The In Vitro Antimicrobial Activity of Natural Infant Fluoride-free Toothpastes on Oral Micro-organisms

Fabíola G. Carvalho, DDS, MS, PhD
Thais De Cássia Negrini, DDS, MS
Luis Victor S. Sacramento, PhD
Josimeri Hebling, DDS, MS, PhD
Denise M.P. Spolidorio, BS, PhD
Cristiane Duque, DDS, PhD

ABSTRACT

Purpose: The objective of this study was to evaluate the antimicrobial activity of six toothpastes for infants: 3 fluoride-free experimental toothpastes--cashew-based, mango-based and without plant extract and fluoride compared with 2 commercially fluoride-free toothpastes and 1 fluoridated toothpastes.
Methods: Six toothpastes for infants were evaluated in this study: (1) experimental cashew-based toothpaste; (2) experimental mango-based toothpaste; (3) experimental toothpaste without plant extract and fluoride (negative control); (4) First Teeth brand toothpaste; (5) Weleda brand toothpaste; and (6) Tandy brand toothpaste (positive control). The antimicrobial activity was recorded against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, and *Candida albicans* using the agar plate diffusion test.
Results: First Teeth, Weleda, mango-based toothpaste, and toothpaste without plant extract presented no antimicrobial effect against any of the tested micro-organisms. Cashew toothpaste had antimicrobial activity against *S mutans*, *S sobrinus*, and *L acidophilus*, but it showed no antimicrobial activity against *C albicans*. There was no statistical difference between the inhibition halo of cashew and Tandy toothpastes against *S mutans* and *L acidophilus*.
Conclusions: Cashew fluoride-free toothpaste had inhibitory activity against *Streptococcus mutans* and *Lactobacillus acidophilus*, and these results were similar to those obtained for fluoridated toothpaste.
(J Dent Child 2011;78:3-8)

Received September 2, 2009; Last Revision January 13, 2009; Revision Accepted January 13, 2009.

KEYWORDS: TOOTHPASTE, ANARCADIUM OCCIDENTALE, MANGIFERA INDICA, PLANT EXTRACTS

Dr. Carvalho is professor, Health and Technology Rural Center, Federal University of Campina Grande, Patos, Paraiba, Brazil; Dr. Negrini is doctorate student, Department of Clinical Analysis, and Dr. Sacramento is professor, Departament of Princípios Ativos e Naturais, both in the School of Pharmaceutical Sciences, Dr. Hebling is professor, Department of Orthodontics and Pediatric Dentistry, Dr. Spolidorio is professor, Department of Physiology and Pathology, both in the Araraquara School of Dentistry, all at the University of São Paulo State, Araraquara, São Paulo, Brazil; Dr. Duque is professor, Department of Pediatric Dentistry, Federal Fluminense University, Nova Friburgo, Rio de Janeiro, Brazil.
Correspond with Dr. Carvalho at fabigalbi@yahoo.com.br

Fluoride toothpastes are the most widely used method of fluoride application in the world, and these toothpastes have made an important contribution to the reduction in caries prevalence in all countries.[1] Excessive ingestion of fluoride during tooth maturation, however, can result in the development of fluorosis. Infants and young children tend to swallow toothpaste, which has been shown to contribute to the increasing incidence of enamel fluorosis observed in the last decade.[2]

The most critical period of fluorosis risk is believed to be from approximately 19 to 26 months of age.[3] Therefore, to avoid inappropriate fluoride intake in this period, studies have suggested the use of small quantities of toothpaste under parental supervision[4] as well as reduction of fluoride (**F**) concentration in toothpaste (500 ppm F).[5] As yet, however, the anticaries efficacy and antimicrobial activity of these low-fluoride-concentration toothpastes has not been proven, and further investigation is required before they can be recommended.[1,4]

There are alternative toothpastes on the market for use by infants, particularly in the critical period of fluorosis risk. These products do not contain fluoride in their composition, but have herbal products or enzymes to produce antiseptic or antimicrobial effects against cariogenic and opportunistic micro-organisms in the oral environment.[6] These toothpastes, however, have shown no antimicrobial effect on individual species grown independently or on biofilms produced in vitro.[6]

The components of natural toothpastes should be as safe as those of conventional toothpastes, provide efficient protection against cariogenic micro-organisms to enhance biofilm control, and prevent dental caries. *Mangifera indica* (mango), originated in Asia, and *Anarcadium occidentale* (cashew), originated in Africa, and are plants whose fruits are traditionally consumed in Brazil. These plants have been used in medical studies and have shown antimicrobial and anti-inflammatory activity.[7-9] Moreover, the extract from the stems and bark of *A occidentale* has shown antimicrobial effect against some of the micro-organisms found in the oral cavity: *Candida tropicallis* and *Candida stellatoidea*,[10] *Streptococcus mutans*, *Streptococcus mitis*, and *Streptococcus sanguis*,[11] and *Staphylococcus aureus*.[12] The acetone extract of *M indica* leaves had an antimicrobial effect against *S aureus*.[13] Furthermore, the water extract of a *M indica* stick showed an antimicrobial effect against *S mutans*, *Streptococcus mitis*, *Streptococcus salivarius*, *and S sanguis*.[14] The antimicrobial effect, however, of *A occidentale* and *M indica* extract, when incorporated into toothpastes, has never been evaluated.

Therefore, the aim of this study was to evaluate the antimicrobial activity against oral micro organisms of 3 fluoride-free experimental toothpastes, cashew-based, mango-based and without plant extract compared with 2 commercially available fluoride-free toothpastes and one fluoride containing toothpaste.

METHODS

Six toothpastes for infants were evaluated in this study: 3 fluoride-free experimental toothpastes—cashew-based, mango-based and without plant extract and fluoride (negative control); 2 commercially available fluoride-free toothpastes—First Teeth (Laclede Professional Products Inc, Gardena, Calif) and Weleda (Weleda D,Schwabisch Gmund, Germany); and 1 fluoridated toothpaste (positive control), Tandy (Colgate-Palmolive Ind. Com. Ltda, São Paulo, Brazil; Table 1).

NATURAL TOOTHPASTE PREPARATION

M indica L var espada fruits and *A occidentale* L pseudo-fruits were collected at Medicinal and Toxic Plant Garden, School of Pharmaceutical Sciences, University of São Paulo State, Araraquara, São Paulo, and botanically identified. The material was air-dried at 40ºC for 72 hours in a heater and triturated to obtain a coarse power. The dried and pulverized material was submitted for extraction procedures via turbolysis technique for 15 minutes at room temperature. It was performed mixing 10 g of each pulverized fruit with 90 g of 70% (v/v) ethanol. After this period, the hydroalcoholic extract was filtered and stored at 5ºC until the gel was manufactured.

The components used in the experimental toothpastes were added in the same concentrations regularly used to formulate common toothpastes: 14% silica; 0.4% carboxymethylcellulose; 72.2% sorbitol; 0.075% saccharine; 0.2% cashew or mango flavor; and 12%

Table 1. Composition of Toothpastes Used in This Study

Toothpaste	Composition
1. Cashew toothpaste (experimental).	Water, silica, extract of cashew, carboxymethylcellulose, sorbitol, saccharine.
2. Mango toothpaste (experimental).	Water, silica, extract of mango, carboxymethylcellulose, sorbitol, saccharine.
3. Toothpaste without plant extract (experimental—negative control).	Water, silica, carboxymethylcellulose, sorbitol, saccharine.
4. Weleda (Weleda D, Schwabisch Gmund, Germany).	Water, esculina, extract of Calendula (Callendula officinalis), silica, algin.
5. First Teeth (Laclede Professional Products Inc, Gardena, Calif.)	Water, glycerol, sorbitol, natural banana, natural apple, xylitol, lactoferrin, lactoperoxidase, glucose oxidase, pectin, propylene glycol, aloe vera.
6. Tandy (Colgate-Palmolive Ind. Com. Ltda, São Paulo, Brazil—positive control).	Water, silica, sodium lauryl sulfate, carboxymethylcellulose, sorbitol, saccharine, sodium fluoride (1,110 ppm), polyethylenoglycol.

hydroalcoholic extracts of cashew or mango. The components were mixed until a gel viscosity was obtained, and then the final product was inserted in code-identified aluminum tubes. The control toothpaste was formulated using the same components without the hydroalcoholic extract.

ANTIMICROBIAL ACTIVITY

The antimicrobial activity of each toothpaste was evaluated against *S mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, and *Candida albicans* using the agar plate diffusion test. S mutans, S sobrinus, and L acidophilus strains were grown in brain heart infusion broth (BHI, Difco Laboratories, Detroit, Mich) for 48 hours at 37°C, and C albicans strain was grown in Mueller Hinton broth (MH, Difco Laboratories), according to the physiological characteristics of each micro-organism. The resultant bacteria and yeast were again placed in 5 mL BHI and MH, respectively, for 24 hours at 37°C to form a suspension (inoculum) containing 1.0 x 107 colony-forming units.

In each sterilized petri dish (20 x 100 mm), a base layer containing 15 mL of BHI agar mixed with 300 μL of each inoculum was prepared. After solidification of the culture medium, 6 wells measuring 4 mm in diameter were made in each plate. One well was left unfilled and served as a negative control of each plate; the other 5 wells were completely filled with one of the test toothpastes listed in Table 1. Ten wells were filled with each material and micro-organism strain. The plates were kept for 2 hours at room temperature for diffusion of the materials and then incubated at 37°C for 24 hours.

Zones of microbial growth inhibition were recorded in millimeters using a digital caliper (Mitutoyo, São Paulo, Brazil). Measurements were taken at the greatest distance between 2 points at the outer limit of the inhibition zone formed around the well. This measurement was repeated 3 times, and the mean was computed for each well. Statistical analysis was performed using ANOVA 2-way and Tukey tests to compare the inhibition zones of the toothpastes against each micro-organism strain at a significance level of 5%.

RESULTS

The inhibition halos of toothpastes tested in this study are given in Table 2. First Teeth, Weleda, mango, and toothpaste without plant extract presented no antimicrobial effects against all micro-organisms tested. The cashew toothpaste showed significant antimicrobial activity against *S mutans* (P=.001), *S sobrinus* (P=.001), and *L acidophilus* (P=.001), but it showed no antimicrobial activity against *C albicans*. This natural toothpaste showed the highest inhibition zone against *L acidophilus* (P=.001). Tandy had antimicrobial activity against all micro-organisms (P=.001) and showed the highest inhibition halo against *C albicans*. There was no statistical difference between the inhibition halo of cashew and Tandy toothpastes against *S mutans* and L *acidophilus* (P<.86). The antimicrobial activity of tooth-pastes tested against *S mutans* is shown in Figure 1.

Table 2. Inhibition Zones Expressed in mm Mean (Standard Deviation) Induced by Toothpastes Against 4 Oral Micro-organisms

Toothpaste	Micro-organism*†			
	Streptococcus mutans	*Streptococcus sobrinus*	*Lactobacillus acidophilus*	*Candida albicans*
First Teeth	0	0	0	0
Weleda	0	0	0	0
Toothpaste without extract	0	0	0	0
Mango	0	0	0	0
Cashew	9.7 (0.2)^{A,a}	9.1 (0.2)^{B,a}	12.1 (0.2)^{C,b}	0
Tandy	9.7 ± 1.8^{A,a}	13 (1.3)^{B,b}	10.9 (1.4)^{A,b}	17.9 (1.4)^{C,a}

* Values=mean±standard deviation.

† Means followed by the same uppercase letters in the rows and lowercase letters in the columns do not differ statistically (Tukey's test; P>.05).

DISCUSSION

Although fluoride is important to prevent dental caries, the swallowing of fluoride toothpaste by infants is associated with an increased risk of fluorosis.[2,3] Fluoride-free toothpastes were developed and sold to promote oral hygiene in infants without risk of developing fluorosis. However, these toothpastes should have an inhibitory activity against cariogenic bacteria and opportunistic micro-organisms to help maintain oral health and protect against caries development.

In the present study, 2 commercially available fluoride-free toothpastes for infants (First Teeth and Weleda) were tested against several oral micro-organisms. The action mechanism of First Teeth is based on the lactoperoxidase enzyme system. In the presence of hydrogen peroxide and the thiocyanate ion (SCN-), lactoperoxidase catalyzes the formation of the hypothiocyanate ion (OSCN-), a compound that reacts quickly with the sulfhydryl group of proteins, leading to disorganization of the bacterial metabolic system.[6] Weleda has an anti-inflammatory, antiseptic, and healing effect (manufacturer's information) due to the calendula extract. Although the composition of these commercial toothpastes may show some antimicrobial activity, the present study did not confirm this hypothesis. Our results agree with those reported by Modesto et al., who demonstrated no antimicrobial effect against any of the micro-organisms evaluated. In an in vivo study, Kirstila et al., verified no effect of toothpaste containing the lactoperoxidase-system, similar to that of First Teeth, after 2 weeks of daily use, on peroxidase activity or any of the monitored oral bacterial counts compared with the placebo toothpaste.

The antimicrobial activity of fluoride-free experimental toothpastes evaluated in the present study showed that only cashew toothpaste had inhibitory activity against some of the oral micro-organisms tested. *A occidentale* extract (12%) showed inhibition halo formation against *S mutans*, *S sobrinus*, and *L acidophilus*. Pereira et al., found an antimicrobial effect of cashew extract at concentrations of 50, 25, 12.5, and 6.25 mg/mL against *S mutans*, *S mitis*, and *S sanguis*. According to these authors, *A occidentale* extract interferes with the synthesis of glucan by glucosyltransferase produced by *S mutans*, interfering in the adherence mechanism of bacteria on the tooth surface. The cashew toothpaste had no antimicrobial activity against *C albicans* only. This yeast usually shows low sensitivity to growth inhibition by plant extracts.[16] Kolaczkowski et al., and Akinpelu tested *A occidentale* extract against *C albicans* and also found no inhibitory activity. According to Kolaczkowski et al., *A occidentale* extract showed potent growth inhibitory activity against *Candida glabrata*. It was, however, inactive against *C albicans*, even at the highest concentrations used.

Interestingly, there was no significant difference between the inhibition halo formation of cashew fluoride-free toothpaste and Tandy against *S mutans* and *L acidophilus*. This result shows that cashew fluoride-free toothpaste is as effective against cariogenic micro-organisms as fluoridated toothpaste. The fluoridated component usually incorporated in toothpastes is sodium fluoride, but in the mid-1990s stabilized stannous fluoride (**SnF_2**) toothpaste formulations were developed. Stannous fluoride toothpaste (0.454% SnF_2) can release fluoride ions and inhibit the microbial metabolism.[17] The divalent metal ion (**Sn_2^+**) appears to bind to pellicle coated dental surfaces and is capable of interacting with negatively charged moieties, such as bacteria, showing antimicrobial activity.[18]

Some clinical studies have shown superior plaque inhibition and anticaries effectiveness of stannous fluoride toothpaste when compared to sodium fluoride.[19-21] The antibacterial activity is greater when the fluoride is associated with Sn_2^+ or with amine.[22] It is known, however, that fluoride can inhibit many bacterial enzymes, including enolase, which is essential to sugar metabolism, in addition to acting as a transmembrane proton transporter. This interferes in the movement of protons out of the cytoplasm and decreases acid tolerance response.[23] Tandy toothpaste contains 1,100 ppm of sodium fluoride and sodium lauryl sulfate (Table 1). Likewise, fluoride and sodium lauryl sulfate, an anionic stable detergent, can inactivate bacterial enzymes associated with sugar transport and the synthesis of extracellular polysaccharides.[24] *A occidentale* extract

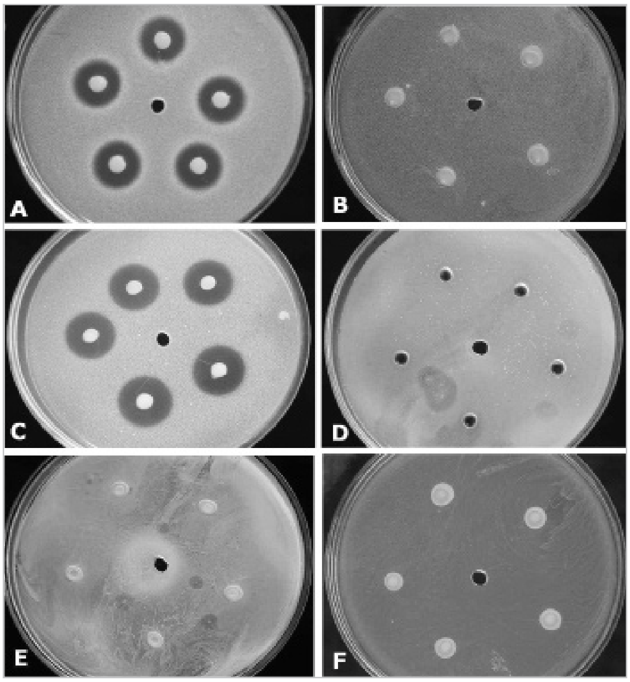

Figure 1. Inhibition zones of each toothpaste against Streptococcus mutans strain. A=cashew; B=mango; C=Tandy; D=First Teeth; E=Weleda, F=Toothpaste without plant extract.

was responsible for the antimicrobial effect of cashew toothpaste, which did not contain fluoride and sodium lauryl sulfate. Moreover, the control toothpaste without natural extract and fluoride showed no antimicrobial activity against the tested micro-organisms.

Some studies have reported the antimicrobial effect of *M indica* extract and leaf.[14,25] In the present study, however, mango toothpaste showed no inhibitory effect on the tested micro-organisms. According to Prashant et al., *M indica* water extract formed an inhibition halo against *S mutans*, *S salivarius*, *S mitis*, and *S sanguis* at 10% and 50% concentrations. The tannin in *M indica* plants has an astringent effect on the mucous membrane, and it can form a layer over enamel, thus providing protection against dental caries.[14] Furthermore, Bairy et al. suggested that mangiferin possesses antibacterial activity in vivo against specific periodontal pathogens, because the subgingival plaque microbiota of subjects that used mango leaves as a home care hygiene device revealed a significant decrease in the proportion of *Prevotella intermedia* and *Porphyromonas gingivalis* when compared with toothbrush users. The antimicrobial activity of *M indica* extract incorporated into toothpastes needs to be further investigated.

Although many plant extracts or derivatives have been incorporated into commercial toothpastes, no data are available on the use of cashew and mango extracts. Thus, it is difficult to make comparisons with other studies. Although this is a preliminary study, it has provided important microbiological data, suggesting further investigations with fluoride-free cashew extract toothpaste. The active components of cashew extract responsible for antimicrobial activity must be fractionated and tested separately. Furthermore, clinical studies also are necessary to prove the efficacy of fluoride-free cashew extract toothpaste, because this toothpaste could be used as an oral hygiene alternative for infants to control enamel fluorosis and biofilm accumulation.

CONCLUSIONS

1. The commercially available fluoride-free toothpastes tested showed no inhibitory activity against the tested micro-organisms.
2. The fluoride-free cashew extract toothpaste had an inhibitory effect against *Streptoccocus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus*, and these results were similar to those obtained for the tested fluoride toothpaste.

ACKNOWLEDGMENTS

The authors wish to thank Cynthia Helena Gontijo Cordeiro, doctorate student of Departament of Princípios Ativos e Naturais, School of Pharmaceutical Sciences, University of São Paulo State, Araraquara, Brazil for laboratorial support during the experimental toothpastes preparation.

REFERENCES

1. Word Health Organization. *Fluorides and Oral Health*. Technical report series. Geneva, Switzerland: WHO; 1994:846.
2. Browne D, Whelton E, O'Mullane D. Fluoride metabolism and fluorosis. J Dent 2005;33:177-86.
3. Evans RM, Darvell BW. Redefining the estimate of the critical period for susceptibility to enamel fluorosis in human maxillary incisors. J Public Health Dent 1995;55:238-49.
4. Ammari AB, Bloch-Zupan A, Ashley PF. Systematic review of studies comparing the anti-caries efficacy of children's toothpaste containing 600 ppm of fluoride or less with high fluoride toothpastes of 1,000 ppm or above. Caries Res 2003;37:85-92.
5. Horowitz HS. Commentary and recommendations for the proper uses of fluoride. J Public Health Dent 1995;55:57-62.
6. Modesto A, Lima KC, Uzeda M. Effects of three different infant dentifrices on biofilms and oral micro-organisms. J Clin Pediatr Dent 2000;24:237-43.
7. Akinpelu DA. Antimicrobial activity of *Anarcadium occidentale* bark. Fitoterapia 2001;72:286-7.
8. Olajide OA, Aderogba MA, Adedapo ADA, Makinde JM. Effects of *Anarcadium occidentale* stem bark extract on in vivo inflammatory models. J Ethnophography 2004;95:139-42.
9. Garrido G, Gonzalez D, Lemus Y, et al. In vivo and in vitro anti-inflammatory activity of *Mangifera indica* L. extract (Vimang). Pharmacol Res 2004;50:143-9.
10. Araújo CRF, Pereira MSV, Higino SJ, Pereira JV, Martins AB. In vitro antifugal activity of Anarcadium occidentale Linn. bark upon leavens of the Candida gender. Arq Odontol 2005;41:193-272.
11. Pereira JV, Sampaio FC, Pereira MCV, Melo AFM, Higino JS, Carvalho AAT. In vitro antimicrobial activity of an extract from *Anacardium occidentale* Linn. on *Streptococcus mitis, Streptococcus mutans,* and *Streptococcus sanguis*. Odontol Clin 2006;5:137-41.
12. Silva JG, Souza IA, Higino JS, Siqueira-Junior JP, Pereira JV, Pereira MCV. Antimicrobial activity of the hydroalcoholic extract of *Anacardium occidentale* Linn. against multi-drug resistant strains of *Staphylococcus aureus*. Braz J Pharm 2007;7:572-7.
13. Doughari JH, Manzara S. In vitro antibacterial activity of crude leaf extracts of *Mangifera indica* Linn. Afr J Microbiol Res 2008;2:67-72.
14. Prashant GM, Chandu GN, Murulikrishna KS, Shafiulla MD. The effect of mango and neem extract on four organisms causing dental caries: *Streptococcus mutans, Streptococcus salivarius, Streptococcus mitis*, and *Streptococcus sanguinis*: An in vitro study. Indian J Dent Res 2007;18:148-51.
15. Kirstila V, Lenander-Lumikare M, Tenovuo J. Effects of a lactoperoxidase system-containing toothpaste on dental plaque and whole saliva in vivo. Acta Odontol Scand 1994;52:346-53.
16. Kolaczkowski M, Kolaczkowska A, Sroda K, et al. Substrates and modulators of the multidrug transporter Cdr1p of *Candida albicans* in antifungal extracts of medicinal plants. Mycoses. In press.
17. White DJ. A "return" to stannous fluoride dentifrices. J Clin Dent 1995;6:29-36.
18. Wade W, Addy M, Hughes J, Milsom S, Doherty F. Studies on stannous fluoride toothpaste and gel: Antimicrobial properties and staining potential in vitro. J Clin Periodontol 1997;24:81-5.
19. Stookey GK, Mau MS, Isaacs RL, Gonzalez-Gierbolini C, Bartizek RD, Biesbrock AR. The relative anticaries effectiveness of three fluoride-containing dentifrices in Puerto Rico. Caries Res 2004;38:542-50.
20. Ramji N, Baig A, He T, et al. Sustained antibacterial actions of a new stabilized stannous fluoride dentifrice containing sodium hexametaphosphate. Compend Contin Educ Dent 2005;26:19-28.
21. White DJ. Effect of a stannous fluoride dentifrice on plaque formation and removal: A digital plaque imaging study. J Clin Dent 2007;18:21-4.

22. van Loveren C. Antimicrobial activity of fluoride and its in vivo importance: Identification of research questions. Caries Res 2001;35:65-70.
23. Marquis RE, Clock SA, Mota-Meira M. Fluoride and organic weak acids as modulators of microbial physiology. FEMS Microbiol Rev 2003;26:493-510.
24. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. J Dent Res 1992;71:1431-8.
25. Bairy I, Reeja S, Siddharth, Rao PS, Bhat M, Shivananda PG. Evaluation of antibacterial activity of Mangifera indica on anaerobic dental microflora based on in vivo studies. Indian J Pathol Microbiol 2002; 45:307-10.