essential oil with mangosteen extract,

propolis extract, guava leaf extract oral spray and chlorhexidine mouthrinse not only affected the growth but also inhibited acid production of *L. casei*.



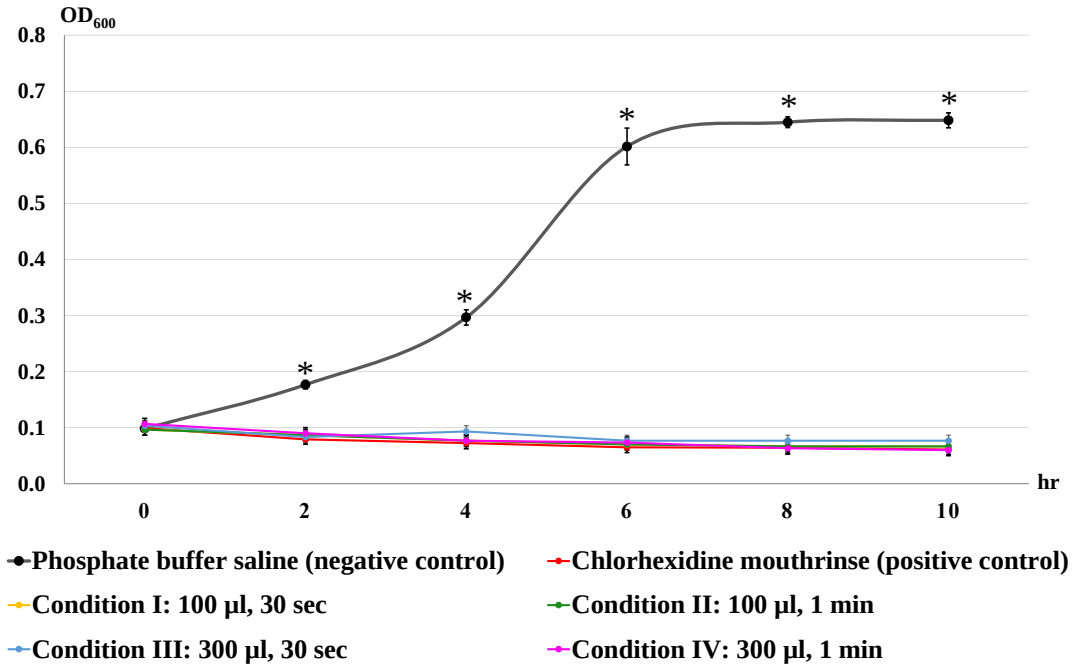
* OD in negative control group is significantly higher than other groups

Figure 11. Growth curve of *L. casei* culture after treatment with essential oil oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of essential oil oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *L. casei* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



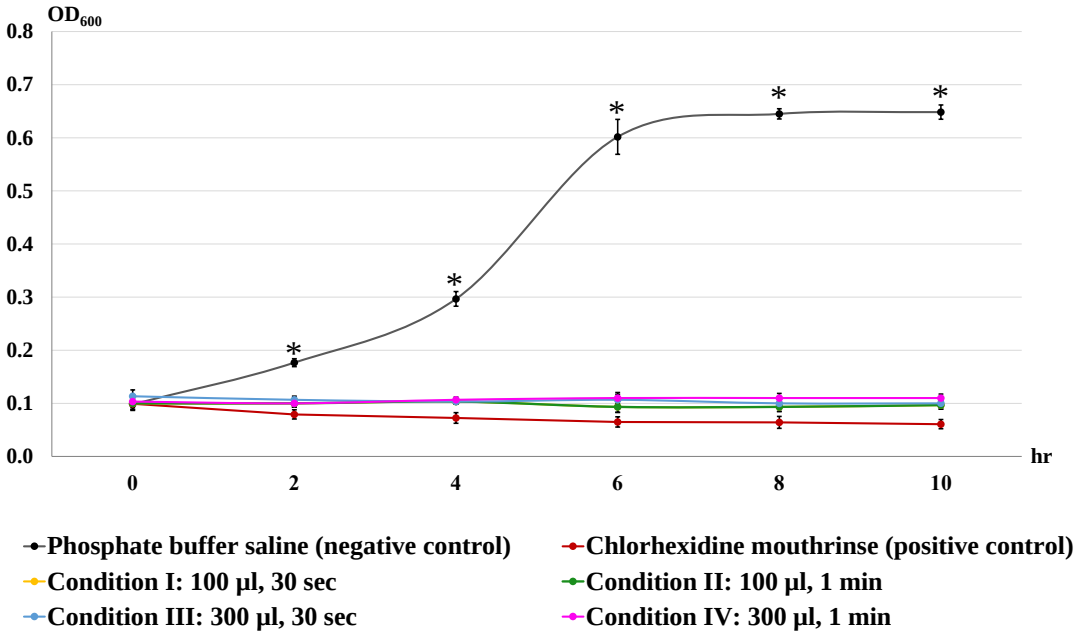
* OD in negative control group is significantly higher than other groups

Figure 12. Growth curve of *L. casei* culture after treatment with essential oil with mangosteen extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil with mangosteen extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of essential oil with mangosteen extract oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *L. casei* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



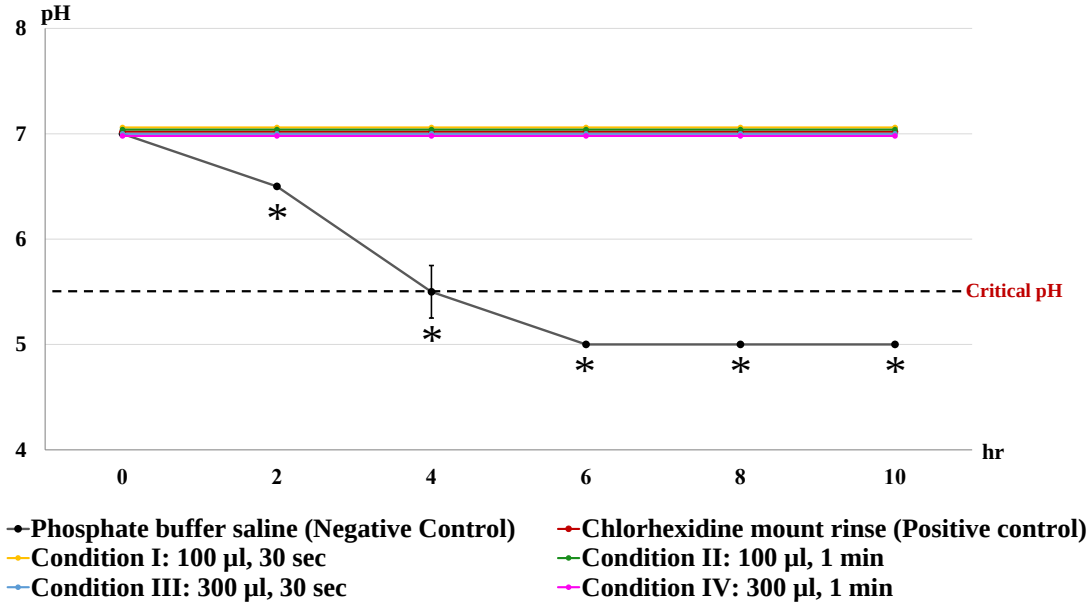
* OD in negative control group is significantly higher than other groups

Figure 13. Growth curve of *L. casei* culture after treatment with propolis extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and propolis oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of propolis oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *L. casei* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



* OD in negative control group is significantly higher than other groups

Figure 14. Growth curve of *L. casei* culture after treatment with guava leaf extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and guava leaf extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. Growth curves show that all conditions of guava leaf extract oral spray have similar effect to chlorhexidine mouthrinse which significantly inhibit growth of *L. casei* (p=0.001) when compared with PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test.



* pH culture in negative control group is significantly lower than other groups

Figure 15. pH of *L. casei* culture after treatment with essential oil oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *L. casei* cultures contacted to essential oil oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).

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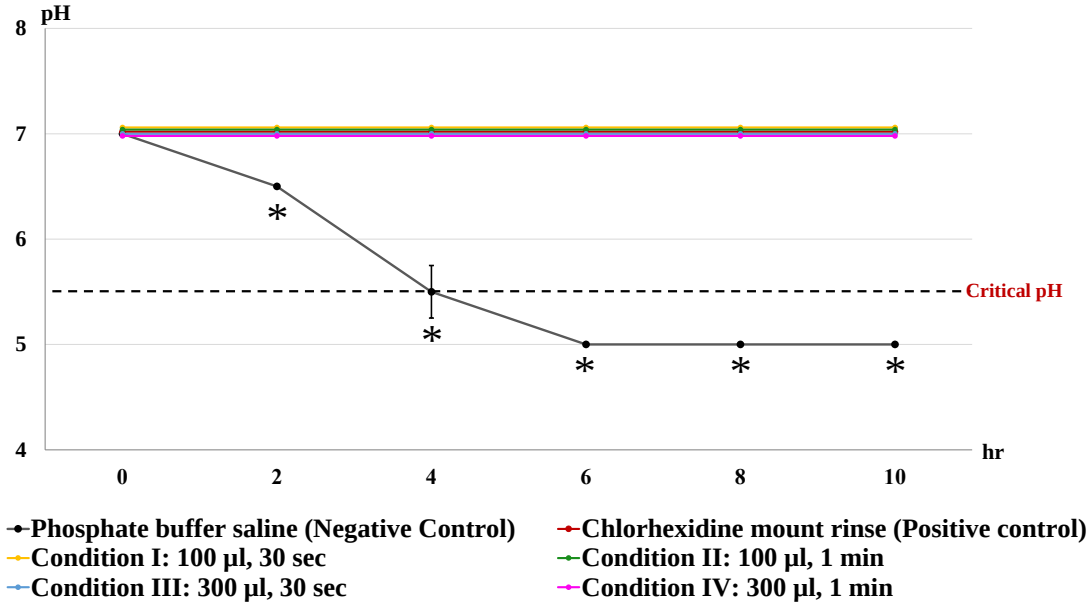
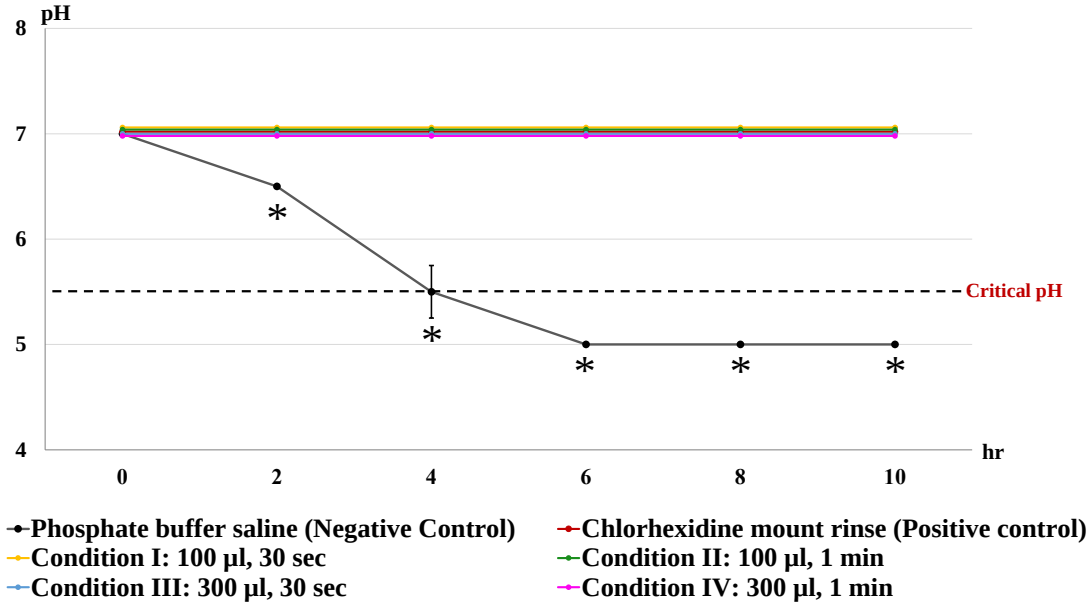


* pH culture in negative control group is significantly lower than other groups

Figure 16. pH of *L. casei* culture after treatment with essential oil with mangosteen extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and essential oil with mangosteen extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *L. casei* cultures contacted to essential oil with mangosteen extract oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).



* pH culture in negative control group is significantly lower than other groups

Figure 17. pH of *L. casei* culture after treatment with propolis extract oral spray. At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and propolis extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *L. casei* cultures contacted to propolis extract oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).

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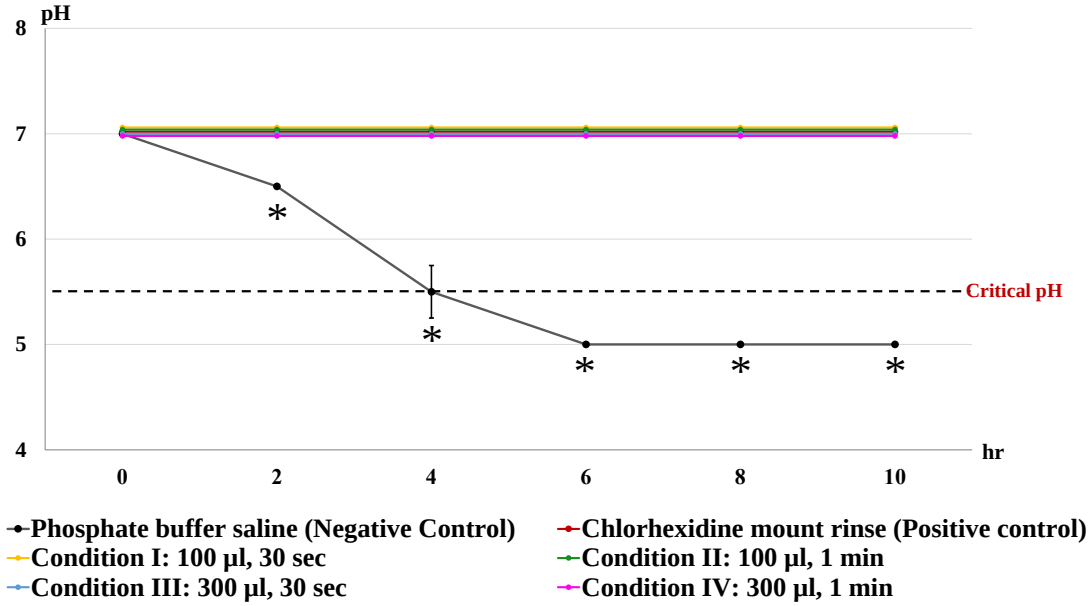

* pH culture in negative control group is significantly lower than other groups

Figure 18. pH of *L. casei* culture after treatment with guava leaf extract oral spray.

At 0 hr, each culture was adjusted to 0.1 OD_{600} after direct contact to PBS, chlorhexidine mouthrinse and guava leaf extract oral spray. The experiment performed measurement every 2 hr for up to 10 hr. One of three independent experiments is shown. Error bars demonstrate standard deviation. The results show that pH of *L. casei* cultures contacted to propolis extract oral spray in all 4 conditions are comparable to chlorhexidine mouthrinse and significantly higher than pH of PBS group (black) analyzed in each time point by one-way ANOVA and Tukey test (p=0.001).

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CHAPTER IV
DISCUSSION AND CONCLUSIONS

This study demonstrates that oral sprays containing essential oils can inhibit the growth of oral pathogens after a direct contact in *in vitro*., The results are consistent with the effect of purified active ingredients or crude extracts reported in previous studies.(13) Variety of essential oils e.g. peppermint oil, eucalyptus oil and menthol which used as a component of oral sprays in this study contained the antibacterial activity against *S. mutans* and *L. casei*, possibly through their isolated constituents such as the sesquiterpene (E, E)-farnesol which is the major compound against *S. mutans*.(25)

The second oral spray used in this study was the mangosteen extract oral spray. Many *in vitro* studies demonstrated non-toxic property and inhibitory effect of mangosteen pericarp crude extract on *S. mutans* growth.(18-21) When oral spray containing magosteen extract had been tested, not only the growth of *S. mutans* was inhibited, but also the inhibitory effect against *L. casei*, the pioneer organism in the progression of the dentine caries.(2, 3, 5, 39)

This study also demonstrated that antibacterial effect against *S. mutans* in oral spray containing propolis was similar to propolis crude extract. According to the study of Koo et al(25), this effect was the result from several compounds in propolis that could inhibit streptococcal glucosyltransferase activities which is the virulence factors in the pathogenesis of dental caries. However, the antibacterial effect of these oral sprays were not derived from only the crude extract because there are other ingredients

that can alter the properties of natural extracts which are claimed as the main ingredient in products.

Apart from the bacterial growth inhibition, acid production of *S. mutans* and *L.casei* after contacted with natural oral sprays is investigated in this study. The the culture pH measurement corresponded with the bacterial growth curves. While optical density showed inhibition of bacterial growth in the group that *S. mutans* or *L. casei* had directed contact with oral sprays consisting of essential oil or essential oil with mangosteen or propolis extract, culture pH maintained at pH 7 from throughout 10-hr culture. This experiment revealed the same effect as the study of Lee that essential oil isolated from turmeric significantly inhibited the growth of *S. mutans* and the pH decrease was substantially inhibited at also close to pH 7.(53)

Recent studies demonstrated the antibacterial effect of natural extract on bacterial growth inhibition by disk diffusion or agar diffusion method. These natural extracts effectively function equally to chlorhexidine mouthrinse which is the gold standard of antiplaque chemical agent.(8, 42) Agar diffusion method is not used in this study because the limited ability of oil diffusion on agar is considered. Nonetheless, the results consistently demonstrated that antibacterial property of oral sprays consisting of essential oils or essential oils with mangosteen extract or propolis extract was comparable to chlorhexidine mouthrinse. Since the use of chlorhexidine mouthrinse may be denied because of its bitter tastes or bacterial-resistant in long-term chlorhexidine using. Therefore, these natural products may be beneficial an alternative choice in patients with special needs.

When the essential oil oral spray with and without mangosteen extract was compared, no additional antibacterial effect is detected in the product with mangosteens

extracts. Whether mangosteen extract has the synergistic antibacterial effect when combined with essential oil in oral spray cannot be concluded from the experiments in this study.

The antibacterial effect of guava leaf extract oral spray appeared more potent against *L. casei* in this study. While guava leaf extract oral spray only inhibited the growth of *S.mutans* in some tested conditions, and the most effective antibacterial effect was with the highest concentration and contact time (300 µl and 1 min). Since the overnight cultures of *L. casei* at 0.1 OD_{600} demonstrated ten times fewer number of *L. casei* than the cultures of *S. mutans* at 0.1 OD_{600}, partial inhibition effect in *S. mutans* cultures may be related to the number of cells at the time in direct contact with guava leaf extract oral spray.

The oral sprays were recommended by its manufacturers to be used as often as preferable. However, the effect of long term use of broad-spectrum antibacterial agent and the disturbance of normal flora cannot be neglected.(34) The further study should also determine whether its antimicrobial activity remains potent in dental biofilm. Because biofilms present in the oral cavity are complex community comprising more than 700 strains of microbial anchored to enamel and microorganisms remaining in the biofilm structure are more resistant to antibiotics than those in the planktonic condition.(54, 55) Moreover, future research should expand to the gram-negative bacteria which is strongly associated with chronic periodontitis. The different cell wall structure; gram-negative bacteria has more complex cell wall than gram-positive bacteria, may give the different in result on antibacterial effectiveness of natural spray.(56, 57)

Conclusions

The essential oil, essential oil with mangosteen extract and propolis extract oral spray show antibacterial effect against *S. mutans* and *L. casei* equally to 0.02% chlorhexidine mouthrinse, in all tested conditions *in vitro*. However, synergistic effect of mangosteen extract when combined to essential oil oral spray was inconclusive. When guava leaf extract oral spray was tested, growth and acid production of *L. casei* was totally inhibited in all tested conditions *in vitro*. By contrast, guava leaf extract oral spray can inhibit *S.mutans* growth and acid production in some tested conditions.

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จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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Statistical analysis of essential oil oral spray against *S. mutans*
(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	2.313	.068
	Within Groups	.001	30	.000		
	Total	.002	35			
hr2	Between Groups	.194	5	.039	32.072	.000
	Within Groups	.036	30	.001		
	Total	.230	35			
hr4	Between Groups	1.729	5	.346	72.500	.000
	Within Groups	.143	30	.005		
	Total	1.873	35			
hr6	Between Groups	4.073	5	.815	210.520	.000
	Within Groups	.116	30	.004		
	Total	4.189	35			
hr8	Between Groups	5.023	5	1.005	412.567	.000
	Within Groups	.073	30	.002		
	Total	5.096	35			
hr10	Between Groups	5.081	5	1.016	442.758	.000
	Within Groups	.069	30	.002		
	Total	5.150	35			

Statistical analysis of essential oil with mangosteen extract oral spray against *S. mutans* (Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	1.497	.220
	Within Groups	.001	30	.000		
	Total	.002	35			
hr2	Between Groups	.193	5	.039	31.519	.000
	Within Groups	.037	30	.001		
	Total	.230	35			
hr4	Between Groups	1.760	5	.352	73.603	.000
	Within Groups	.143	30	.005		
	Total	1.903	35			
hr6	Between Groups	4.115	5	.823	211.951	.000
	Within Groups	.116	30	.004		
	Total	4.232	35			
hr8	Between Groups	5.063	5	1.013	412.828	.000
	Within Groups	.074	30	.002		
	Total	5.136	35			
hr10	Between Groups	5.100	5	1.020	440.998	.000
	Within Groups	.069	30	.002		
	Total	5.170	35			

Statistical analysis of propolis extract oral spray against *S. mutans*
(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	1.988	.109
	Within Groups	.001	30	.000		
	Total	.002	35			
hr2	Between Groups	.194	5	.039	31.925	.000
	Within Groups	.036	30	.001		
	Total	.231	35			
hr4	Between Groups	1.723	5	.345	72.047	.000
	Within Groups	.143	30	.005		
	Total	1.866	35			
hr6	Between Groups	4.045	5	.809	205.079	.000
	Within Groups	.118	30	.004		
	Total	4.164	35			
hr8	Between Groups	4.949	5	.990	342.919	.000
	Within Groups	.087	30	.003		
	Total	5.035	35			
hr10	Between Groups	4.947	5	.989	252.400	.000
	Within Groups	.118	30	.004		
	Total	5.064	35			

Statistical analysis of guava leaf extract oral spray against *S. mutans*
(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.001	5	.000	1.868	.130
	Within Groups	.002	30	.000		
	Total	.003	35			
hr2	Between Groups	.163	5	.033	24.232	.000
	Within Groups	.040	30	.001		
	Total	.204	35			
hr4	Between Groups	1.455	5	.291	56.119	.000
	Within Groups	.156	30	.005		
	Total	1.611	35			
hr6	Between Groups	3.313	5	.663	131.035	.000
	Within Groups	.152	30	.005		
	Total	3.465	35			
hr8	Between Groups	4.104	5	.821	223.322	.000
	Within Groups	.110	30	.004		
	Total	4.214	35			
hr10	Between Groups	4.582	5	.916	207.149	.000
	Within Groups	.133	30	.004		
	Total	4.715	35			

Statistical analysis of essential oil and essential oil with mangosteen extract oral spray against *S. mutans* (pH measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.000	1.000
	Within Groups	.002	30	.000		
	Total	.002	35			
hr2	Between Groups	4.500	5	.900	35.923	.000
	Within Groups	.752	30	.025		
	Total	5.252	35			
hr4	Between Groups	18.000	5	3.600	67500.000	.000
	Within Groups	.002	30	.000		
	Total	18.002	35			
hr6	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			
hr8	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			
hr10	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			

Statistical analysis of propolis extract oral spray against *S. mutans*
(pH measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.000	1.000
	Within Groups	.002	30	.000		
	Total	.002	35			
hr2	Between Groups	4.500	5	.900	35.923	.000
	Within Groups	.752	30	.025		
	Total	5.252	35			
hr4	Between Groups	18.000	5	3.600	67500.000	.000
	Within Groups	.002	30	.000		
	Total	18.002	35			
hr6	Between Groups	31.410	5	6.282	1119.998	.000
	Within Groups	.168	30	.006		
	Total	31.578	35			
hr8	Between Groups	31.410	5	6.282	1119.998	.000
	Within Groups	.168	30	.006		
	Total	31.578	35			
hr10	Between Groups	30.972	5	6.194	278.083	.000
	Within Groups	.668	30	.022		
	Total	31.640	35			

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Statistical analysis of guava leaf extract oral spray against *S. mutans*
(pH measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.000	1.000
	Within Groups	.002	30	.000		
	Total	.002	35			
hr2	Between Groups	3.500	5	.700	28.000	.000
	Within Groups	.750	30	.025		
	Total	4.250	35			
hr4	Between Groups	14.076	5	2.815	506.750	.000
	Within Groups	.167	30	.006		
	Total	14.243	35			
hr6	Between Groups	26.139	5	5.228	470.500	.000
	Within Groups	.333	30	.011		
	Total	26.472	35			
hr8	Between Groups	28.243	5	5.649	1016.750	.000
	Within Groups	.167	30	.006		
	Total	28.410	35			
hr10	Between Groups	29.076	5	5.815	1046.750	.000
	Within Groups	.167	30	.006		
	Total	29.243	35			

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Statistical analysis of essential oil oral spray against *L.casei*

(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.512	.765
	Within Groups	.003	24	.000		
	Total	.003	29			
hr2	Between Groups	.051	5	.010	8.279	.000
	Within Groups	.030	24	.001		
	Total	.081	29			
hr4	Between Groups	.324	5	.065	5.564	.002
	Within Groups	.279	24	.012		
	Total	.603	29			
hr6	Between Groups	1.228	5	.246	7.807	.000
	Within Groups	.755	24	.031		
	Total	1.983	29			
hr8	Between Groups	1.358	5	.272	7.334	.000
	Within Groups	.889	24	.037		
	Total	2.247	29			
hr10	Between Groups	1.417	5	.283	7.602	.000
	Within Groups	.895	24	.037		
	Total	2.312	29			

Statistical analysis of essential oil with mangosteen extract oral spray against *L.casei* (Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.745	.597
	Within Groups	.003	24	.000		
	Total	.003	29			
hr2	Between Groups	.051	5	.010	8.315	.000
	Within Groups	.030	24	.001		
	Total	.081	29			
hr4	Between Groups	.328	5	.066	5.633	.001
	Within Groups	.279	24	.012		
	Total	.607	29			
hr6	Between Groups	1.241	5	.248	7.897	.000
	Within Groups	.755	24	.031		
	Total	1.996	29			
hr8	Between Groups	1.335	5	.267	7.203	.000
	Within Groups	.889	24	.037		
	Total	2.224	29			
hr10	Between Groups	1.389	5	.278	7.450	.000
	Within Groups	.895	24	.037		
	Total	2.284	29			

Statistical analysis of propolis extract oral spray against *L.casei*
(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.534	.748
	Within Groups	.002	24	.000		
	Total	.003	29			
hr2	Between Groups	.046	5	.009	7.411	.000
	Within Groups	.030	24	.001		
	Total	.076	29			
hr4	Between Groups	.319	5	.064	5.477	.002
	Within Groups	.280	24	.012		
	Total	.599	29			
hr6	Between Groups	1.238	5	.248	7.876	.000
	Within Groups	.754	24	.031		
	Total	1.992	29			
hr8	Between Groups	1.383	5	.277	7.468	.000
	Within Groups	.889	24	.037		
	Total	2.272	29			
hr10	Between Groups	1.421	5	.284	7.622	.000
	Within Groups	.895	24	.037		
	Total	2.316	29			

Statistical analysis of guava leaf extract oral spray against *L.casei*
(Optical density measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.612	.691
	Within Groups	.003	24	.000		
	Total	.004	29			
hr2	Between Groups	.037	5	.007	6.000	.001
	Within Groups	.030	24	.001		
	Total	.067	29			
hr4	Between Groups	.274	5	.055	4.718	.004
	Within Groups	.279	24	.012		
	Total	.554	29			
hr6	Between Groups	1.132	5	.226	7.199	.000
	Within Groups	.755	24	.031		
	Total	1.887	29			
hr8	Between Groups	1.259	5	.252	6.797	.000
	Within Groups	.889	24	.037		
	Total	2.148	29			
hr10	Between Groups	1.286	5	.257	6.897	.000
	Within Groups	.895	24	.037		
	Total	2.181	29			

Statistical analysis of essential oil, essential oil with mangosteen extract, propolis extract and guava leaf extract oral spray against *L.casei* (pH measurement)

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
hr0	Between Groups	.000	5	.000	.000	1.000
	Within Groups	.002	30	.000		
	Total	.002	35			
hr2	Between Groups	4.500	5	.900	35.923	.000
	Within Groups	.752	30	.025		
	Total	5.252	35			
hr4	Between Groups	18.000	5	3.600	67500.000	.000
	Within Groups	.002	30	.000		
	Total	18.002	35			
hr6	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			
hr8	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			
hr10	Between Groups	32.000	5	6.400	120000.000	.000
	Within Groups	.002	30	.000		
	Total	32.002	35			

VITA

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