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Efficacy of *Citrus hystrix* sprays in decontaminating *Streptococcus mutans* on children's toothbrushes

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ABSTRACT: Toothbrushes are sometimes contaminated by micro-organisms that can cause infection. This study evaluates the efficacy of *Citrus hystrix* sprays compared with 0.12% chlorhexidine gluconate (CHX) for disinfecting bacteria on children's toothbrushes. The participants were 61 children with a high caries risk aged 8–11. Each subject received two toothbrushes to be used continually for 7 consecutive days. The first set of toothbrushes was used as a baseline without any disinfectant. The others were divided into 5 groups and sprayed with distilled water (control), 0.12% CHX, 6% makrut oil (MO), 10% MO, or 13% MO. Toothbrushes were placed vertically into sterile test tubes containing 8 ml of TYS20B medium and incubated overnight. Then we performed 10-fold serial dilutions from 10^{-1}–10^{-5}, and spread them on BHI or MS agars for investigating facultative bacteria, oral streptococci, and *Streptococcus mutans*. Results showed that for total facultative bacteria, 10% MO and 13% MO gave the highest reduction rate (100%) > CHX (88%) > 6% MO (81%). For *S. mutans*, 10% MO and 13% MO also showed the highest reduction rate (100%) > 6% MO (90%) > CHX (88%). For oral streptococci, 13% MO gave the highest reduction rate (100%) > 10% MO (91%) > CHX (88%) > 6% MO (69%). There was no significant difference between oral sprays and 0.12% CHX in the reduction of total facultative bacteria, oral streptococci, and *S. mutans* ($p > 0.05$).

KEYWORDS: toothbrush contamination, makrut, chlorhexidine, disinfection

INTRODUCTION

Early childhood caries is one of the most common chronic diseases in the world including Thailand[1,2]. Children who have dental caries often present with low body mass index which eventually affects growth development[3]. It is a multifactorial disease and one of the most important factors is caries-associated bacteria in dental plaque[4]. Dental plaque or oral biofilm is a complex microbial community composed of numerous microorganisms aggregated by surrounded extracellular polymeric matrix that attaches to tooth surfaces[5]. Mature biofilm is highly tolerant to antimicrobial therapeutics[4]. Ecological plaque hypothesis suggested that an imbalance of microorganisms in oral biofilm results in a pathological condition such as dental caries and periodontitis[6].

Numerous studies have demonstrated a strong association between *Streptococcus mutans* and caries in regard to prevalence and transmission, as identical strains of *S. mutans* have been isolated from mothers and their children[4,6–10]. Two important characteristics of *S. mutans* relates to plaque development: firstly, its ability to produce water-insoluble polymers (glucan) which aid in the persistent adhesion and colonization on tooth surfaces by the action of a glucosyltransferases (GTFase) enzyme. Secondly, it can synthesize intracellular polysaccharide (IPS) which sustains continual acid production during low exogenous substrate[4,6]. The critical step in dental plaque development is also an essential pathway in caries development. It is of great importance to prevent the presence of *S. mutans* for more effective prevention of dental caries.

Toothbrushes are a potential source of infection. Previous studies showed a correlation between contaminated brushes and oral disease. Patients with oral disease have a substantial decrease in both initial and recurrent symptoms when they change toothbrushes[11–15]. Studies have shown that toothbrush sharing is a risk factor for the transmission of hepatitis B[15]. Previous studies reported that *S. mutans* was found in both children's and adults' toothbrushes[12,13]. Not only oral streptococci were found on toothbrush bristles, but also herpes simplex virus type I, *Pseudomonas aeruginosa*, and other

opportunistic pathogens[16–18]. Reducing contamination of toothbrushes may therefore be important to control of a wide range of oral diseases. One solution might be single-use brushes, or at least renewing brushes regularly. Since, this might have important economic and environmental consequences, an alternative approach might be preferable. It is not feasible to sterilize toothbrushes between uses but a decontamination procedure that reduces the infectious burden might be acceptable[17].

Many studies have demonstrated the efficiency of various methods in disinfecting oral microorganisms on toothbrushes such as UV radiation, microwave radiation, ozone inhibition, and chemical disinfection[12–14,17,19–23]. In spite of novel methods of decontamination, a method that is highly effective and user-friendly is using chemical agents[11]. Glass and colleagues reported that there was no oral bacteria on toothbrushes after subjects had used an antibacterial mouthwash[16]. Caudry and colleagues showed that there was a significant reduction of oral bacteria after immersing toothbrushes in Listerine mouthwash for 20 min[20]. Mehta and colleagues demonstrated that there was a significant reduction of oral bacteria after soaking toothbrushes in 0.12% chlorhexidine gluconate (CHX) mouthwash overnight[18]. There were also few studies which reported the efficiency of 0.12% CHX in a different preparation such as mouthwash and spray in disinfection of children's toothbrushes[13,23].

The use of natural products proven to have anti-cariogenic properties has been growing[24–27]. *Citrus hystrix* DC (makrut) is a common type of lime native of SE Asia[28]. A previous study showed that 3 volatile oils extracted from herbs (lemon grass, betel, and makrut oils) have an antibacterial effect against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* Typhimurium[28]. Later studies successfully developed a readily-dissolved edible makrut film for suppression of bad breath and antibacterial activities against various respiratory pathogens including multi-resistant bacteria were reported[28,29]. Moreover, our previous study on the efficacy of makrut oil capsules and spray—10% makrut oil (MO) and 12% makrut leaf oil (MLO)—on inhibiting *S. mutans* (ATCC 25175) growth and biofilm formation showed satisfactory results (unpublished data). The efficiency of these oil formulations on the inhibition of growth and biofilm formation along with the susceptibility test of *S. mutans* standard trains (ATCC 25175) and clinical isolates from Thai children should therefore be tested before future clinical trial. From our preliminary study, we tested for biofilm susceptibility of oral spray formulations: 6% MO, 10% MO, 13% MO, 4% MLO and 8% MLO, and they showed promising results in inhibiting 3 h and 6 h formed biofilm of *S. mutans* (ATCC 25175) and some clinical isolates from Thai children (unpublished data).

The objectives of this study were to evaluate the efficiency of three *C. hystrix* oil oral spray formulations (6% MO, 10% MO, 13% MO) in decontaminating *S. mutans* on children's toothbrushes compared with 0.12% CHX as a toothbrush disinfectant.

MATERIAL AND METHODS

Essential oil preparation

Makrut oil (batch no. 5 209 234/1009; density = 0.87 g/ml) was purchased from Thai-China Flavours and Fragrances Co., Ltd. The components of the essential oils were analysed using GC-MS (Auxs) as described previously[28]. The main component in makrut peel is L-limonene (40%). Three oil formulations (6% MO, 10% MO, and 13% MO) were prepared. Degradation and taste experiments were performed[28].

Study design

This cross-sectional study was approved by the Ethical Human Research Committee, Faculty of Dentistry and the Faculty of Pharmacy, Mahidol University, Thailand (MU-DT/PY-IRB 2013/037.0708). Consents were signed from all child parents/legal guardians. All subjects were free to withdraw from the study at any time. The sample size was calculated based on previous studies with $\alpha = 0.05$ and power of 80%, using the software package Primer of Biostatistics (McGraw-Hill, NY, USA). A minimum of 27 children was required[21,22].

Subject selection and clinical examination

Subjects were recruited from 3rd to 5th grade students aged between 8 and 11 years old from the Suan Missakawan school, Bangkok, Thailand. Total of 61 students who showed high level of salivary *S. mutans* ($> 5 \times 10^5$ colony-forming units (CFU)/ml) were selected to participate in this study. Salivary *S. mutans* levels were determined using Saliva-Check Mutans kit (GC Cooperation, JAPAN) following the instruction of the manufacturer. All subjects were healthy. None of them had a professional prophylaxis or fluoride application within the last 3 months or taking any kind of antibiotics during the study period.

Before the study began, all subjects were taught a tooth brushing technique by examiners using a scrub method. Before the beginning of this study, 4 undergraduate dental students attended lectures to familiarize themselves with the diagnostic criteria of dental caries and plaque deposition. All lectures were given by a dentist (KM) using a document and visuals describing the criteria and illustrating the caries condition. Oral examination was done under the natural light based on the criteria published by the World Health Organization[30]. Kappa statistic values for the decayed, missing, and filled teeth and plaque index were 0.85 and 0.88, respectively. The strength of agreement was good. The weighted kappa coefficient was 0.83 for total score ($p < 0.001$) showed an excellent agreement. The plaque deposit was assessed according to what was visible to the naked eye after all participants have chewed one tablet of a disclosure agent (Fuchsin Basic, Merck, USA) using Debris Index in simplified oral hygiene index[31] and modified for mixed dentition[31,32]. The six index teeth (surface) were 55 (B), 51 (La), 65 (B), 75 (Li), 71 (La), and 85 (Li). In the absence of either of these anterior teeth, the primary central incisor on the opposite side of the midline was substituted. In the absence of either of the second primary molar, the first primary molar in the same quadrant was substituted. The area of each tooth was assigned a score from 0–3. A score of 0 indicates no debris or stain present, 1 indicates soft debris covering not more than one third of the tooth surface, or the presence of extrinsic stains without other debris regardless of surface area covered, 2 indicates soft debris covering more than one, and 3 indicates soft debris covering more than two-thirds of the exposed tooth surface. To determine a total plaque index for each subject, the scores for each tooth were summed and divided by the number of teeth examined. All participants were given 2 toothbrushes and advised for washing and storing method using only tap water by gently rinsing thoroughly for 10 s and storing open-air without touching other toothbrushes. In the first week, each subject received Mdent toothbrush and toothpaste (1000 ppm NaF, Mdent brand is a product of the Faculty of Dentistry, Mahidol University, Bangkok, Thailand) which was used for 7 consecutive days. Reminders were given to parents or caretakers to supervise their children to brush twice a day according to the method taught. After 7 days, the toothbrushes were collected. Then a second set of toothbrushes was distributed to all subjects and used for another 7 days.

Microbiological procedures

The first set of toothbrushes was used to determine a baseline of bacteria level from each subject without any disinfectants. The second set of toothbrushes was randomly divided into 4 disinfectant groups: Group 1 (0.12% CHX gluconate and 0.15% benzidamine mouthwash, $n = 8$), Group 2 (6% MO, $n = 16$), Group 3 (10% MO, $n = 16$), and Group 4 (13% MO, $n = 16$). Unused toothbrushes ($n = 8$) were used as a sham control. Toothbrushes were fixed vertically in a closed container and let them dried. The toothbrushes were then sprayed with disinfectants 6 times on the bristles at a distance of 5 cm (approximately 0.6 ml of solution per toothbrush) in the following areas: (1) right side, (2) left side, (3) top, (4) bottom, (5) front, and (6) back of the toothbrush head[22]; gently hitting the toothbrush against the sink to remove excess of antimicrobial solution. After disinfection for 2 h, the toothbrushes were placed vertically into 25×150 mm sterile test tubes containing 8 ml TYS20B medium and vortexed for 1 min[22,33]. Ten-fold serial dilution from 10^{-1}–10^{-5} was performed to determine cell densities. Aliquot of 20 µl from each dilution was spread on brain-heart infusion and Mitis Salivarius agars to identify total facultative bacteria, oral streptococci, and *S. mutans*. The plates were incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 h. Each sample was done in triplicate.

Statistic analysis

Data is expressed as mean of colony forming units per millilitre (CFU/ml). Percentage reductions of the total facultative bacteria, oral streptococci and *S. mutans* were calculated and compared to that of the control group. For oral streptococci and *S. mutans*, a stereo microscope was used in helping colony determination. Bactericidal effect was considered if it had killed 99% of the bacteria. Comparison of the efficacy of each oral spray with 0.12% CHX was done using χ^2 ($p < 0.05$) and analysed with SPSS 14.0 software for Windows.

RESULTS

Total of 61 children participated in this study, 39% were boys and 60% were girls (mean age = 10.0 ± 0.7 years old). Mean DMFT, DMFT and plaque index were 2.11 ± 0.69, 5.6 ± 1.9, and 1.75 ± 0.59, respectively. Total facultative bacteria, oral streptococci and *S. mutans* found on the first set of toothbrushes as baseline showed no difference

between the 4 groups. Eighteen percent of subjects did not have oral streptococci and 41% of the participants did not have *S. mutans*.

Total facultative bacteria (TFB) found in distilled water group before and after spraying ranged from 7.5×10^3–5.5×10^6 and 3.5×10^3–1.35×10^6 CFU/ml, respectively. Table 1 shows the number of TFB found in 6% MO, 10% MO, 13% MO, and 0.12% CHX. TFB found in 6% MO group before and after spraying ranged from 10^3–10^7 and 0–2×10^4 CFU/ml, respectively. Treatment with 6% MO showed 81% reduction of TFB. TFB found in 10% MO group before and after spraying ranged from 0.05×10^3–1.8×10^7 and 0–3.25×10^3 CFU/ml, respectively. 10% MO showed 100% reduction of TFB. TFB found in 13% MO group before and after spraying ranged from 2×10^3–6.5×10^6 and 0–0.025×10^4 CFU/ml, respectively. Treatment with 13% MO showed 100% reduction of TFB. TFB found in 0.12% CHX group before and after spraying were ranged from 10^5–9×10^6 and 0–3×10^6 CFU/ml, respectively. Treatment with 0.12% CHX showed 88% reduction of TFB. Three oral sprays and 0.12% CHX show no significant reduction rate of TFB ($p = 0.201$).

Table 2 shows number of oral streptococci found in 6% MO, 10% MO, 13% MO, and 0.12% CHX. Oral streptococci found in 6% MO group before and after spraying ranged from 0.1×10^3–2.5×10^6 and 0–2.6×10^4 CFU/ml, respectively. Treatment with 6% MO showed 69% reduction of oral streptococci. Oral streptococci found in 10% MO group before and after spraying ranged from 0.025×10^3–3.5×10^6 and 0–0.775×10^3 CFU/ml, respectively. 10% MO showed 91% reduction of oral streptococci. Oral streptococci found in 13% MO group before and after spraying ranged from 0.025×10^3–2.5×10^5 and 0–0.05×10^3 CFU/ml, respectively. 13% MO showed 100% reduction of oral streptococci. Oral streptococci found in 0.12% CHX group before and after spraying ranged from 0.75×10^3–2×10^4 and 0–3×10^3 CFU/ml, respectively. 0.12% CHX showed 88% reduction of oral streptococci. Three oral sprays and 0.12% CHX showed no different significance in reduction rate of oral streptococci ($p = 0.121$).

Table 3 shows number of *S. mutans* found in 6% MO and 10% MO, 13% MO, and 0.12% CHX. *S. mutans* found in 6% MO group before and after spraying were ranged from 0.025×10^3 to an uncountable number (mean = 5×10^3 CFU/ml) and 0–0.075×10^3 CFU/ml, respectively. Treatment

Table 1 Number of total facultative bacteria and percentage reduction on toothbrushes treated with 6% MO, 10% MO, 13% MO, and 0.12% CHX.

Sample	Total facultative bacteria (CFU/ml)		Reduction after treatment (%)
	Before	After	
6% MO			
2	3.5×10^6	1.5×10^3	99.96
13	5×10^6	10^3	99.98
21	10^7	2×10^4	99.80
22	6.75×10^5	0.025×10^3	99.99
24	2.5×10^4	10^5	no reduction
29	4.37×10^5	0.025×10^3	99.99
47	7×10^5	0	100
48	10^5	1.5×10^3	98.64
56	5.2×10^6	1.1×10^4	99.79
59	8×10^4	0	100
71	4.75×10^5	0	100
78	2.75×10^5	0.525×10^3	99.81
79	3.75×10^3	0	100
85	10^3	0.95×10^3	5
86	4×10^4	0	100
87	8.5×10^5	0.125×10^3	99.99
10% MO			
1	2.75×10^5	0	100
7	1.2×10^7	0	100
12	2.9×10^4	0	100
14	6.5×10^5	0.275×10^3	99.96
19	7.5×10^4	0	100
35	9.75×10^5	0.2×10^3	99.98
37	1.3×10^6	2.5×10^3	99.81
39	6×10^5	0	100
40	0.05×10^3	0	100
41	5.2×10^6	0	100
55	1.7×10^5	0	100
58	1.8×10^7	3.25×10^3	99.98
66	3.75×10^5	2.75×10^3	99.27
68	3.7×10^6	0	100
83	1.5×10^6	0	100
90	1.8×10^4	0	100
13% MO			
3	3.5×10^4	0	100
4	1.6×10^6	0	100
5	1.1×10^6	0.125×10^3	99.99
8	3.7×10^5	0	100
11	6.5×10^5	0	100
17	1.37×10^6	0.75×10^3	99.95
18	7.7×10^4	0	100
23	5.5×10^4	0	100
33	6.5×10^6	0.75×10^3	99.99
36	7×10^3	0.025×10^4	99.64
43	2.2×10^5	0	100
61	4.5×10^5	0.5×10^3	99.89
65	3.1×10^4	0.025×10^3	99.92
76	2×10^3	0	100
77	4×10^4	0	100
88	2.4×10^6	0	100
0.12% CHX			
6	6.5×10^6	25×10^3	99.62
9	7×10^6	0	100
16	4×10^6	3×10^6	25
45	2.1×10^6	0.1×10^3	99.99
49	9×10^6	0.3×10^3	99.99
51	1.1×10^5	0.3×10^3	99.73
72	4.75×10^5	1.25×10^3	99.74
73	10^5	0.05×10^3	99.95

Table 2 Number of oral streptococci and percentage reduction on toothbrushes treated with 6% MO, 10% MO, 13% MO, and 0.12% CHX.

Sample	Oral streptococci (CFU/ml)		Reduction after treatment (%)
	Before	After	
6% MO			
2	2.5×10^6	0.025×10^3	99.99
13	2.5×10^6	0	100
21	2.65×10^5	2×10^4	92.45
22	9.5×10^3	0	100
24	2.25×10^3	2.6×10^4	no reduction
29	2.5×10^3	0	100
47	0.7×10^3	0	100
48	3.25×10^3	0.625×10^3	80.77
56	1.5×10^4	1.5×10^4	4.76
59	10^3	0	100
71	3.75×10^4	0	100
78	0.05×10^3	0	100
79	0.55×10^3	0	100
85	0.1×10^3	0	100
87	3.75×10^3	0	100
10% MO			
1	2.5×10^3	0	100
7	1.8×10^4	0	100
12	1.15×10^3	0	100
19	0.625×10^3	0	100
37	4.75×10^3	0	100
41	5.25×10^4	0	100
58	3.5×10^6	0.775×10^3	99.97
66	0.35×10^3	0.525×10^3	no reduction
68	2.375×10^3	0	100
83	3.1×10^6	0	100
90	0.025×10^3	0	100
13% MO			
3	2.5×10^5	0	100
4	0.8×10^3	0	100
5	6.5×10^3	0	100
8	2.2×10^5	0	100
18	10^3	0	100
23	0.25×10^3	0	100
36	4.25×10^3	0	100
43	3.75×10^3	0	100
61	1.8×10^5	0.05×10^3	99.97
65	0.125×10^3	0	100
76	0.025×10^3	0	100
77	0.125×10^3	0	100
88	4.5×10^3	0	100
0.12% CHX			
6	5.75×10^3	0	100
9	5×10^3	0	100
16	2×10^4	3×10^3	85
45	9.8×10^3	0	100
49	22.5×10^3	0	100
51	0.85×10^3	0	100
72	2×10^3	0	100
73	0.75×10^3	0	100

Table 3 Number of *S. mutans* and percentage reduction on toothbrushes treated with 6% MO, 10% MO, 13% MO, and 0.12% CHX.

Sample	*S. mutans* (CFU/ml)		Reduction after treatment (%)
	Before	After	
6% MO			
2	4.5×10^3	0.075×10^3	98.33
21	5×10^3	0	100
22	3.75×10^3	0	100
47	0.025×10^3	0	100
56	$>3\times10^8$	0	100
59	0.05×10^3	0	100
71	$>3\times10^8$	0	100
78	0.5×10^3	0	100
79	0.5×10^3	0	100
87	0.25×10^3	0	100
10% MO			
7	10^5	0	100
12	0.2×10^3	0	100
14	0.475×10^3	0	100
19	6.5×10^3	0	100
37	5.5×10^3	0	100
41	0.1×10^3	0	100
55	0.575×10^3	0	100
66	0.175×10^3	0	100
83	$>3\times10^8$	0	100
13% MO			
4	0.57×10^3	0	100
5	1.275×10^3	0	100
11	0.075×10^3	0	100
18	0.5×10^3	0	100
36	2.5×10^3	0	100
77	0.2×10^3	0	100
88	4×10^3	0	100
0.12% CHX			
6	8.25×10^3	0	100
9	3.25×10^4	0	100
16	$>3\times10^8$	3.7×10^3	no reduction
45	2.05×10^3	0	100
51	0.4×10^3	0	100
72	10^3	0	100
73	0.2×10^3	0	100

with 6% MO showed 90% reduction of *S. mutans*. *S. mutans* found in the 10% MO group before and after spraying ranged from 0.1×10^3 to an uncountable number and no *S. mutans* was detected, respectively. Treatment with 10% MO showed 100% reduction of *S. mutans*. *S. mutans* found in the 13% MO group before and after spraying were ranged from 0.2×10^3–4×10^3 (mean $= 4\times10^3$) and no *S. mutans* was detected, respectively. Treatment with 13% MO showed a 100% reduction of *S. mutans*. *S. mutans* found in 0.12% CHX group before and after spraying were ranged from an uncountable number to 0.2×10^3 (mean $= 1\times10^3$) and 0–3.7×10^3 CFU/ml, respectively. Treatment with 0.12% CHX showed 88% reduction of *S. mutans*. All oral sprays and 0.12% CHX showed no significant difference of reduction rates of total facultative bacteria, oral streptococci and *S. mutans* ($p = 0.22$).

DISCUSSION

The American Dental Association suggests that to reduce dental plaque or oral biofilm accumulation, toothbrushes should be cleaned after each use and should be replaced every 3–4 months[34]. Copious amount of bacteria has been detected on toothbrushes even after 24 h, and mutans streptococci (MS) has been found on toothbrushes soaked in sterile tap water for 20 h[13]. Another group of researchers reported the detection of MS on toothbrushes soaked in sterile saline for 2 h[35]. The hypothesis is that if we reduce the biofilm formation on toothbrush, the chance of re-infection would be reduced. Most of soaking time in antibacterial agents was at least 2 h. There was only a study done by Nelson-Filho et al which compared an efficiency of two methods of disinfecting MS on toothbrushes which were soaking and spraying of 0.12% CHX and result showed that there was no significant difference between those methods[22]. In this study, we found that three oral sprays and CHX showed a significant reduction of total facultative bacteria, oral streptococci and *S. mutans* on toothbrush when compared with distilled water. From our preliminary study, five oral spray formulations (6% MO, 10% MO, 13% MO, 4% MLO, 8% MLO) successfully inhibited biofilm formation of *S. mutans* (ATCC 25175) in vitro. This study confirmed that three oral spray formulations (6% MO, 10% MO, 13% MO) effectively reduced facultative bacteria, oral streptococci, and *S. mutans* and was not significantly different from a gold standard antibacterial agent (0.12% CHX) when spraying and left for 2 h.

CHX was ranked as a gold standard antibacterial agent effective in reducing most bacteria on toothbrushes. Likewise, this study also show the effectiveness of CHX in a spray form[16,20–24,35]. Nevertheless, CHX is an antiseptic agent which effectively killed bacteria by penetrating into cell cytoplasm and lyse cell component[36]. Previous report showed that bacteria might developed a resistant mechanism, if used for a long time. Most researchers are trying to use herbal extraction as an alternative[36,37]. The mechanism by which essential oils inhibits biofilm formation is unknown. It has been proposed however that it could inhibit bacterial growth, acid production, or substrate adhesion. The active ingredients that have an antibacterial property in *C. hystrix* essential oils are α-terpineol and citronellal, but their mechanism of inhibitory effect is unknown. However, there are other ingredients that have a bactericidal effect on *S. mutans* such as tt-farnesol. It has no bactericidal effect directly, but it interferes with the adhesion and polysaccharide production that relates to the biofilm formation[35]. The hydrophobicity of essential oils might enable them to partition the lipid component of bacterial cell membranes, rendering them permeable and leading to leakage of bacterial cell contents. As the mechanism of *C. hystrix* essential oil is still unclear, further study would be needed.

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