Article

Efficacy of guaco mouthwashes (*Mikania glomerata* and *Mikania laevigata*) on the disinfection of toothbrushes

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Abstract: Guaco *Mikania glomerata* Spreng. and *M. laevigata* Sch. Bip. ex Baker, Asteraceae, has antimicrobial activity and may be helpful in reducing the incidence of oral diseases. This double-blinded randomized clinical trial aimed to evaluate the efficacy of guaco mouthwashes on the disinfection of toothbrushes used by preschool children, tested positive for mutans streptococci (MS), as well as the quantification of its coumarin contents by high performance liquid chromatography. Ethanol extracts were obtained by percolation. The mouthwashes were prepared with 2.5% g/mL *M. glomerata* and *M. laevigata* ethanol extracts, standardized for their coumarin content (% mg/mg). Antimicrobial effect of the mouthwashes and extracts were assessed *in vitro* against *Streptococcus mutans* (ATCC 25175™), using 2.4 to 500 µg/mL to calculate the minimum inhibitory concentration (MIC). For the *in vivo* study, 24 patients were randomly assigned to a 4-stage changeover system with a one-week interval between each stage. All solutions were used in all stages by a different group of children. After brushing without toothpaste, toothbrushes (n=96) were sprayed with water and solutions of *M. glomerata* (2.5%), *M. laevigata* (2.5%) and chlorhexidine (0.12%). Microbiological analysis was carried out after 4 h and 30 days, respectively. MIC values were 400, 125 and 14 µg/mL, respectively, for both crude ethanol extracts, mouthwashes of *M. glomerata* and *M. laevigata*. Statistical analysis showed that all solutions decreased contamination of toothbrushes by mutans streptococci (chlorhexidine 50.7±17.7%; *M. glomerata* 37.3±23.7% and *M. laevigata* 28.7±25.1% of inhibition). Treatment with chlorhexidine and *M. glomerata* were statistically similar (*p*>0.05). *M. glomerata* mouthwash could be useful in herbal strategy programs against mutans streptococci and the marker coumarin may be not related to the activity observed.

Introduction

Severe early childhood caries (ECC) is a condition observed worldwide which could trigger the loss of the deciduous dentition as well as abscesses, pain and malocclusion if left untreated (Kumarihamy et al., 2011; Tanner et al., 2011). The high prevalence of ECC in children is related to parental care, eating habits, oral health education in family and at school (Kumarihamy et al., 2011; Paula et al., 2012). ECC is a multifactorial, complex infectious disease derived from a dynamic process resulting from the prolonged interaction between biofilm, carbohydrate, saliva and cariogenic microorganisms (Kumarihamy et al., 2011; Paula et al., 2012). The streptococci mutans are strongly associated with ECC (Tanner et al., 2011).

The mechanical removal of biofilm by brushing, assisted by the use of dental floss, represents the most effective, affordable and widespread maintenance of oral health, which makes brushing the primary method of oral hygienics (Spolidorio et al., 2003). There is evidence that toothbrushes, after being used, can be contaminated with different types of bacteria, viruses, yeasts and intestinal parasites (Ditmyer et al., 2011; Kennedy et al., 2003; Paula et al., 2012), and it may serve as a source for inoculation/reinoculation of potential pathogenic microorganisms such as *Streptococcus mutans* (Chaves et al., 2007; Taji & Rogers, 1998). In places such as daycare centers, preschools and other institutions with young children,

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the salivary contact between individuals is hard to avoid or to control. Besides the toothbrush can be changed and/or shared inadvertently (Kumarihamy et al., 2011; Malmberg et al., 1994).

To prevent toothbrush from becoming a reservoir of microorganisms, the ideal is that it should be stored in an appropriate location and subjected to frequent disinfection (Devine et al., 2007). Therefore, research is necessary to find practical, efficient and inexpensive ways to control toothbrushes contamination by streptococci mutans, in order to prevent cross-infection and re-inoculation of microorganisms (Devine et al., 2007). Several methods of brushing are widely described, but the disinfection of toothbrushes is rarely discussed.

Chemical agents have been described for its disinfection such as hydrogen peroxide, essential oils, cetylpyridinium chloride, dentifrices containing triclosan, alcoholic solution of 0.12% to 77% chlorhexidine and 1% sodium hypochlorite. The 0.12% chlorhexidine was described as the most effective (Devine et al., 2007; Sato et al., 2004; Nelson-Filho et al., 2011).

Medicinal plants and herbal medicines have been used as an alternative treatment for dental conditions described in several studies (Ngueyem et al., 2008; Oliveira et al., 2007; Pereira et al., 2006). Species such as pomegranate (*Punica granatum* L.), sage (*Salvia officinalis* L.), marigold (*Calendula officinalis* L.), *Bridelia grandis* Pierre ex Hutch (Euphorbiaceae) and bacupari (*Rheedia brasiliensis* Planch. & Triana) showed activity against oral microorganisms, especially mutans streptococci (Ngueyem et al., 2008). However, there are no studies reporting the effectiveness of solutions made of natural products for the disinfection of toothbrushes.

Species of guaco (*Mikania laevigata* Schultz Bip. ex Baker and *M. glomerata* Sprengel, Asteraceae) present various popular indications. It is commonly used as antiseptic, for fever, syphilis, eczema and itchy skin treatment as well as a healing product (Botsaris, 1997; Matos, 1998). Guaco activity against oral microorganisms involved in the development of caries (*S. mutans* and *S. sobrinus*) was evaluated in several studies (Duarte et al., 2005; Santos et al., 1998; Yatsuda et al., 2005). The ethanol extracts of both *Mikania* showed the same bactericidal activity, with minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC), respectively 25 and 50 µg/mL (Sá et al., 2003; 2010). The pharmacology of these herbal preparations was also evaluated in studies of toxicity *in vivo*. The effect of hydroalcoholic extract of *M. glomerata* was evaluated on the reproductive system of mice for ninety days, with a daily dose of 3.3 g/kg body weight (Sá et al., 2010). No significant change was observed on the reproductive system, indicating that the hydroalcoholic extract of *M. glomerata* neither showed toxic effect nor interfered in the fertility of mice, at the evaluated dose (Sá et al., 2010).

For these reasons, this double-blinded randomized clinical trial aimed to evaluate the efficacy of guaco mouthwashes on the disinfection of toothbrushes used by preschool children, tested positive for mutans streptococci as well as the quantification of its chemical constituents by high performance liquid chromatography.

Materials and Methods

Plant material

Mikania glomerata Sprengel and *Mikania laevigata* Schultz Bip. ex Baker, Asteraceae, were grown under controlled conditions of temperature, humidity and light in the experimental field (21º14'S, 45º 00'W, 918 m altitude) of the Division of Medicinal Plants of the Department of Agriculture, Federal University of Lavras (Castro et al., 2006). Plants were identified by Dr. Mara Ritter from the Institute of Biosciences, Universidade Federal do Rio Grande do Sul where voucher specimens are deposited under registration numbers ICN 141992 and ICN 141990.

The plant material was dried at 40 °C and blended. The water matter, the total ash content and the yield of ethanol extracts from dried plant material were determined as described by the WHO (1998).

The dried plant materials of *M. glomerata* (260 g) and *M. laevigata* (100 g) were subjected to percolation with ethanol. Extracts were concentrated in rotary evaporator at 50 °C under reduced pressure, to the residue, affording respectively, 55 g of *M. glomerata* ethanol extract and 35 g of *M. laevigata* ethanol extract.

Mouthwash preparation

Preparation of the mouthwash solutions of *M. glomerata* and *M. laevigata* was performed according to the commercial formulation of solutions, with modifications. Briefly, 2.5 g of the ethanol extract of each species were diluted in 20 mL of ethanol (Dynamic, Diadema, São Paulo, Brazil). One mL of polysorbate 20 (Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 200 mg of sucrose (Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 10 mL of glycerol (Dynamic, Diadema, São Paulo, Brazil) and ultrapure water (18 Ω, Elga Purifier) *qsp* 100 mL were added to the solutions. The solutions were stored at 4 °C until the moment of use.

Analysis of extracts and solutions by high performance liquid chromatography (HPLC)

HPLC analysis was conducted on a Waters 1515 separation module, equipped with binary pump, and detector UV/VIS (model 2489), and the data were processed by Breeze software. Reverse phase Xbridge® column C18 (150 x 4.6 mm id, 3.5 μm, Waters), was used combined with the pre-column XBridge® C-18 (20 x 4.6 mm id, 3.5 μm, Waters), and flow rate of 0.5 mL/min. The analysis was performed at room temperature with detection at 274 nm. Isocratic elution was used (MeOH: water, 47:53) (Bolina et al., 2009). The solvent used was HPLC grade (Merck, Darmstadt, Germany), ultrapure water (ELGA, 18.2 Ω) and it was degassed by ultrasonic bath before use. Reference solution coumarin (Sigma, St. Louis, MO, USA) and samples (EMG, EML and solutions) were solubilized in MeOH, HPLC grade (Merck, Darmstadt, Germany). The final concentration of samples was 1 mg/mL and the reference solutions were 0.003 to 0.06 mg/mL. Samples and reference solutions were centrifuged at 8400 g for 5 min. Sample and standard solutions were manually injected (20 μL) in the device. Analyses were performed in triplicate. In the quantification of coumarin by HPLC, the regression equation of the standard curve was y=14245586x-6574.0. The linear range was 0.5 to 80 μg/mL, showing detection limit of 2.33 μg/mL and limit of quantitation of 7.76 μg/mL. The correlation coefficient (R) obtained was 0.9967.

In vitro evaluation of antimicrobial activity of solutions of the extracts of M. glomerata *and* M. laevigata *against* S. mutans

Antimicrobial activity was performed as described by Spolidorio et al. (2011) and Taji & Rogers (1998) with modifications to the final reading. The standard strain *Streptococcus mutans* ATCC 25175™ was used. Serial dilutions of the extracts and solutions were carried out with a concentration range from 2.4 to 500 μg/mL, in order to calculate the MIC. To each well, there was added 100 μL of bacitracin sucrose broth, selective enrichment broth (CaSaB20), 100 μL of the inoculum and 50 μL of test solution. Chlorhexidine (0.12%, Periogard, Colgate-Palmolive Ind. Brasileira, Osasco, SP, Brazil) was used as positive control and 10% dimethylsulfoxide (Sigma, St. Louis, MO, USA) as a negative control. The entire solution matrixes (without the addition of extracts) were evaluated in the presence of the inoculum. Coumarin was analyzed in the range of 3.1 to 50.0 μg/mL. The microbiological control of the solutions was also performed by incubating only the test solutions in CaSaB 20. Plates were sealed and incubated at 37 °C for 48 h. After

incubation, 10 μL solution of methyl 3-(4,5-dimethyl-tioazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/mL) (Sigma, St. Louis, Mo, USA) were added to each well and subsequently incubated for additional 3 h. Subsequently, 100 μL of an isopropanol solution acidified with 40 μM HCl (Merck, Darmstadt, Germany) was added to solutions of the experimental and control treatment.

Reading was performed in microplate reader (Reader-TP, Thermoplate) at 595 nm. The assay was performed in triplicate. The MIC was defined as the lowest concentration of test samples which inhibited visible growth of the microorganism tested.

Clinical trial of the use of solutions of M. glomerata *and* M. laevigata *on the disinfection of toothbrushes*

This research project was approved by the local Ethical Research Committee of University of Vila Velha (process #193-2009), and written informed consent was obtained from the parents or legal representatives. Children from a private school located in the city of Vitória (ES, Brazil) were pre-selected to participate in this study. Fifty patients of both genders aged 2 to 5 years old, who had complete primary dentition and good health were enrolled in this study. Children who were using antimicrobial mouthwashes and those who had used antibiotics in the previous three months were excluded from the study.

In addition to these inclusion criteria, children formally tested positive for mutans streptococci in saliva. The determination of the presence of for mutans streptococci in the children's saliva was performed using the Kit Dentocult® SM Strip mutans (Orion Diagnostica Oy, Espoo, Finland). It is a method based on adherence and growth of mutans streptococci in plastic strips. The kit contains tubes with selective medium, bacitracin discs and plastic strips. Saliva samples were collected on the strips directly from the oral cavity of children. Strips were fixed on the lid of the tube and submerged in culture medium with bacitracin. Tubes were identified and incubated at 37 °C for 48 h. After incubation, the growth of mutans streptococci was identified by counting colonies/biofilms present on the strip. Thirty eight children tested positive for mutans streptococci.

Children were randomly assigned using a table of random numbers, to a 4-stage changeover system with a one-week interval between each stage. The four solutions were used in all stages, but each of them was used by a different group of children in each stage to minimize the occurrence of variables that could interfere with the results. At each stage, children were subjected to toothbrushing during 1 min performed by a single professional using Sanifil Leader® kids toothbrush (Facilit Odontológica e Perfumaria Ltda)

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with soft bristles and small head, previously coded.

Four solutions were evaluated in this study: Sterile tap water, 0.12% chlorhexidine gluconate solution (Periogard), 2.5% solution of *M. glomerata* ethanol extract; and 2.5% solution of *M. laevigata* ethanol extract. The solutions were placed in individual plastic trigger spray bottles (Elyplast, São José dos Campos, SP, Brazil) under aseptic conditions.

For the randomized disinfection protocol, toothbrushes (n=96) were maintained in a fixed position at 5 cm from the bottle containing each test solution, which was sprayed on all bristles of the toothbrush, five times on each side (totaling approximately 0.5 mL of solution per toothbrush).

Toothbrushes were maintained in a closed vessel for 4 h at room temperature for drying, simulating the average interval between brushings (Warren et al., 2001), and then sent to microbiological processing, to evaluate the bacterial viability and count the number of colonies/biofilms of mutans streptococci adhered to the bristles.

After the drying time, toothbrushes of each stage were placed individually in Falcon tubes type (TPP Trasadingen, Switzerland) containing 50 mL of CaSaB20 culture medium in an upright position, having the bristles being totally submerged in the culture medium, incubated for four days at 37 °C. After the incubation period, aliquots from each tube were analyzed for viability of the biofilm by means of the MTT colorimetric assay, according to Spolidorio et al. (2011) and Taji & Rogers (1998), with modifications. Afterward, toothbrushes were incubated for another 26 days at 37 °C for analysis of the number of colonies on the bristles.

Briefly, three aliquots (100 μL) of each tube were transferred to a 96-well plate. Ten μL solution of MTT (5 mg/mL) (Sigma Chemical, St. Louis, MO, USA) were added and subsequently incubated for additional 3 h. The final procedure was identical to the one previously described in the in vitro study.

After thirty days, toothbrushes were analyzed for the presence or absence of developing biofilm on the surface of the bristles, with a stereoscopic microscope (Nikon-Japan), under reflected light. Toothbrushes were stained with 0.1% safranin solution (Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil) for 15 min, washed with PBS and air dried. The numbers of MS colonies/biofilms on the toothbrush bristles after microbial culture were counted by three calibrated observers and expressed according to a ranked scale, as suggested by Nelson-Filho et al. (2000): 0, no MS colonies/biofilms or no bacterial growth; 1, 1-50 MS colonies/biofilms; 2, 51-100 MS colonies/biofilms; 3, >100 MS colonies/ biofilms (intense bacterial growth, with confluent colonies, not allowing accurate counting of the colonies/biofilms).

As additional control, five toothbrushes were removed from their original containers and subjected to microbiological processing, without being used. This procedure was performed in order to verify that the toothbrush did not have any contamination from the manufacturing process and packaging industry.

At the end of the experiment, all children received new toothbrushes and toothpaste without fluoride. It was also given educational lectures on mechanical control of the biofilm and the care of toothbrushes. The examiners were blinded to the groups during microbiologic analyses.

Statistical analysis

Data from the *in vitro* antimicrobial effect of extracts and solutions of *Mikania* as well as data from the clinical trial for disinfection of toothbrushes were expressed as standard error of the mean (SEM). The presence of normal distribution was estimated using the Shapiro-Wilk test. For analysis of the in vitro antimicrobial effect and for the concentration of coumarin, one way analysis of variance (ANOVA) was applied. The statistical significance of inhibiting bacterial growth was determined by Student's t test. Kruskal-Wallis one-way ANOVA followed by Dunn's test were applied to compare treatments for disinfection of toothbrushes. Differences were considered significant when $p<0.05$. The statistical non-parametric Friedman test was applied to verify possible differences between the solutions relating to inhibition or formation of cariogenic biofilm on toothbrush bristles after thirty days. Statistical analyzes were carried out using free software Tanagra and the software GraphPrism® (Prism 5 for Windows, version 5, 2007).

Results

Water matter, ashes and ethanol extractable matter analysis were performed for the plant material and results are shown in Table 1. Results for coumarin quantification of in the various samples are shown in Table 2.

Table 1. Results from pharmacognostic analysis of *Mikania glomerata* and *M. laevigata*.

Assay	*M. glomerata*	*M. laevigata*
Water matter (% g/g)	8.4±0.2	7.7±0.2
Ash (% g/g)	2.2±0.2	7.9±0.2
Extractable matter with ethanol (% g/g)	47.3±2.1	44.0±1.3

Table 2. Coumarin content in plant raw material, ethanol extracts and solutions of *Mikania glomerata* and *M. laevigata,* by high performance liquid chromatography (HPLC).

Sample	*M. glomerata*	*M. laevigata*
plant raw material	< LOQ	0.12±0.02 %
ethanol extract	< LOQ	0.32±0.05 %
ethanol extract mouthwash (2,5%)	< LOD	0.04±0.00 %

MIC values for the ethanol extract and solution of *M. glomerata* were 400 and 125 µg/mL, respectively, whereas those for the ethanol extract and solution of *M. laevig*ata were 400 and 14 µg/mL, respectively. The MIC for coumarin was 15 µg/mL.

Out of the 38 children initially enrolled in the study, 24 participated (63.2% of children); fourteen children did not attend all stages due to the absence in the school during the days of the study, being excluded from the trial. Ninety six toothbrushes were evaluated.

Colonies/biofilms of MS were present in the culture medium of all toothbrushes after a single brushing. Toothbrushes of the additional control (n=5) showed no microbial contamination. The results of microbiological analysis of toothbrushes after disinfection are shown in Figure 1. All tested mouthwash solutions were able to reduce the formation of mutans streptococci: chlorhexidine 50.7±17.7%; *M. glomerata* 37.3±23.7% and *M. laevigata* 28.7±25.1%, compared to the group that received the normal cycle of brushing and disinfection with water (Figure 1). There was no difference among the treatments with guaco solutions (*M. glomerata* and *M. laevigata*). However, treatment with 0.12% chlorhexidine was statistically similar to *M. glomerata* solution but different from the treatment with the solution of *M. laevigata* in the confidence level of 95%.
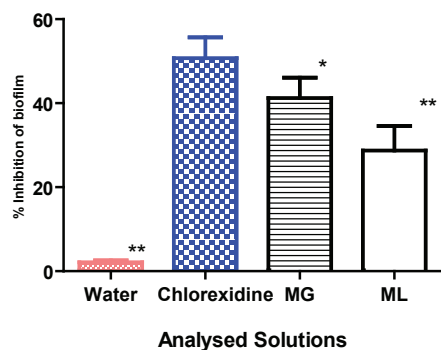

Figure 1. Microbiological evaluation of toothbrushes after use of the mouthwash solutions. MG (*Mikania glomerata* 2.5%) *similar to chlorhexidine (*p*>0.05), ML (*Mikania laevigata* 2.5%) and water **differs from chlorhexidine (*p*>0.05).

After 30 days of incubation, MS colonies/biofilms were observed on all (100%) toothbrushes and there was no statistical difference among treatments

(Figure 2). The microbiological processing of the five new toothbrushes showed no turbidity of the medium within 30 days of incubation.
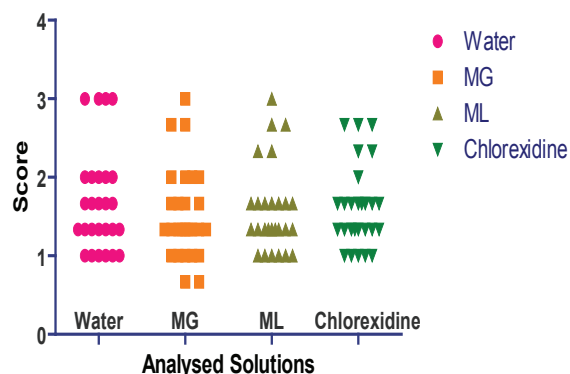

Figure 2. Evaluation of colonies/biofilms on toothbrushes after thirty days of the treatment with mouthwashes. MG (*Mikania glomerata* 2.5%) ML (*Mikania laevigata* 2.5%).

Discussion

Many studies have reported contamination of toothbrushes after a single brushing, by different bacteria, viruses and yeasts from the oral cavity or from the environment (Nelson-Filho et al., 2000; Quirynen et al., 2001; Quirynen et al., 2003; Sato et al., 2004; Yoshikawa et al., 2007). Therefore, mechanisms and substances for disinfecting toothbrushes have been evaluated in order to avoid, under normal storage conditions, inoculation/reinoculation of opportunistic or pathogenic microorganisms in the oral cavity (Yoshikawa et al., 2007; Devine et al., 2007; Nelson-Filho et al., 2004; Nelson-Filho et al., 2011).

Severe early childhood caries (ECC) in preschool age is related to a rich microbiota, with *S. mutans* among the main species associated with the development of this disease (Tanner et al., 2011). Since the control of *S. mutans* is essential for the maintenance of oral health (Rosa & Sanches, 2000), the contamination of toothbrushes by this group of microorganisms was evaluated.

The high percentage of children tested positive for mutans streptococci in this study may be related to the transmission of cariogenic microbiota. This can occur directly through saliva or through objects such as toothbrushes, pacifiers, dentures or removable orthodontic appliances (Caufield et al., 1993; Köhler & Bratthall, 1978; Tedjosasongko & Kozai, 2002) that may serve as a source for inoculation/reinoculation of microorganisms with pathogenic potential.

Therefore, the disinfection of toothbrushes could prevent cross-infection and reinoculation of microorganisms and reduce the contamination of surfaces, especially in the period called by Caufield et al. (1993) as the first "window of infectivity". For

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this reason, the age group of 2-5 years old was chosen in order to evaluate the microbial contamination of toothbrushes after use.

It is also important to say that the reduction in contamination of the toothbrush may occur due to the presence of toothpastes during brushing, which can have in their composition antibacterial agents such as fluorine and triclosan (Quirynen et al., 2003; Nelson-Filho et al., 2004). For this reason, toothpaste was not used during brushing, in order to avoid interference in the contamination of toothbrushes by mutans streptococci, and to evaluate the effectiveness of proven solutions for disinfection.

The present study aimed to evaluate the contamination of toothbrushes by mutans streptococci and the efficacy of four solutions (water, 0.12% chlorhexidine, 2.5% ethanol extract of *M. glomerata* and 2.5% ethanol extract of *M. laevigata* solutions) in the disinfection of toothbrushes in children 2-5 years. Chlorhexidine (0.12%, Periogard) was used as positive control and water as negative control.

Although some studies have shown promising results when using chlorhexidine to disinfect toothbrushes (Nelson-Filho et al., 2011), the results of the present study showed that chlorhexidine reduced by 50.7±17.7% the formation of mutans streptococci. This could be explained by the different method used in the study, since MTT colorimetric assay provides the presence of all viable bacteria present in brushes, even if there is not a colony formed yet. The MTT assay is a fast colorimetric method with high accuracy.

The *M. glomerata* mouthwash had the same efficacy of chlorhexidine, considered the gold standard. Given the disadvantages of chlorhexidine after prolongued oral use (teeth/tongue staining, increased tartar, change in taste, burning mouth sensation, mouth irritation), a mouthwash of *M. glomerata* could be useful in herbal strategy programs against mutans streptococci.

The result of 100% positive cultures in the toothbrushes of the control group of this study, which was sprayed only with water, increases the need to disinfect the brush after use, since the interval of four hours drying time between brushing is not sufficient for removal of microorganisms.

M. glomerata and *M. laevigat*a species showed water matter and ashes within the limits described in Brazilian Pharmacopoeia (2005), that report maximum values of 10% and 15%, respectively, for *M. laevigata*. Coumarin levels in samples of *M. laevigat*a (Table 2) complied with the required by the Brazilian Pharmacopoeia (2005) for guaco-cheiroso (min. 0.1%). For the extract and solution no data were found for comparison. Coumarin was detected in samples of *M. glomerata* (Table 2), but in concentrations bellow the

limit of quantification established for the method. The presence of coumarin in samples of *M. glomerata* has been reported in several articles (Castro et al., 2006), as well as its absence (Bertolucci et al., 2009).

The effectiveness of *in vitro* antimicrobial activity of guaco (*M. glomerata* and *M. laevigata*) of this study was lower than previously reported (Yatsuda et al., 2005), which may be related to chemical composition and method of preparation of the extracts and solutions. Both 2.5% *M. laevigata* mouthwash and coumarin showed a MIC value 8-fold smaller than 2.5% *M. glomerata* mouthwash. However, the efficacy of the solutions of guaco mainly prepared with *M. glomerata*, as disinfecting agents of toothbrushes is first reported by this study. This discrepancy could be explained by the variation of strains of *S. mutans* which depend on the oral/ dental environment of each individual (Holbrook & Magnúsdóttir, 2012). The antimicrobial activity of *M. glomerata* reported by Yatsuda et al. (2005) was not correlated with the presence of coumarin in the extract, which was also observed in this study, since this compound could not be quantified in the *M. glomerat*a samples (Table 2). Nevertheless, the effect of 2.5% M. laevigata mouthwash could be related with coumarin. Both *guaco* solutions reduced the number of colonies/ biofilms on toothbrushes incubated for thirty days, suggesting that the effect of these solutions remain during the storage time of the brushes during normal use.

Based on the results of this study, it may be concluded that the solution of 2.5% of *M. glomerata* and 2.5% of *M. laevigata* decreased contamination of toothbrushes by mutans streptococci and that *M. glomerata* had the same efficacy of 0.12 % chlorhexidine (gold standard). Therefore, it can be concluded that *M. glomerata* could be useful in herbal strategy programs against mutans streptococci and that the marker coumarin may be not related to the activity observed.

Acknowledgment

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