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Stability and disinfecting proprieties of the toothbrush rinse of the essential oil of *Protium heptaphyllum*

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***Protium heptaphyllum* (PH) is rich in essential oil, has anti-inflammatory properties and has no toxic potential. However, data is unavailable about its antiseptic effect against bacteria that cause caries. This study aimed to evaluate the antiseptic effect of the toothbrush rinse of essential oil of PH as well as its chemical stability. The toothbrush rinse was prepared with 1% essential oil of PH. The minimum inhibitory concentration (MIC) of the essential oil and toothbrush rinse were evaluated against *Streptococcus mutans* (ATCC 25175TM). The *ex vivo* study was double-blinded and randomised; the children were divided into three groups, each participating in a crossover design where all solutions (water, toothbrush rinse (1%) and chlorhexidine (0.12%) were used in all stages by different groups of children. The chemical composition of the essential oil and toothbrush rinse were analysed by gas chromatography coupled to mass spectrometry. The stability was evaluated at three time points. The essential oil and toothbrush rinse exhibited antimicrobial activity against *S. mutans*, MIC = 0.125 and 2.4 µg/ml. The toothbrush rinse showed the same effect as chlorhexidine on disinfecting the toothbrushes contaminated with mutans streptococci (pH = 57.3 ± 5.3%; chlorhexidine 55.5 ± 13.3%; water 39.4 ± 5.8%; p > 0.05). Chromatographic analysis showed that the essential oil contained monoterpenes as a major component, and the toothbrush rinse possessed the same constituents as the pure essential oil, except for α-terpineol. Storage did not cause chemical degradation of the toothbrush rinse, but decreased the concentration of the chemical constituents. The toothbrush rinse of essential oil of *P. heptaphyllum* showed antiseptic properties and exhibited antimicrobial activity against mutans streptococci.**

Key words: Mutans streptococci, *Protium heptaphyllum*, antimicrobial, monoterpenes, chemical composition, contamination of toothbrushes.

INTRODUCTION

Oral diseases such as caries and periodontitis in children and adolescents have an impact on the quality of life of

these individuals (Paula et al., 2012; Gherunpong et al., 2004). Dental caries is a multifactorial disease following Keyes's trilogy, which is based on substrate (teeth), microorganisms and diet (Keyes, 1960; Kumarihamy et al., 2011). The frequency and times when these factors of Keyes's trilogy interact determine the severity of the injury due to the effect of the acid on the mineral forming the tooth, resulting in the destruction of the tooth (Carounanidy and Sathyanaraynan, 2009). When left untreated, tooth decay can cause abscesses, pain and malocclusion as well as the loss of deciduous teeth (Kumarihamy et al., 2011).

There are more than 500 microbial species in the oral cavity (Paster et al., 2001). The tooth has an ideal environment for those species, and they live in synergism and antagonism in the biofilm in a form of homeostasis (Marsh and Devine, 2011). The primary microorganisms on the surface of the tooth belong to the mutans streptococci group, *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus mitis* and *Streptococcus mutans* (Carounanidy and Sathyanaraynan, 2009). The most common activities used to reduce the oral microbial load are brushing one's teeth with toothpaste, rinsing with mouthwash containing antiseptic solutions and dipping the brush in antiseptic solutions, such as chlorhexidine, xylitol and fluorine (Nelson-Filho et al., 2011; Subramaniam and Nandan, 2011; Efstratiou et al., 2007; Mehta et al., 2007). The latter is needed to avoid re-contamination, mainly because the brush rinse reduces the degree of contamination but also because the residual pathogens remain active. To minimise contamination on a toothbrush, the disinfection process should be initiated immediately after it is unpacked, and a daily routine of applying antiseptics should be maintained to prevent the formation of bacterial biofilms (Neal and Rippin, 2003; Nascimento et al., 2012).

Antiseptic solutions are capable of preventing the adhesion of bacteria on the surface of the teeth and their subsequent colonisation; hence, they inhibit bacterial growth (Subramaniam and Nandan, 2011). However, only a few studies of natural products in dentistry have been conducted, though these natural products have recently received more attention. The primary objective of those published studies was to find, identify and evaluate substances exhibiting antibacterial and antifungal activities (Santos et al., 2009).

A study by Santos et al. (2009) reports several plants used by a population for dental use, such as the pomegranate (*Punica granatum* L.), purple cashew (*Anacardium occidentale* L.), juá (*Zizyphus joazeiro* Mart),

mint leaf (*Plectranthus amboinicus* (Lour) Sprengel) and mastic (*Schinus terebinthifolius* Raddi). These plants are used because they are known for their analgesic, anti-inflammatory and wound healing activities (Santos et al., 2009). However, despite being used as a medicinal plant by many people, the majority of these plants have not yet been evaluated sufficiently in terms of their bactericidal activity. A study by Ramos et al. (2009) reported the use of essential oils and plant extracts, such as *S. mutans*, for inhibiting the growth of fungi and bacteria. Recently, our group reported the effectiveness of using a mouthwash containing guaco (*Mikania glomerata* Sprengel and *Mikania laevigata* Sch. Bip. ex Baker) to disinfect toothbrushes (Lessa et al., 2012).

The species *Protium heptaphyllum* (Aubl) March (Burseraceae family) is popularly known as putty or pitch black and is distributed in various regions of Brazil, such as the North, Northeast, Southeast and Midwest areas (Pinto et al., 2008; Vieira-Junior et al., 2005). In those regions, this species is popularly used for healing as well for an expectorant, anti-ulcerogenic and anti-inflammatory (Vieira-Junior et al., 2005). The anti-nociceptive and anti-inflammatory activities of individual components of resin *P. heptaphyllum*, such as triterpenes α - and β -amyrin, were evaluated in animal models, which had been induced with periodontitis and gingivitis (Pinto et al., 2008).

In this study, we hypothesised that the essential oil extracted from the resin of *P. heptaphyllum* and the toothbrush rinse produced from the oil exhibited antimicrobial activity and that the formulation would be stable. However, the use of toothbrush rinse prepared with essential oil extracted from the resin of *P. heptaphyllum* for the disinfection of toothbrushes has not yet been evaluated. To improve the potential for clinical application of the *P. heptaphyllum* essential oil toothbrush rinse, a formulation was prepared, and its chemical stability was evaluated for 12 months. Thus, the aim of this study was to evaluate the effect of the *P. heptaphyllum* essential oil toothbrush rinse in the disinfection of toothbrushes and to evaluate the chemical stability of the formulation over 12 months.

MATERIALS AND METHODS

Plant

The stem exudate of the *P. heptaphyllum* (Aubl.) species was collected in Guriri, São Mateus, Espírito Santo, Brazil, in May, 2010. Voucher specimens of the samples were prepared for subsequent botanical identification in the herbarium of the University Vila Velha

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Table 1. Experimental design of the crossover study (n=7).

Solutions	Week 1	Week 2	Week 3	Week 4	Week 5
Water	Group I		Group III		Group II
Rinse <i>P. heptaphyllum</i> 1%	Group II	Wash-out period	Group I	Wash-out period	Group III
Chlorhexidine 0.12%	Group III		Group II		Group I

- UVV (UVV / ES 1802).

Extraction of the essential oil

The extraction of the essential oil was performed using hydrodistillation, maintaining a minimum temperature required for boiling; the method used was based on that published by Skrubis (1982) and Ming et al. (1996), with modifications. The extraction process took two hours to complete. The hydrolate was kept in a dark environment at 4°C. The oily fraction was filtered using anhydrous magnesium sulphate. The oil was stored at 4°C in amber vials under critical cooling.

Preparation of essential oil toothbrush rinse *P. heptaphyllum*

The toothbrush rinse containing the essential oil of the species *P. heptaphyllum* was prepared by following the formulation methods of commercial mouthwashes, with modifications. Briefly, 10 g of essential oil were weighed and added to 10 ml polysorbate 20 (Vetec-Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 2 g sucrose (Vetec-Sigma-Aldrich, Duque de Caxias, Rio de Janeiro, Brazil), 100 ml glycerine (dynamic, Diadema, São Paulo, Brazil) and ultrapure water (18 W, Elga Purifier) qsp 1,000 ml. The toothbrush rinse was separated into aliquots of 100 ml. One aliquot was submitted to microbiological assays and *in vivo* testing of disinfection, and the remaining aliquots underwent evaluation of stability and were stored at different temperatures and locations. The samples used to evaluate the biological activity were stored at 4°C until analysed.

Evaluation of *in vitro* antimicrobial activity of the essential oil toothbrush rinse and *P. heptaphyllum* against *S. mutans*

The antimicrobial activity analysis was performed as described by Zarai et al. (2011), with modification. The standard strain used was ATCC 25175™ *S. mutans*. We performed a serial dilution of the toothbrush rinse with a concentration range of 100 to 0.01 µg/ml to calculate the minimum inhibitory concentration (MIC) (Lessa et al., 2012). In total, 100 µl bacitracin sucrose broth (SB20), a selective enrichment broth (Davey and Rogers, 1984), 100 µl of the inoculum and 50 µl of the test solution were added to each well. As a positive control, we used 0.12% chlorhexidine solution (Periogard, Colgate, São Paulo, São Paulo, Brazil) and 10% dimethyl sulphoxide (Sigma, St. Louis, Mo., USA) as a negative control. We also evaluated the entire toothbrush rinse solution matrixes (without the addition of essential oil) in the presence of inoculum. The microbiological control solutions were also prepared by incubating the test solution in only SB20. The plates were sealed and incubated at 37°C for 48 h. After incubation, a 10 µl solution of methyl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT, 5 mg/ml) (Sigma, St. Louis, Mo, USA) was added to each well and incubated for an 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 treatments. The analysis was performed using a microplate reader (Reader-TP, Thermoplate) at 595 nm. The assay was performed in triplicate. The MIC was defined as the lowest concentration of the tested samples to inhibit visible growth of the microorganism tested.

Clinical trial for the use of *P. heptaphyllum* toothbrush rinse to disinfect toothbrushes

This research project was approved by the local Research Ethics Committee at the University of Vila Velha (CAAE N° 03487312.2.0000.5064), and informed consent was obtained from parents or legal guardians. Children of a private school located in the city of Vitória (ES, Brazil) were preselected to participate in this study. Fifty subjects were included in this study, which consisted of both sexes, 2 to 5 years of age, who had complete primary dentition and were in good health. Children who were using antimicrobial mouthwash, who had used antibiotics in the last three months or who were not present in all phases of the study were excluded from the study. The inclusion criteria were that children should have mutans streptococci in their saliva. The presence of mutans streptococci in children was identified using the kit Dentocult® SM Strip mutans (Orion Diagnostica Oy, Espoo, Finland), which is specific for confirming the identity of this group of microorganisms in saliva (Lessa et al., 2012).

Children were randomly assigned to one of three groups, using a table of random numbers. The study consisted of a crossover design with a washout period of one week. The test was conducted in three stages, where the three solutions (sterile tap water, chlorhexidine 0.12% and toothbrush rinse of *P. heptaphyllum* 1.0%) were used in all stages, but each of them was rotated to a different group of children in each stage to minimise the occurrence of variables that could interfere with the results (Table 1). Shortly before the evaluation, the solutions were placed in individual plastic trigger spray bottles (Elyplast, São José dos Campos, SP, Brazil) under aseptic conditions, wrapped with aluminium foil and identified with numbers. The identity of each solution was only known by the person who prepared the solutions, who did not participate in any stage of the clinical experiment.

At each stage, the children received new toothbrushes with soft bristles and small heads (Infantil Leader Macia, Sanifil) that were properly coded. The children were subjected to tooth brushing, which was performed by a single professional without dentifrice in a position suggested by Starkey (1961), always following the same sequence. The brushing time was one minute, and time was kept with a standardised digital stopwatch (Lessa et al., 2012). After brushing, the toothbrushes were carefully rinsed in tap water, and the excess water was removed from the bristles by slightly beating the handle of the toothbrush against the edge of the sink. Then the

practitioner suspended the toothbrushes in a vertical position with the head facing up and at a distance of, at most, 5 cm between the brush head and the bottle. Each solution was sprayed on the bristles five times, totalling approximately 0.5 ml per toothbrush (Lessa et al., 2012).

The toothbrushes were kept in a closed vessel for 4 h while drying, simulating the average interval between brushings (Warren et al., 2001). After drying, the toothbrushes were kept in 50 ml SB20 culture medium in an upright position with the bristles totally submerged in the culture medium; the toothbrushes were incubated for four days at 37°C. After the incubation time, aliquots from each tube were submitted for an analysis of bacterial viability. Three aliquots (100 µl) of each tube were transferred to a 96-well plate, and 10 µl solution of MTT (5 mg/ml) (Sigma Chemical, St. Louis, MO, USA) was added; the solution was subsequently incubated for an additional 3 h. The final procedure was identical to the one previously described in the *in vitro* study.

As an additional control, five toothbrushes were removed from their original containers and subjected to microbiological processing without being used. This procedure was performed to verify that the toothbrushes were not contaminated from the manufacturing process and industrial packaging (Nascimento et al., 2012).

Analysis of the chemical constituents

Analysis of the chemical constituents of the oil and the toothbrush rinse of *P. heptaphyllum* was performed using a gas chromatograph (Trace Ultra, Thermo Scientific®) coupled to a mass spectrometer (MS) (DSQII, Thermo Scientific®). The samples were separated using a capillary column DB-5 ms (30 m × 0.25 mm id × 0.25 µm, J & W Scientific, Folsom California, USA). The temperature program was initiated at 60°C and increased to 240°C by a temperature ramp of 3°C/min. The final temperature was maintained for 5 min. Helium gas was used with a constant flow rate of 1 ml/min. The injector temperature was maintained at 220°C, and the temperature of the interface (GC/MS) was maintained at 250°C. The mass detector operated by ionisation with electron impact (+70 eV) using the scan mode and was held at 35 to 450 m/z. The detector voltage was set to 1.6 kV. The samples were injected into the GC/MS in duplicate and diluted in hexane (2 mg/ml), and the injected volume was 2.0 µl. The identification of the substances contained in the oil was performed by comparing similarities between data obtained in this study with mass spectra obtained from the literature (Adams, 1995). The relative percentages of these compounds were calculated from the mean areas of the chromatograms obtained.

Evaluation of the stability of essential oil toothbrush rinse of *P. heptaphyllum* with respect to time and temperature

The aliquots of toothbrush rinse *P. heptaphyllum* were submitted to different treatments: (i) extract control (CE), where the toothbrush rinse was immediately extracted and analysed at the rate it was produced; (ii) storage under controlled temperature (A2), where the temperature remained between 18 and 23°C and relative humidity between 35 and 50% (the analysis was performed after two months of storage); (iii) negative control (NC), consisting of aliquots prepared and stored in a refrigerator for one hour at a controlled temperature (2 to 8°C); (iv) positive control (PC), where aliquots were subjected to 50°C in a double boiler for two hours; and (v) storage control at room temperature (A12), where the samples were stored for 12 months at room temperature (18 to 23°C and relative

humidity between 35 and 50%). After these treatments, the samples were subjected to the extraction process consisting of adding 2 ml of hexane (high performance liquid chromatography (HPLC) grade), vigorously shaking and separating by centrifugation at 1588 × *g*. The supernatant was removed from an aliquot of 100 ml and added to a vial containing 890 ml of HPLC grade hexane, 10 L of internal standard and a blend of C7 to C32 alkanes (Sigma). The samples were injected using the method used to identify the constituents present in the essential oil. The stability of the substances was assessed by examining the ratio of the area of each substance and its internal standard area (Aa/Ai). Among the substances identified in the essential oil, five were selected according to their relative percentage. The substances selected, the internal standards and the ions used to obtain the area are described in Table 2.

Statistical analysis

Data from the study on the antimicrobial effect of the essential oil toothbrush rinse and *P. heptaphyllum in vitro* and the data from the toothbrush rinse treatment of *P. heptaphyllum* and chlorhexidine in disinfecting brushes were expressed as the mean ± standard error of the mean (SEM). We applied the Shapiro-Wilk normality test in the data sets. The differences were considered significant at $p < 0.05$. Comparisons between treatments for disinfecting brushes were applied using the Kruskal-Wallis one-way analysis of variance (ANOVA) followed by the Dunn's test. The differences were considered significant at $p < 0.05$. The statistical non-parametric Friedman test was applied to investigate possible differences between the solutions with respect to the inhibition or absence of cariogenic biofilm formation on the bristles of the brushes. To compare the ratio of the Aa/Ai's stability, a one-way ANOVA test was applied followed by Tukey's test. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using free software and Tanagra GraphPrism® software (Prism 5 for Windows, version 5.00, 2007).

RESULTS

The MIC values against *S. mutans* for the essential oil and the toothbrush rinse of *P. heptaphyllum* were 0.13 and 2.4 µg/ml, respectively. The base toothbrush rinse showed no antimicrobial activity when in the presence of the inoculum; nor did the base toothbrush rinse show microbial growth when evaluated only in the presence of the growth medium. Of the 50 children initially enrolled in the study, 39 (78% children) tested positive for mutans streptococci (MS). However, the study was completed with 21 children, because 18 children did not attend all stages of the study (school absence during study days); thus, these students were excluded from evaluation, which was the main limitation of the study. In total, sixty-eight brushes were evaluated.

Contamination of the brushes was observed in all toothbrushes after a single brushing. The additional control toothbrushes ($n = 5$) showed no microbial contamination. The toothbrush rinse containing the essential oil of *P. heptaphyllum* was able to reduce the formation of MS. The results of the microbiological ana-

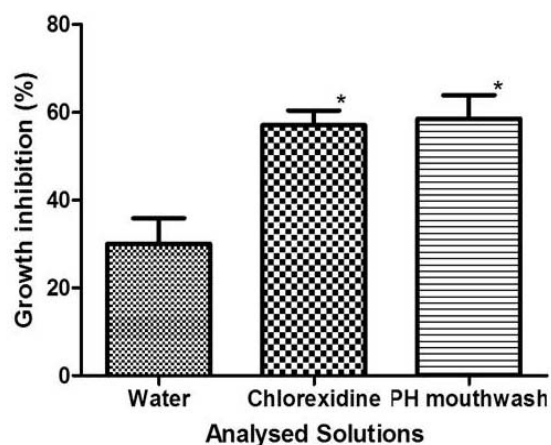


Figure 1. Microbiological evaluation of toothbrushes contaminated with mutans streptococci after use of toothbrush rinse containing the essential oil of *P. heptaphyllum* 1%. PH (*Protium heptaphyllum* 1%)* difference compared to water ($p < 0.05$).

Table 2. List of substances used for monitoring the stability of the toothbrush rinse, the internal standard and the ions (m/z).

Compound	Ions	Internal standard	Ions
p-Cymene	119	Nonane (C9)	57
Terpinolene	93	Nonane (C9)	57
p-Cymen-8-ol	135	Decane (C10)	57
α -Terpineol	121	Decane (C10)	57
Carvenone	95	Undecane (C11)	57

lysis of the toothbrushes after disinfection are shown in Figure 1. The essential oil composition of *P. heptaphyllum* showed predominant monoterpenes, with terpinolene as the primary constituent (35.1%), followed by p-cymene (26.66%), tricyclene (11.05%) and p-cymen-8-ol (10.12%) (Table 2). The toothbrush rinse containing the essential oil of *P. heptaphyllum* 1% consisted of the same constituents, except for the α -terpineol. The toothbrush rinse of *P. heptaphyllum* was a clear, colourless liquid, with the characteristic odour of pure oil. To test the stability of the toothbrush rinse, some substances were monitored, such as terpinolene, p-cymene, p-cymen-8-ol, α -terpineol and carvenone, because of their larger relative area (Table 3). Because the substance tricyclene was the first peak on the chromatogram, it was not monitored. In addition, the extraction process interfered with the relative area of α -terpineol as it was eliminated in this process (Figure 2A).

The relative amount of p-cymene was not altered by

the extraction process or the change in temperature. However, storage for 2 to 12 months resulted in a decrease of its relative area (Figure 2B). For substances p-cymen-8-ol, terpinolene and carvenone, the extraction process led to losses of these products (Figure 2C, D and E), and storage for 2 and 12 months influenced their volatility. Therefore, a period of 12 months in storage had the greatest influence on the loss of p-cymen-8-ol (Figure 2D). The overlap of all of the chromatograms obtained showed the same chromatographic profile for all samples with reduced peak areas (Figure 3).

DISCUSSION

The present study demonstrated the antimicrobial activity of essential oil extracted from the resin of *P. heptaphyllum* and a toothbrush rinse containing 1% of the essential oil. The data indicated that the chemical constituents of the essential oil of *P. heptaphyllum* exhibit antimicrobial activity against gram-positive *S. mutans*, the primary cariogenic bacteria tested. The use of this toothbrush rinse was also evaluated as an alternative treatment to disinfect toothbrushes. Several studies have evaluated substances that could be used as disinfectants for toothbrushes, seeking an easy, economical and safe use for disinfecting them and thereby maintaining oral health (Quintas et al., 2015; Nelson-Filho et al., 2011; Zarai et al., 2011; Juiz et al., 2010; Efstratiou et al., 2007; Mehta et al., 2007).

The present study showed the *in vitro* and *ex vivo* effects of *P. heptaphyllum* mouthrinse against the gram positive bacteria group MS. The experiment was designed in order to establish the MIC of pure essential oil, which was 0.013%, and thereafter, to estimate the mouthwash concentration, which was about 80-fold concentrated (1.0%), based on the predictable loss of activity and bioviability during the formulation. The MIC of the pH mouthwash was 0.24%, that is about 19-fold bigger than the pure essential oil. It is also important to highlight that with 1% essential oil of *P. heptaphyllum*, the components of the oil will be diluted in a concentration similar to a commercial mouthwash such as listerine (menthol 0.042%, thymol 0.064%, methyl salicylate 0.06% and eucalyptol 0.092%) (Pan et al., 2010; Oyanagi et al., 2012). There are some studies about essential oils mouthwash, several using a 0.2 to 1.4% concentration of essential oil (Kothiwale et al., 2014; Lobo et al., 2014; Quintas et al., 2014) and others in which the final concentration is omitted (Batista et al., 2014). An antiseptic agent to be used in children should not be harmful to the mucosa; its toxicity should be low if ingested accidentally, and it should be free from sugar and alcohol (Subramanian and Nandan, 2011). The toxicity of *P. heptaphyllum* has been evaluated by Siane

Table 3. Composition (shown as a relative percentage) of the essential oil resin of *P. heptaphyllum* and toothbrush rinse prepared with the essential oil of *P. heptaphyllum* and the IK data.

Substance	Relative percentage	IK	RT (min)
Tricyclene	11.05	926	5.6
α-Pinene	4.35	939	7.67
2-Δ-Carene	1.09	1001	7.97
p-Cymene	26.66	1026	8.4
Terpinolene	35.81	1088	10.50
p-Cymen-8-ol	10.12	1183	14.59
α-Terpineol	2.29	1189	15.19
2-Methoxy-α-methylbenzyl alcohol	1.08	NR	16.61
Carvenone	1.27	1252	18.4
Total	96.61	-	-

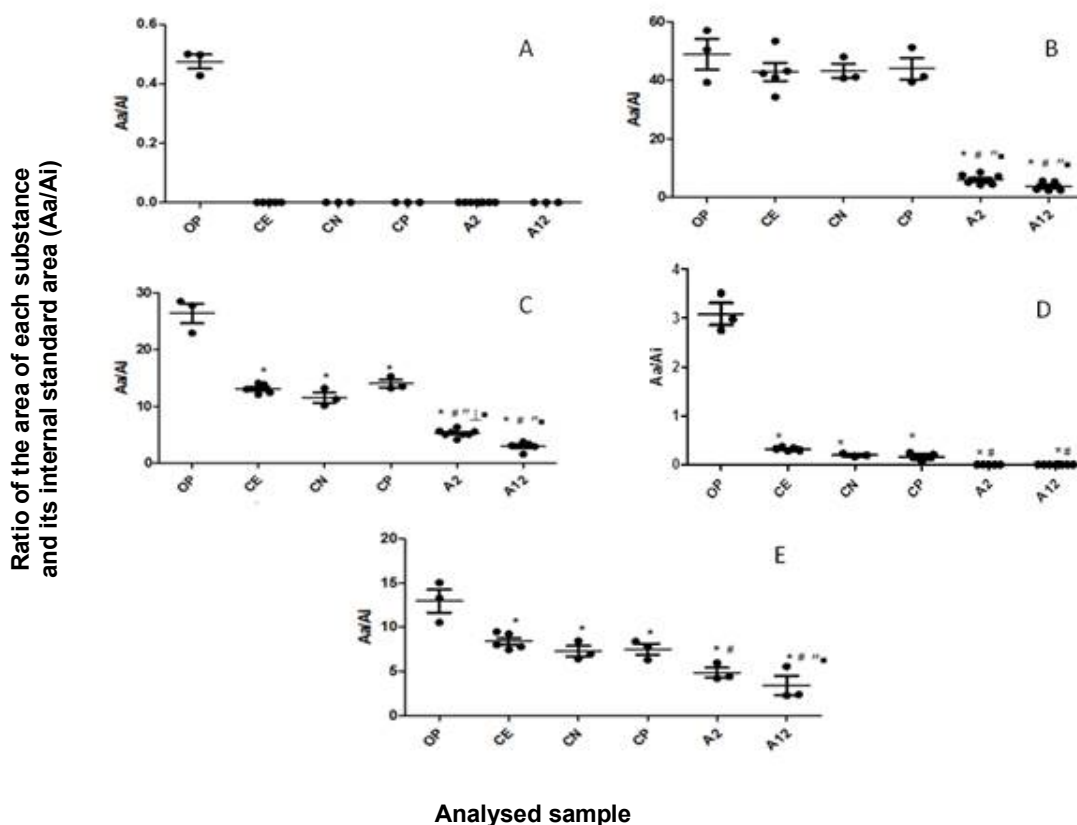


Figure 2. Evaluation of the stability of the oil *Protium heptaphyllum* (OP), the essential oil toothbrush rinse *P. heptaphyllum* (1%) (CE), kept under refrigeration (CN), subjected to heating (CP), stored at room temperature for two months (A2) and 12 months (A12). x-Axis: ratio of the area of each substance and its internal standard area (Aa/Ai) and y-axis: analysed sample. A: α-terpineol. B: p-cymene. C: p-cymen-8-ol. D: carvenone. E: terpinolene. It differs from pure oil *, # EC, ^CN, CP □ A12. p < 0.05.

et al. (1999), who described the resin of *P. heptaphyllum* as showing no toxic potential. Polysorbate 20, a

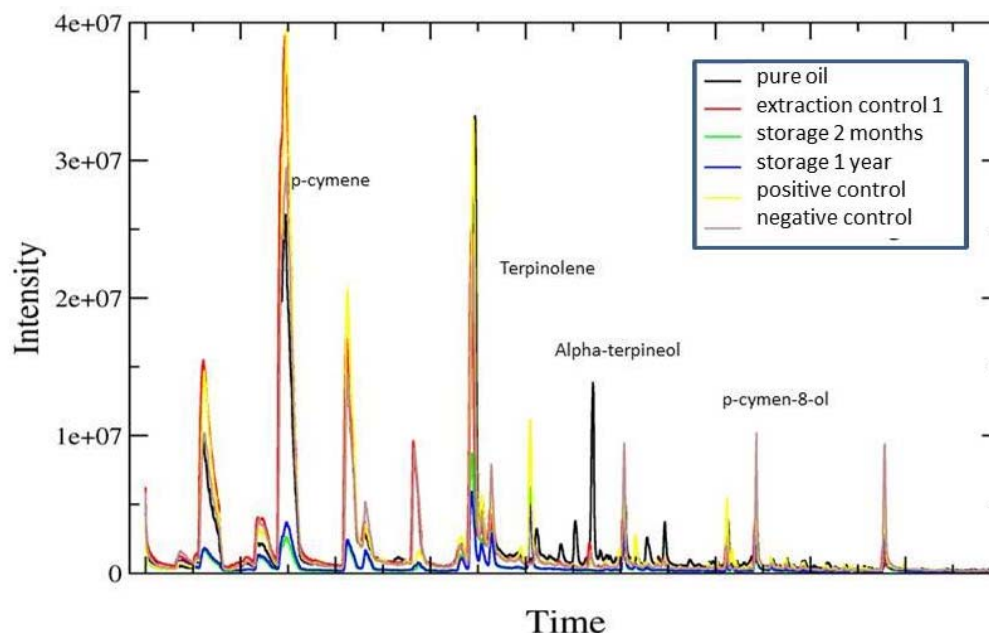


Figure 3. Overlay of chromatograms of aliquots of *Protium heptaphyllum* oil (PO), toothbrush rinse essential oil of *P. heptaphyllum* (1%) (EC), kept under refrigeration (NC), and subjected to heating (PC), stored at room temperature for two months (A2) and 12 months (A12)

solubilising agent, was used in the formulation of the toothbrush rinse because it had no toxic or irritant properties (Cordeiro et al., 2006). Previous trials evaluating mouthwash to disinfect toothbrushes specified that supervised participants used clean and new brushes, which only after brushing were placed in contact with the analyte (do Nascimento et al., 2015; Nelson-Filho et al., 2011; Mehta et al., 2007). This procedure was also adopted in this study.

Contamination of the brushes was observed in all toothbrushes after a single brushing. The toothbrush rinse essential oil of *P. heptaphyllum* showed the same efficacy in disinfecting toothbrushes contaminated with MS as did the gold standard, chlorhexidine. Both treatments were more effective than water (Figure 1). Chlorhexidine is known as the most effective commercial solution and is used as the gold standard in controlling plaque, because it is effective on gram-negative and gram-positive yeast (Nelson-Filho et al., 2011; Subramanian and Nandan, 2011; Juiz et al., 2010; Nelson-Filho et al., 2000). There are several studies which compare either the effectiveness or effect of chlorhexidine-containing mouthrinses and different products on dental plaque, gingivitis and caries prophylaxis, using a one-week period of washout (Charangundla et al., 2014; Lobo et al., 2014; Venu et al., 2013; Barnes et al., 2011; Franco Neto et al., 2008). The

studies showed that chlorhexidine significantly reduced the mutans streptococci group or other microorganisms' levels, but these levels returned to baseline (Lobo et al., 2014). The effectiveness of chlorhexidine after a 12 h application was also evaluated (Tomás et al., 2013), showing that after 12 h, the effect decreases. Since in the studies with one week washout, a difference between groups (for example, water and chlorhexidine) was observed, it is reasonable to use this period of washout in the present study. At low concentrations, chlorhexidine is bacteriostatic, while in high concentrations, it is bactericidal (Subramanian and Nandan, 2011). However, the use of chlorhexidine is associated with detrimental effects, such as a darkening of dental enamel, hyperplasia of the tongue papillae and loss of sense of taste (Juiz et al., 2010). Due to these effects, a search for other agents that have beneficial effects similar to those of chlorhexidine has been increasing.

Cordeiro et al. (2006) described the importance of using medicinal plants to support other therapies and routine prophylaxis. Batista et al. (2014) reported the use of mouthwashes of chamomile and pomegranate extracts and suggested that both extracts have anti-inflammatory and antimicrobial properties. In a recent study conducted by our group (Lessa et al., 2012), a mouthwash known as guaco was prepared with ethanolic extracts of *Mikania laevigata* and *Mikania glomerata*. It showed antimicrobial

activity against *S. mutans* and was evaluated as a toothbrush sanitiser; mouthwash prepared with *M. glomerata* had the same efficacy as the mouthwash with chlorhexidine 0.12% (gold standard).

A search for chemical markers is essential for assessing and maintaining quality. The results found for the essential oil of *P. heptaphyllum* (Table 3) are consistent with work previously described, where the components α -pinene, terpinolene, p-cymen-8-ol and α -cymene were also reported as major components of the *P. heptaphyllum* oil (Marques et al., 2010; Siane et al., 1999). However, some differences were observed in relative percentages and may be related to genetic factors, the nutritional status of the plant and the soil and climatic conditions (Figueiredo et al., 2012); medicinal plants that are derived from the same species may exhibit significant differences in quality when collected at different sites (Asghari et al., 2012). The temperature did not seem to be a factor that influenced the degradation of the substances; however, volatilisation of the chemicals might have contributed to losses, since the overlap of all chromatograms obtained observes the same chromatographic profile for all samples, with a reduction in peak area (Figures 2 and 3).

A surfactant was added to the formulations in this study (polysorbate 20) because essential oils are notoriously volatile and quickly evaporate off of surfaces (Rojo et al., 2012). However, the presence of this fixative at a concentration of 1% did not appear to be adequate in maintaining the composition for more than two months. A change in the amount of this surfactant would not be indicated, because higher concentrations of this product exhibit potential antimicrobial activity (Cordeiro et al., 2006).

Based on the results of this study, the essential oil of *P. heptaphyllum* and the toothbrush rinse made from this oil exhibited antimicrobial activity against the causative agent of caries, *S. mutans*. The effectiveness of the toothbrush rinse *P. heptaphyllum* is equal to that of chlorhexidine (0.12%). The stability of the toothbrush rinse *P. heptaphyllum* was not influenced by temperature, but was influenced by the storage time. However, the substances were volatilised instead of degraded.

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Conflict of interest

There is no conflict of interest as regard this study.

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