Original Article

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Antimicrobial activity of neem toothpaste against caries-associated microorganisms

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Objective: The aim of this study was to evaluate the antimicrobial potential of neem toothpaste against various oral microorganisms.

Materials and Methods: Toothpaste containing chloroform extract of neem leaf was prepared by TISTR, Ministry of Science and Technology, Thailand. Two commercial herbal toothpastes were selected for assessment of their antimicrobial activities compared to neem toothpaste. The toothpaste solution was prepared by adding 0.66 g of toothpaste to 1 mL of sterile deionizing water and mixed thoroughly. The suspensions of *Streptococcus mutans* KPSK$_2$, *Lactobacillus casei* ATCC 393, and *Candida albicans* ATCC 10231 were prepared and added to toothpaste solutions. Subsequent to 50 sec, the final solutions were brought to D/E neutralizing broth and cultured on Brain Heart Infusion agar, Rogosa agar and Sabouraud dextrose agar for the quantitation of *S. mutans, L. casei* and *C. albicans*, respectively at 37 °C for 24-48 h. A control was performed using sterile distilled water. The number of colonies was counted after incubation and expressed as colony forming unit (CFU)/ml. A statistical analysis was done by Kruskal-Wallis and Mann-Whitney tests.

Results: The result showed that all three herbal toothpastes had a significant suppressive effect against tested microorganisms compared with a control. Neem toothpaste was the most active against *L. casei* and *C. albicans* whereas other commercial toothpaste, tea tree oil toothpaste, was the most active against *S. mutans* and *L. casei*.

Conclusion: Information from the present study is scientific evidence to demonstrate that neem toothpaste could be selected as natural antimicrobial toothpaste for the prevention of caries-associated microorganisms. With appropriate tooth brushing, it is a simple way to gain good oral and dental health.

Keywords: Neem toothpaste, *Streptococcus mutans* KPSK$_2$, *Lactobacillus casei*, *Candida albicans*

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Introduction

Dental caries is a chronic disease that has widely affected many populations all over the world. It is accepted to be the second most common infectious diseases affecting human' health [1]. The disease arises as a result of acid producing bacteria such as *Streptococcus mutans* and *Lactobacillus* spp. in the oral biofilm or dental plaque on the tooth surface that metabolize fermentable carbohydrates leading to demineralization and the destruction of the tooth [2, 3]. *S. mutans* can synthesize a range of extracellular polysaccharides, including water-soluble and non-water-soluble glucan and fructan from sucrose. These polysaccharides support colonization of bacteria and are significant virulence components in the development of

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dental caries. *S. mutans* has long been considered as the main causative agent of dental caries due to their adhesive ability, acid generation, acid resistance, and water-soluble glucan formation [4, 5]. For lactobacilli, the noticed determinants of cariogenicity in lactobacilli are their ability to generate acids and their capacity to multiply and remain in acidic condition. These bacteria can cause a decrease in environmental pH as far as values are less than 4.5 and they are able to survive in a pH as low as 2.2 [6]. Lactobacilli are considered secondary invaders which involve in the progression of carious lesions rather than the primary initiators of caries, as *S. mutans*. This could be attributed to the fact that lactobacilli have a difficulty in attaching themselves to the tooth surfaces and they need mechanical retentive areas, after the lesion is formed, where aciduric environment can be established.

Apart from streptococci and lactobacilli, Candida species are considered as caries-associated microorganism and often used to evaluate the caries activity [7, 8]. Previous studies have shown the relationships between high levels of salivary Candida and the severity of caries in both children and adults [9, 10]. The most frequently noticed candida species is *Candida albicans*, which is a common opportunistic infection occurs in the oral cavity causing candidiasis. The *Candida albicans*-associated disease is generally seen in infants and elderly persons, and also in those with local and systemic immunological suppression such as diabetes, malnutrition, radiation therapy, chemotherapy, the use of systemic corticosteroids, human immunodeficiency virus (HIV) infection, and leukemia.

Azadirachta indica or neem is an evergreen tree, commonly found in India, Pakistan, Bangladesh, Nepal, and Southeast Asian countries including Thailand. It is in the family of Mahogany, Meliaceae. This plant has been used for the treatment of acute and chronic diseases. Various parts of neem have been applied to cure human illness since early time such as flowers for the prevention and curing of bile diseases, leaf for ulcers, and bark for central nervous system, paralysis, and psychiatric illness. In addition, the prevention of infectious diseases, including smallpox, and plasmodium infection has been documented. Furthermore, the plant had been used in patients who were suffering from urinary tract disorders, gastrointestinal problems, ulcers, diabetes, and high blood pressure. In Nigeria, India and other Asian countries, neem has been used to treat malaria [11].

In dentistry, herbal toothpastes consisting of neem extract were revealed to have significant antimicrobial properties against *Streptococcus mutans*, *Staphylococcus aureus, Escherichia coli,* and *C. albicans* [12, 13]. *In vitro* studies on dental caries demonstrated that petroleum ether and a chloroform extract of neem had a strong antimicrobial effect against caries-associated bacteria, *S. mutans* [14]. Furthermore, mouth rinse containing neem was demonstrated to have an equal anti-gingivitis effect as chlorhexidine [15]. Therefore, this study aimed to evaluate the antimicrobial potential of toothpaste containing neem extract against caries-associated oral microorganisms.

Materials and methods

Toothpastes

Toothpaste containing chloroform extract of neem leaf was developed by the Thailand Institute of Scientific and Technological Research (TISTR), Ministry of Science and Technology, Thailand. Two commercial herbal toothpastes, commercial toothpaste I (tea tree oil toothpaste) and commercial toothpaste II (aloe vera toothpaste)

purchased from the department store in Bangkok, Thailand were selected for assessment of their antimicrobial activities compared to neem toothpaste. Toothpaste solution was prepared by adding 0.66 g of toothpaste to 1 ml of sterile deionizing water and mixed thoroughly for the use in the following experiment.

Microorganisms

Pure cultures of *Streptococcus mutans* $KPSK_2$, *Lactobacillus casei* ATCC 393, and *Candida albicans* ATCC 10231 acquired from the culture collection of Oral Microbiology Department, Faculty of Dentistry, Mahidol University was maintained in freeze drying condition. The bacteria and fungi were subcultured on Brain Heart Infusion (BHI) agar and incubated at 37°C for 24 h. Then, the bacterial colonies were suspended in 15% glycerol, divided into small aliquots and kept frozen at -20°C. In each experiment, an aliquot of each microorganism was thawed and cultured on Brain Heart Infusion (BHI) (Difco Laboratories, Detriot, MI) agar. Few colonies from the agar were transferred to BHI broth and suspension of each microorganism was prepared in BHI broth. The suspensions of 10^8CFU/ml of bacteria and 10^7CFU/ml of fungi (equivalent to 0.5 MacFarland Standard) were used for antimicrobial evaluation.

Antimicrobial assay

One ml of the prepared microbial suspension was mixed with the toothpaste solution to make a final concentration of 22%. This was equal to the initial concentration of toothpaste in normal tooth brushing [16]. Subsequent to 50 sec, 10 µl of the solution was brought to 190 µl of D/E neutralizing broth (Difco Laboratories, Detriot, MI), in addition, 10-fold diluted and cultured on BHI agar, Rogosa agar and Sabouraud dextrose agar (Difco Laboratories, Detriot, MI) for the quantitation of *S. mutans, L. casei* and *C. albicans*, respectively at 37°C for 24-48 h. A control was performed using sterile distilled water. The number of colony forming units (CFU) grown on agar plate was counted after incubation and expressed as CFU/ml.

Statistical analysis

A statistical analysis was performed by Kruskal-Wallis and Mann-Whitney tests to compare the effect of different toothpastes on microorganisms and to compare the effect among different toothpastes. All tests were carried out in triplicate of three independent experiments. A *p*-value of <0.05 was set as a significance level. For illustration, the data are displayed as means and standard deviations of Log CFU/ml.

Results

The results from our experiments showed that all three toothpastes had a significant inhibitory effect on all tested microorganisms compared with a control (Table 1). Maximum suppression was observed on *L. casei*. Neem toothpaste was the most active against *L. casei* and *C. albicans* whereas commercial toothpaste I, which contained tea tree oil as a major component, was most active against *S. mutans* and *L. casei*.

Discussion

Nowadays, the treatment of infectious diseases is done by using several chemical agents that are commercially available, however, this can disturb the balance of microbial ecology in the oral cavity, induce resistant strains, and bring about unwanted adverse effects for instance, hypersensitivity, immunosuppressive and allergic conditions [17-19]. Due to this, the exploration for new herbal antimicrobial substances used for

Table 1 Inhibitory effect of different toothpastes on the tested microorganisms

Toothpastes	Microorganisms		
	S. mutans	*L. casei*	*C. albicans*
Control	6.03±0.15	7.18±0.12	5.75±0.06
Neem	2.68±0.55*	0	0
Commercial I (Tea tree oil)	0	0	1.99±1.01*
Commercial II (Aloe vera)	4.31±1.92*	0	0.63±0.06*

data presented as mean±SD of Log colony forming unit (CFU)/ml

* Significant difference from control at $p < 0.05$

curing the infections by these microorganisms is required. Plant products or natural products have been revealed to have a significant role in prevention and treatment of diseases via the induction of antioxidant function [20], suppression of microbial growth [11-13], and alteration of genetic pathways [21]. Several natural compounds, phytochemicals, isolated from plants are recognized as great possible choices. Moreover, the World Health Organization (WHO) has estimated that about 80% of the population worldwide still have a need for herbal medicines for the treatment of several diseases by reason of simple accessibility, low cost, and minor side effects [22].

Dental disease, for example, dental caries, is primarily caused by complex microbial communities in oral biofilm or dental plaque. Poor oral hygiene is one of the factors influences the accumulation of microorganisms and their harmful activities [12]. In this regard, proper mechanical cleaning of teeth by tooth brushing with toothpaste is recommended to prevent the formation of oral biofilm. Recently, a number of toothpaste preparations containing plant ingredients have made significant contributions to oral prophylaxis in improving oral health as a result of their antimicrobial and anti-inflammatory effects.

In this study, we have made a toothpaste with chloroform extract of neem leaf and tested against pathogenic oral microorganisms associated with dental caries. Since chloroform extraction of neem has been noticed to provide a strong antimicrobial potency against oral *Streptococcus* spp. compared with petroleum ether, ethanol, and distilled water extraction [14]. Regarding the tested microorganisms, two bacteria (*S. mutans* and *L. casei*) and fungus (*C. albicans*) were incubated with 0.33 g/ml of each toothpaste. This is equivalent to 22% of the initial toothpaste concentration found in the oral cavity during normal tooth brushing [16]. The significant microbial suppressive effects of neem toothpaste were demonstrated in this study. The most efficient result was indicated on *L. casei* and *C. albicans*. Similar to previous studies, a chloroform extract of neem leaf has been revealed to have antibacterial property against caries associated streptococci including *S. mutans, S. mitis, S. sanguinis*, and *Lactobacillus* spp. [14, 23, 24]. The stronger inhibitory effect was observed against gram-positive bacteria than gram-negative bacteria [25]. Furthermore, aqueous extract of neem leaf demonstrated to affect the cell surface hydrophobicity, adherence, and biofilm formation of oral yeast, *Candida albicans* [26]. When considering the overall effect on all caries-associated microorganisms, neem toothpaste showed a potent inhibitory effect similar to commercial herbal toothpaste containing tree tea oil (commercial toothpaste I).

Previous study performed to investigate the antibacterial efficacy of extracts from several parts of neem on oral bacteria reported that bark and leaf extracts showed potent antibacterial property against all the tested bacteria [27]. On the other hand, extracts from seed and fruit exhibited inhibitory effect only at higher concentrations [27]. In endodontic treatment of dental patients, root canal irrigants prepared from neem leaf extracts and grape seed extracts provided significantly greater zones of microbial inhibition compared with 3% sodium hypochlorite, the standard irrigant [28].

Although this study elucidated the antimicrobial effect of toothpaste containing neem extracts, further study is necessary to identify the active ingredients and their concentrations from a harvested neem leaf. Since neem and its ingredients have been reported to play a key role in the inhibitory effect on the growth of a wide range of microorganisms including viruses, bacteria, and fungi [26, 27, 29], it is likely that the important active components are azadirachtin, nimbolinin, nimbin, nimbidin, nimbidiol, nimbolide, sodium nimbinate, gedunin, salannin, and quercetin [30]. In addition, quercetin and beta-sitosterol, polyphenolic flavonoids, purified from fresh leaves of neem were also demonstrated to have antibacterial and antifungal activities [31-33]. Possible mechanism of antimicrobial action is proposed to be the effect on bacterial and fungal cell wall lysis [34, 35]. However, further investigations are still needed for better understanding mechanisms of its antimicrobial action, and the effect on cariogenic biofilm, as well as the study in clinical trials.

Conclusions

All three herbal toothpastes had a significant inhibitory effect against tested microorganisms compared with a control. Neem toothpaste was the most active against *L. casei* and *C. albicans* whereas other commercial toothpaste (tea tree oil toothpaste) was the most active against *S. mutans* and *L. casei*. Results from the present study are scientific information to prove that neem toothpaste could be assigned as natural antimicrobial toothpaste against caries-associated microorganisms.

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