Original Research
Periodontics

Anti-biofilm and anti-inflammatory effect of a herbal nanoparticle mouthwash: a randomized crossover trial

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Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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https://doi.org/10.1590/1807-3107bor-2019.vol33.0062

Submitted: May 22, 2018
Accepted for publication: September 18, 2019
Last revision: October 9, 2019

Abstract: Laboratory evidence has demonstrated the antimicrobial effect of *Melaleuca alternifolia* (MEL) against oral microorganisms. This randomized, double-blind, crossover clinical trial, compared the anti-biofilm and anti-inflammatory effects of MEL nanoparticles with 0.12% chlorhexidine gluconate (CHX) on biofilm-free (BF) and biofilm-covered (BC) surfaces. Before each experimental period, the participants refrained from all oral hygiene practices for 72 hours. The 60 participants were randomly assigned to professional prophylaxis in two quadrants (Q1–Q3 or Q2–Q4), and rinsed with MEL or CHX for four days. The Quigley & Hein plaque index (QHPI), gingival crevicular fluid (GCF) volume, and participants' perceptions were assessed. CHX showed significantly lower mean QHPI on BF (2.65 ± 0.34 vs. 3.34 ± 0.33, $p < 0.05$) and BC surfaces (2.84 ± 0.37 vs. 3.37 ± 0.33, $p < 0.05$). Intragroup comparisons indicated reductions in GCF in all the groups, with significant differences only for CHX on BF surfaces ($p < 0.05$). Intergroup comparisons revealed no significant differences ($p > 0.05$). Based on individual perceptions, CHX had better taste and biofilm control, but resulted in a greater change in taste. Nevertheless, MEL demonstrated anti-inflammatory effects similar to those of CHX. Further clinical trials testing different protocols, concentrations and follow-up periods are required to establish its clinical application.

Keywords: Biofilms; Periodontal Diseases; Melaleuca.

Introduction

The oral environment has hundreds of bacterial species incorporated into an extracellular matrix rich in polysaccharides, known as multi-species biofilm. When pathological biofilm develops on tooth surfaces, the result may be caries, periodontal disease, peri-implant disease, root canal infection and even the worsening of potentially fatal systemic diseases.[1] These progressive processes may range in severity, but all have a significant impact on function and esthetics, eventually leading to tooth loss, strongly associated with a negative impact on quality of life.[2] Although the mechanical control of dental biofilm is the most widespread form of oral hygiene,[3] lack of motivation, difficult access and inadequate oral hygiene skills can render this control

ineffective.[4] Thus, mouthwashes have taken on a complementary role, and are widely employed in the oral hygiene process.[5]

In recent years, there has been an increase in the search for natural compounds that exert an effect on biological mechanisms. *Melaleuca alternifolia* (MEL) is a herbal product with antiseptic, antibacterial, antifungal, antiviral and anti-inflammatory properties.[6] *In vitro* studies on MEL have reported its ability to inhibit the growth and adhesion of mono-species biofilms of periodontopathogens and cariogenic bacteria,[7] but clinical trials have demonstrated a lower anti-biofilm effect compared with that of chlorhexidine (CHX).[8] This characteristic is probably due to its low clinical stability, low degree of water miscibility, high volatility and difficulties in penetrating dental biofilm.[6] Nanotechnology would reduce these problems, resulting in a product with better therapeutic effectiveness, and greater stability and substantivity.[9] A reduction in MEL size would allow the product to penetrate bacterial biofilm, thus improving release and selectivity of the product, and lowering its toxicity to the patient.[10]

Despite the benefits of nanotechnology, no clinical trials have been performed to investigate the influence of MEL nanoparticles on biofilm reduction, gingival crevicular fluid volume and individual perceptions. Therefore, the aim of the present study was to compare the anti-biofilm and anti-inflammatory effects of CHX and MEL nanoparticles on biofilm-free (BF) and biofilm-covered (BC) tooth surfaces.

Methodology

Study design

This randomized, crossover, double-blind clinical trial was conducted in accordance with the principles stipulated in the Declaration of Helsinki, and received approval from the Human Research Ethics Committee (certificate number: 52275816.0.0000.5346). The study protocol was registered at ClinicalTrials.gov: NCT02695901. Informed consent was given by all participants prior to any clinical procedure. The study was conducted between August 2015 and November 2017 at the Department of Stomatology of the Federal University of Santa Maria, Brazil.

Participants

Sixty healthy participants were enrolled from the School of Dentistry of the university, as a convenience sample. The sample size was determined based on the primary outcome (Quigley & Hein Plaque Index). In a previous study, the mean index values in the MEL and CHX groups were 2.75 ± 0.60 and 2.33 ± 0.66, respectively.[11] Considering the crossover design, a significance level of 5% and a possible 20% dropout rate, 60 patients were required in order to provide a power of 80%.

The inclusion criteria were age ≥ 18 years, adequate systemic health (not undergoing any medical treatment) and having at least six teeth per quadrant.[12] The following exclusion criteria were applied: allergy to CHX or MEL, recent use of CHX or other antiseptic, use of fixed and/or removable prostheses, use of orthodontic appliance, dental caries, maladapted restorations, lesions involving the oral mucosal,[13] active infectious foci (endodontic or periodontal abscesses), history of periodontitis characterized as clinical attachment loss > 3 mm in two or more nonadjacent teeth,[14] marginal gingival bleeding > 15%,[15] any systemic condition that could affect gingival health (pregnancy, breastfeeding, tobacco use), and having undergone local or systemic antimicrobial treatment in the last 90 days.[12]

The randomization sequence was generated using an online computer-based program (Sealed Envelope Ltd., London, England). Sequentially numbered opaque envelopes were used to ensure that allocation would remain concealed. Randomization was performed by a researcher (L.M.O.) not involved in the data collection process. Both the examiner and the participants were unaware of the treatment applied. The mouthwashes were placed in coded opaque bottles to maintain blindness.

Test rinses

Chlorhexidine gluconate (0.12%) (Periogard®, Colgate-Palmolive®, São Paulo, Brazil) and MEL nanoparticles (0.3%) were purchased from Colgate-Palmolive® (São Paulo, Brazil) and Inventiva® (Porto Alegre, Brazil), respectively. The nanostructured lipid carriers were prepared with 7.5% weight/volume (w/v) of MEL, using high-pressure homogenization,

following the proprietary method (Inventiva®, Porto Alegre, Brazil). Acetyl palmitate and polysorbate 80 were used as the solid lipid and the surfactant, respectively. Total solid content was 18.6% (w/v). The characterization of the nanoparticles and the nanoparticle tracking analysis are described below. [16]

Intervention

All participants underwent a 14-day pre-experimental period, during which they received professional prophylaxis, and were instructed to perform meticulous biofilm control until gingival health was achieved.[15] The experimental design is summarized in Figure 1.

After enrollment, each participant was randomized into an intervention group: Group 1 – chlorhexidine gluconate 0.12% (CHX); Group 2 – *Melaleuca alternifolia* nanoparticles 0.3% (MEL). At baseline, the participants received comprehensive professional prophylaxis, and were instructed to refrain from all mechanical control measures for three days. On Day 3, gingival crevicular fluid (GCF) was collected and the participants were randomly assigned to professional prophylaxis on two randomized contralateral quadrants (Q1–Q3 or Q2–Q4), leaving surfaces that were both BF and BC. The participants were given mouthwash, and instructed to rinse with 15 ml for 60 seconds twice a day (every 12 hours), without performing any

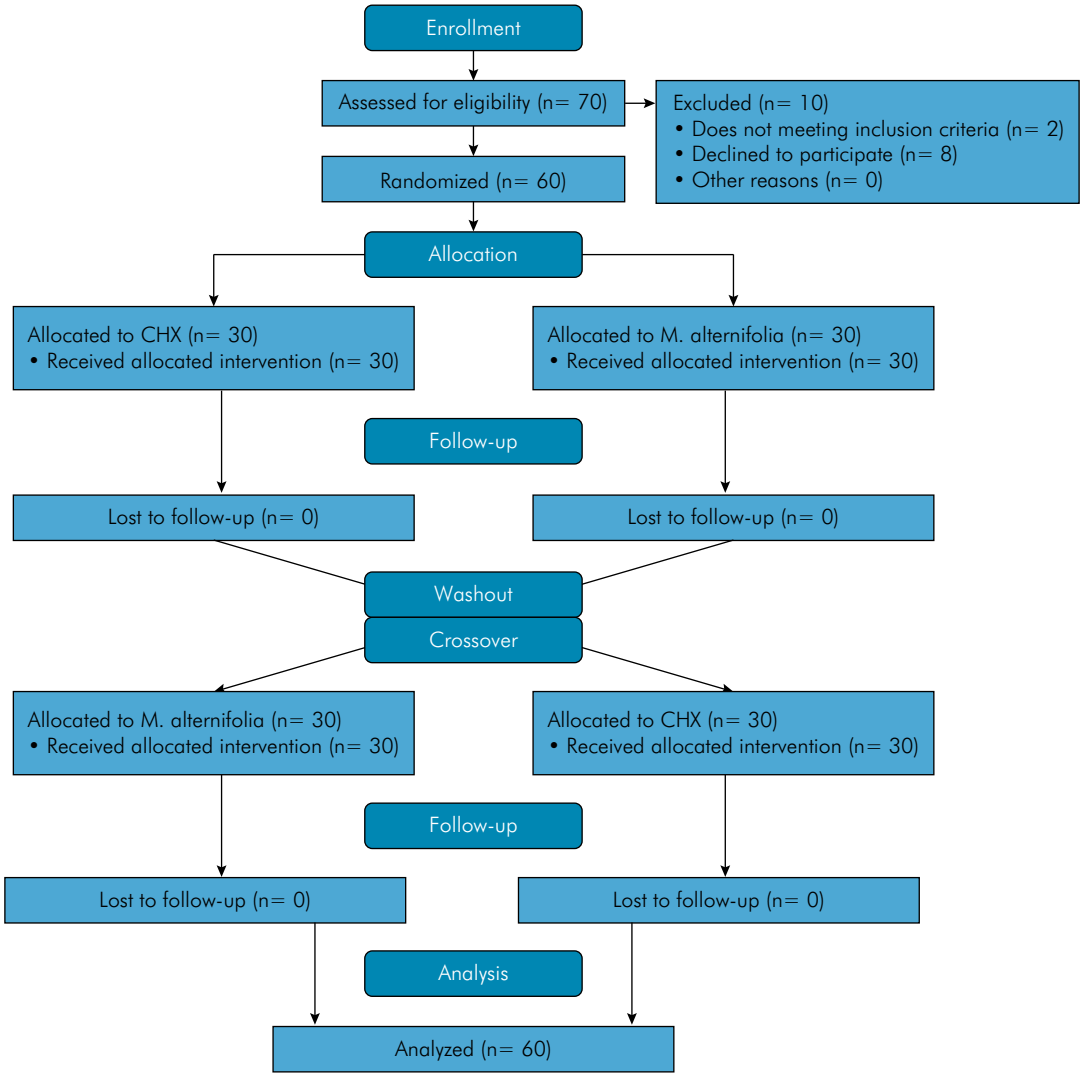


Figure 1. Flow diagram.

additional oral hygiene measures. The randomization process and professional prophylaxis were performed by another researcher (L.M.O.). On Day 7, GCF was sampled again and the plaque index was scored using disclosing solution. All participants then received comprehensive professional prophylaxis, and were instructed to resume their normal oral hygiene practices. Because of the crossover design, the participants were instructed to return after a washout period of 21 days, and the experimental procedures were repeated using the other product. Participant compliance was assessed by measuring the amount of mouthwash in the bottles returned.

Periodontal parameters

The amount of biofilm was assessed on Day 7 for each of the 2 periods (for CHX and MEL), at six sites and on all the teeth (except third molars). Biofilm was evaluated using the Quigley & Hein Plaque Index (QHPI) modified by Turesky et al.,[17] after topically applying two-tone disclosing solution (Young Dental, Earth City, USA), followed by gentle air-drying.[18] All clinical measurements were recorded by the same examiner (J.P.), who had undergone training and calibration exercises for the QHPI Index (k = 0.78), and who was blinded to both the group allocation and the quadrant prophylaxis.

GCF samples were obtained from the mesiobuccal site of the maxillary and mandibular first premolars. After removal of the supragingival biofilm (only on Day 7), the surfaces were air-dried and isolated by cotton rolls. Then, a sterile Periopaper® strip (Oralflow®, New York, USA) was carefully inserted into the gingival sulcus for 30 seconds. The GCF volume was recorded using a calibrated Periotron 8000® (Oralflow®, New York, USA). Strips visually contaminated with blood were promptly discarded. Sampling was performed in a climate-controlled room (20 ± 1°C) to minimize the possible impact of temperature and humidity.[13]

Questionnaire/Follow up

On Day 7, the participants of both interventions (CHX and MEL) received a questionnaire addressing taste of the product, duration of taste, change in taste, application time, comfort of use and perception of biofilm control. The questionnaire was designed by Slot et al.,[18] translated and adapted to Brazilian Portuguese. Each item was answered by marking a point on a Visual Analogue Scale (VAS) from 0 (negative extreme) to 10 (positive extreme).

Assessment of intraexaminer reproducibility

Before beginning the trial, the examiner (J.P.) underwent training and calibration exercises for assessment of clinical parameters, collection of GCF and administration of the questionnaire. Intraexaminer reproducibility for the plaque index was assessed in a sample of six individuals (representing 10% of the total sample), who were not included in the main study. After signing the statement of informed consent, the participants refrained completely from oral hygiene procedures for a period of 96 hours.[19] All the teeth were evaluated twice on the same day, with a minimum interval of 1 hour between evaluations. The kappa coefficient for intraexaminer agreement on the plaque index was 0.78.

Statistical analysis

Descriptive analysis of the plaque index was expressed as mean ± standard of deviation. The Kolmogorov-Smirnov test was used to determine the distribution (normal or non-normal) of the data. Differences within and between the groups, and comparisons between baseline and endline were determined using the Wilcoxon test. A p-value < 0.05 was considered indicative of statistical significance. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 23.0, Chicago, USA).

Results

The study design is shown in Figure 1. No participants were lost to follow-up. The female sex accounted for 63.7% of the sample. Mean age was 24.7 ± 5.73 years, and 92.7% of the participants were white. Based on the amount of mouthwash returned, the participants showed good compliance with the instructions. No serious adverse events or side effects were reported.

Table 1 shows the mean QHPI in all the group. Intergroup comparisons revealed statistically lower mean values for whole-mouth biofilm with CHX compared with MEL on BF (2.65 ± 0.34 *vs.* 3.34 ± 0.33, $p < 0.05$) and BC (2.84 ± 0.37 *vs.* 3.37 ± 0.33, $p < 0.05$) surfaces. Similar results were found in the separate analyses of the anterior and posterior teeth, as well as the buccal/palatal and proximal surfaces. In the intragroup comparisons, MEL resulted in a similar effect on both BF and BC surfaces (3.34 ± 0.33 *vs.* 3.37 ± 0.33, $p > 0.05$), whereas statistically higher mean values were found for BC surfaces in the CHX group (2.65 ± 0.34 *vs.* 2.84 ± 0.37, $p < 0.05$).

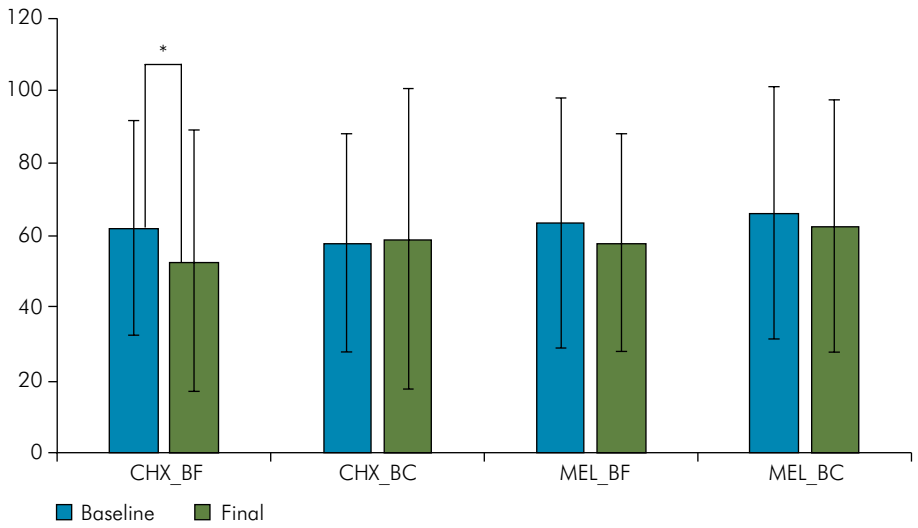
In the intragroup comparison, lower GCF volumes were found over time in almost all the groups (Figure 2). However, the differences were only significant in relation to teeth with BF surfaces submitted to CHX ($p < 0.05$). In the intergroup comparison, no significant differences were found between CHX and MEL ($p > 0.05$).

Regarding the results of the questionnaire addressing the participants' perceptions, CHX had better taste ($p < 0.001$), greater change in taste ($p < 0.001$) and greater biofilm control ($p < 0.001$) (Table 2). No statistically significant differences were found for duration of taste, application time or comfort of use.

Table 1. Mean values of QHPI (SD) in final examination, using experimental mouthwashes in plaque-free and plaque-covered surfaces (n = 60).

Variable	QHPI - Mean (SD)							
	WM		Anterior teeth		Posterior teeth		Buccal and palatal/lingual surfaces	Proximal surfaces
CHX BF	2.65	(0.34) ^{A*a**}	2.35	(0.37) ^{Aa}	2.80	(0.34) ^{Aa}	2.35 (0.37) ^{Aa}	2.80 (0.34) ^{Aa}
MEL BF	3.34	(0.33) ^{Ba}	3.08	(0.34) ^{Ba}	3.47	(0.33) ^{Ba}	3.08 (0.34) ^{Ba}	3.47 (0.33) ^{Ba}
CHX BC	2.84	(0.37) ^{Ab}	2.55	(0.42) ^{Ab}	2.99	(0.36) ^{Ab}	2.55 (0.42) ^{Ab}	2.99 (0.36) ^{Ab}
MEL BC	3.37	(0.33) ^{Ba}	3.10	(0.35) ^{Ba}	3.51	(0.34) ^{Ba}	3.10 (0.35) ^{Ba}	3.51 (0.34) ^{Ba}

QHPI (SD): Quigley & Hein Plaque Index modified by Turesky (Standard Deviation); WM: Whole mouth; CHX: Chlorhexidine; MEL: *Melaleuca alternifolia*; BF: Biofilm-free surfaces; BC: Biofilm-covered surfaces; *Uppercase letters refer to the comparison between CHX biofilm-free *vs* MEL biofilm-free, and between CHX biofilm-covered *vs* MEL biofilm-covered; **Lowercase letters refer to the comparison between CHX biofilm-free *vs* CHX biofilm-covered, and between MEL biofilm-free *vs* MEL biofilm-covered; Different letters demonstrate statistically significant differences (Wilcoxon test; $p < 0.05$).



SD: Standard deviation; PU: Periotron units; Base: Baseline; CHX: Chlorhexidine; MEL: *Melaleuca alternifolia*; BF: Biofilm-free surfaces; BC: Biofilm-covered surfaces; *Wilcoxon test ($p < 0,05\%$).

Figure 2. Intergroup comparisons revealed no significant difference ($p > 0.05$)

Table 2. Complete set of questions from Visual Analogue Scale questionnaire for taste perception, change in taste, and perception of biofilm control (scored from 0 to 10) (n = 60).

Variable		VAS extremes		Experimental mouthwashes				p-value*
		Negative	Positive	CHX		MEL		
Taste perception	How did the product taste?	Very bad	Very good	6.48	(2.77)	2.36	(2.49)	< 0.001
Change in taste	How was your taste of food and drinks affected?	Negative change	Positive change	5.18	(2.45)	3.61	(2.04)	0.001
Duration of taste	How long did the taste remain?	Very short	Very long	4.04	(2.59)	3.67	(2.39)	0.606
Time of application	What is your opinion of the product application time?	Very short	Very long	6.14	(1.95)	5.55	(1.80)	0.096
Comfort of use	What is your opinion of ease in using the product?	Not easy	Very easy	8.58	(2.28)	8.63	(2.14)	0.599
Plaque control	What was your perception of plaque control during these 3 days?	Insufficient	Very efficient	6.52	(2.81)	2.71	(2.54)	< 0.001

CHX: Chlorhexidine; MEL: 3 test days; p-value*: Wilcoxon test.

Discussion

The crossover, short-term, plaque re-growth model demonstrated a significantly lower anti-biofilm effect regarding the MEL groups, compared with the CHX groups, whereas no statistically significant differences were found among the groups, in regard to GCF on BF or BC surfaces.

There has been an increasing demand for alternative products in recent years. The short-term model has been advocated as the screening method, owing to the advantage of allowing effective assessment of an antimicrobial agent independently of tooth brushing.[18] The use of a standard agent as a control also helps determine the properties of the tested formulation.[18] A washout period of 21 days or more reduces the carryover effect,[20] and the crossover design eliminates between-subject variability, thereby reducing the influence of confounding covariates.[21]

Despite the anti-bacterial effect described in laboratory studies,[7] the present findings demonstrated a lower anti-biofilm effect of MEL compared with CHX. The divergent results may be related to the biofilm formation method. Although *in vitro* models have been extensively used to study dental biofilms, these models are limited in regard to simulation of the oral environment and *in vivo* conditions. During chronic infections, the interplay between host and pathogens is complex, marked by species that do not mix directly, but that reside within their own ecological space, a behavior that is not easily replicated and that leads to observable differences between *in vitro* and *in vivo* "chronic infections."[22]

The contrasting biofilm scores reported in clinical studies may be attributed to the different concentrations and applications tested. In some studies, 1.5% MEL solution[11] and 2.5% MEL gel[8] led to a lower reduction in biofilm scores, compared with CHX, corroborating the present findings. On the other hand, studies evaluating periodontal treatment with and without 5% MEL gel found a reduction in the biofilm scores between baseline and endline.[23,24] However, no differences were found between the groups.

Regarding the anti-inflammatory effect, a reduction in GCF volume occurred in all the groups. To the best of our knowledge, this is the only study evaluating the effect of MEL nanoparticles on GCF volume, which is considered a predictor of gingival inflammation.[25] After two to four days of biofilm accumulation, vasculitis, alterations in perivascular collagen, edema and neutrophil migration to the gingival sulcus can be observed in the histopathology of gingivitis.[26] Considering that GCF originates in the microcirculation of gingival tissues, a correlation has been found between GCF volume and gingival inflammation.[27] Clinically, an increase in exudation has been reported to be the initial inflammatory response to 96 hours of biofilm accumulation.[28]

Despite methodological differences, similar anti-inflammatory effects have been found in previous studies. A 2.5% MEL gel demonstrated a consistent

reduction in papillary and gingival indices,[8] and a 1.5% solution significantly reduced ($p < 0.001$) the gingival bleeding index (GBI).[11] However, compared with CHX, the overall differences between the groups were non-significant. Disregarding the CHX group, a highly significant reduction in GBI scores was found for the 5% gel compared with the control group.[23,24]

The present findings show that, though not inhibiting biofilm formation, the MEL nanoparticles exhibited important anti-inflammatory properties. The biofilm structure acts as a barrier to the diffusion of antimicrobial agents, retarding passage into the biofilm matrix. The similar anti-inflammatory effect of CHX and MEL could be attributed to the nanometric size of the particles, which facilitate penetration of MEL oil into the polymer matrix of the biofilm, thereby enhancing the antimicrobial properties of the oil and improving its anti-inflammatory effect.[16] Studies have confirmed that MEL nanoparticles improve the antimicrobial activity of the oil across a wide range of microorganisms.[29,30]

CHX displayed better taste and biofilm control than MEL, in addition to a longer effect of the altered taste. In contrast with the findings of previous studies,[11,31] the unpleasant taste of MEL was the only reported side effect in the present investigation. Moreover, despite individual perceptions, compliance was good with both interventions.

The MEL nanoparticles could have failed due to the lack of substantivity. In a previous study investigating its effects on total bacterial counts, MEL was found to be effective immediately after application. However, bacterial levels nearly returned to normal after a short period of time, unlike the outcome with CHX.[32] Another reason could be the low concentration applied. Indeed, it is known that higher concentrations are needed to effectively inhibit bacterial biofilm versus planktonic bacteria.[33]

This study has limitations that should be addressed. The participants constituted a convenience sample of dental students, thus limiting extrapolation of the findings to the general public. Moreover, although the mouthwashes were placed in coded opaque bottles, blindness may have been compromised due to the participants' knowledge.

Conclusion

Despite our results and the findings described above, the literature on the application of MEL in the oral cavity remains scarce. Furthermore, it has not yet been established whether MEL can be used effectively as a complement to mechanical biofilm control. Most clinical studies have incomplete statistic analyses and small samples, and fail to investigate the perceptions of individuals, thus impeding its clinical properties from being fully understood. Although the attempt to improve MEL substantivity by using nanotechnology seems to have failed, the mouthwash containing 0.3% MEL nanoparticles showed anti-inflammatory effects similar to those of CHX. Thus, further clinical trials testing different protocols and concentrations are needed to clarify the clinical relevance of this product.

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