**In vitro Microbiological Analysis on Antibacterial, Anti-inflammatory, and Inhibitory Action on Matrix Metalloproteinases-8 of Commercially Available Chlorhexidine Digluconate Mouth rinses**

**Abstract**

**Aim:** Chlorhexidine (CHX) mouthrinses are known to have a beneficial effect in the management of periodontal disease. The present study was designed to investigate the antibacterial, anti-inflammatory, and matrix metalloproteinases-8 (MMP-8) inhibition efficacy of eight commercially available CHX mouthrinses from the Dominican Republic. **Methods:** The study samples are categorized into two categories, eight commercially available CHX mouthrinses were case sample group, and positive and negative controls used in the study are categorized as control sample group. Antibacterial activity of the samples was evaluated on bacterial stains obtained from American Type Culture Collection (ATCC, Rockville, MD USA) which were *Porphyromonas gingivalis, Fusobacterium nucleatum, Eikenella corrodens,* and *Aggregatibacter actinomycetemcomitans.* **Results:** The study samples 1, 2, 3, 5, and 6 showed higher antibacterial efficacy with no bacterial colonies formation in dilution assay method, whereas sample 8 showed larger colonies of bacterial growth. The halo diameter found to be average in sample 8 with 13 mm, whereas sample 9 showed 12.5 + 3.48 mm, sample 1 was with a mean of 11.79 + 3.51 mm. The smaller halo diameter and minimal antibacterial activity were observed in samples 4 (mean of 3.5 + 5.95 mm) and 7 (3.5 + 7.70 mm). All eight samples showed statistically significant higher MMP-8 inhibition activity with P < 0.0001. **Conclusion:** Commercially available CHX digluconate mouthrinses showed the difference in plaque inhibition with 0.12 and 0.15% concentration.

**Keywords:** Chlorhexidine, antimicrobial(s), dental plaque, Dominican Republic, matrix metalloproteinases-8, mouthrinses

**Introduction**

Periodontal diseases are the most common chronic infection of periodontium, and periodontitis accounts for higher morbidity of periodontal apparatus. Increasing global prevalence of periodontal disease has also been posing a challenge to clinicians, researchers, and organizations. Conventional periodontal therapy for chronic gingivitis and periodontitis includes supragingival plaque control with concurrent subgingival debridement and root planing based on the depth of disease involvement. Traditionally, periodontal care has been with mechanical removal of both supra- and subgingival biofilms using toothbrush, interdental brush, dental floss, mouthrinse, or irrigator. Global use of antimicrobial agents has been continuously increasing, and currently, a variety of both natural and synthetic forms of antimicrobials are available in the market.\(^1,2\)

Chlorhexidine (CHX) is a bisbiguanide chemical substance that provides an effective, nonirritating antiseptic used in the control of plaque and inflammation. Clinicians widely prescribe CHX as the choice for its antiplaque action and reduction of gingivitis.\(^3,4\) CHX mouthrinses are usually recommended twice daily for 30 s. Clinicians and pharmaceutical instructions advice that CHXs are not to be ingested and must be expectorated on rinsing.\(^5\) The side effects of CHX have been well documented in the literature and classified as localized and reversible. Common side effects of CHX mouthrinse are reversible dryness of mouth, yellow-brown discoloration of teeth and dourness of tongue, mild-to-moderate irritation of oral mucosa, alteration in taste sensation, and burning mouth condition.\(^5\) Studies have evaluated the efficacy of various concentrations of CHXs (0.05%, 0.06%, 0.12%, and 0.15%).

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0.1%, 0.12%, and 0.2%) in reduction of plaque and gingivitis and confirmed significant antimicrobial potential.[4,6-10] Serrano et al. documented systematic review on efficacy of adjunctive antiplaque formulations in the management of gingivitis and concluded that formulations with specific agents for chemical plaque control had demonstrated significant improvements in gingival bleeding and plaque indices.[11] On the contrary, some studies have evidently shown that plant extracts as viable alternative to routine mechanical and antimicrobial periodontal therapy in reduction of supragingival biofilm growth and gingival inflammation.[12,13] In addition, Pistorius et al. published that subgingival irrigation with herbal-based mouthrinse led to a significant reduction in both sulcus and gingival indices.[11] Dhingra et al. documented systematic review on the effectiveness of Azadirachta indica (neem)-based herbal mouthrinse in a significant reduction of plaque and restoring gingival health and results were similar to CHX mouthrinse.[14] Another in vitro study published by Verkait et al. stated that supernatants from herbal- and chitosan-based toothpastes have comparable immediate and ongoing antibacterial efficacies similar to CHX.[15]

Although periodontitis is of an infectious disease origin, pathogenic alterations observed in supporting periodontal tissue are resultant of host tissue immune response against subgingival biofilm.[16] Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that mediate tissue destruction in periodontal diseases. Research studies evidence that MMP-8 is the most predominant collagenolytic agent identified in periodontal tissue and gingival crevicular fluid of periodontitis patients. Currently, research studies have documented that MMP-8 is the most promising proteomic biomarker of periodontitis and that serves as a diagnostic and prognostic biomarker.[17,18] The accuracy of MMP-8 as a diagnostic biomarker in periodontitis condition is dependent on the severity of disease, probing depth, attachment loss, and bone destruction. Studies observed a significant association of reduced MMP-8 levels and favorable prognosis; on the contrary, studies also reported that elevation of MMP-8 levels is significantly associated with disease progression and higher severity of periodontitis.[19-21]

Strong research evidence has been published on comparing various commercially available antimicrobial products on antiplaque and anti-inflammatory activities. Although stronger and consistent results have been published on efficacy of commercially available antimicrobial products, criticism is placed on funding agencies/researchers due to affiliation with commercial products. To the best of our knowledge, no study has been published from the independent researcher/research institution that focused on comparative results of commercially available formulation of CHX 0.12% and 0.15% concentrations. The aim of the present study was to evaluate the in vitro effects of eight commercially available CHX mouthrinses from the Dominican Republic on antiplaque and anti-inflammatory properties.

**Materials and Methods**

In this in vitro study, eight different commercially available CHX mouthrinses were evaluated: CHX 0.12% (a), CHX 0.12% + Xylitol 1.00 g (b), CHX 0.12% (c), CHX 0.12% (d), CHX 0.12% + NaF (e), CHX 0.12% + Xylitol (f), CHX 0.15% + H2O2 2% + extract of Caesalpinia coriaria 5%, CHX 0.12%(g), and Xylitol 1.0 g + zinc acetate 0.34 g (h). Each product or antiseptic tested is listed in Table 1 and were assigned with a number and called for their active compounds. CHX 0.12% + CCP 0.05 g and sterile saline, served as positive [Figure 1] and negative [Figure 2] control, respectively. Antibacterial activity was assessed by the dilution plate assay, diffusion method, and short interval killing test (SIKT) against four bacteria including Porphyromonas gingivalis, Fusobacterium nucleatum, Eikenella corrodens, and Aggregatibacter actinomycetemcomitans. All experiences were performed in duplicate.

**Bacterial stains and culture condition**

All bacterial stains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). *F. nucleatum* (ATCC 25586), *A. actinomycetemcomitans* (ATCC 29523), *P. gingivalis* (ATCC 33277), and *E. corrodens* (ATCC 23834) were stored at −80°C. Strains of *E. corrodens*, *F. nucleatum*, and *P. gingivalis* were grown on Columbia agar (5% defibrinated sheep blood supplemented with 5 mg/mL hemin-menadione) at 37°C in anaerobic atmosphere, for 1 week. *A. actinomycetemcomitans* was plated on selective agar TSBV (Trypticase Soy, supplemented with 10% horse serum, bacitracin 75 μg/mL, and vancomycin 5 μg/mL). TSVB plates were incubated at 5–10% CO₂ for up to 3 days. A few colonies selected from each strain were grown in brain–heart infusion (BHI,

| Table 1: Case and control sample constituents |
|------------------------------------------------|
| **Case sample constituents** | **Control sample constituents** |
| Sample 1: 0.12% CHX (1) | Figure 1: Positive control: 0.12% CHX and CPC with *Porphyromonas gingivalis* plated. |
| Sample 2: 0.12% CHX and xylitol 1.00 gram (2) | | |
| Sample 3: 0.12% CHX (3) | Figure 2: Negative control – sterile saline with *Porphyromonas gingivalis* plated. |
| Sample 4: 0.12% CHX (4) | | |
| Sample 5: 0.12% CHX and sodium fluoride (5) | | |
| Sample 6: 0.12% CHX and xylitol (6) | | |
| Sample 7: 0.15% CHX, hydrogen peroxide and Cc (7) | | |
| Sample 8: 0.12% CHX, xylitol 1.0 gram and zinc acetate 0.34 gram (8) | | |

CHX=Chlorhexidine, CPC=Cetylpyridinium chloride
Collins, et al.: Efficacy of chlorhexidine mouthrinses available at the Dominican Republic

Oxoid) in anaerobic atmosphere at 37°C for 72 h. After grown in broth, the optical density for each bacterium was calculated to standardize the amount of bacteria present before each trial beginning to achieve an optical density between 0.5 and 1.0, measured at a wavelength of 600 nm.

**Testing antimicrobial activity**

Diffusion plate assay was employed to determine antimicrobial efficacy of mouth rinses to inhibit the growth of bacteria. The efficacy was tested through the evaluation of inhibition zone. Each strain was suspended in 0.5 mL of phosphate-buffered saline (pH 7.4) to achieve an optical density between 0.5 and 1.0, measured at a wavelength of 600 nm. Using a sterile cotton swab, the plates were streaked to form a bacterial lawn, and 10 uL of each antimicrobial agent and controls was added over the lawn. Plates were incubated up to 5 days under conditions described before and the inhibition zones were evaluated by at least two different diameters measures (mm). The result was determined by averaging at least two measurements.

The dilution plate assay\(^{[22]}\) was performed using culture media indicated for each strain tested, as described above. After autoclaving, the medium was cooled to 52°C, and the antisepsics solutions were added in a concentration of 10% v/v. Media were poured and dried in a laminar airflow hood for 30 minutes. Tenfold dilution series of each bacterial culture were prepared, and aliquots of 100 uL of \(10^2\) and \(10^3\) were transferred onto their respective agar plates and spread using a sterile Drigalski loop. Colonies were counted after corresponding time and conditions of incubation.

To determine the antimicrobial activity of the eight mouthrinses described, after a short exposure time, the SIKT was slightly modified, where the assay was performed with three anaerobic bacterial strains \(P.\) gingivalis, \(F.\) nucleatum, and \(E.\) corrodens.\(^{[23]}\) All of them were cultured in BHI broth the cultures were harvested at exponential growth and mixed to a total volume of 10 mL with increasing amounts of 8 mouthrinses and a positive/negative control, ranging from 5, 20, and 35% (v/v). The mixtures of mouthrinse and bacterial cells were incubated at 37°C by slow rotation, after incubation for, 1, 3, and 5 min, tenfold dilution series were prepared, and aliquots of 10 uL were spotted onto agar plates. Anaerobic incubation of the plates was performed as described. The survival of the bacteria after exposure to the chemical solutions was calculated as proportions of the control expressed in percentages of survival.

**Matrix metalloproteinases-8 activity assay**

MMP assay was conducted using a commercial MMP fluorometric assay kit (AnaSpec, San Jose, CA, USA) using indications provided by manufacturer. 10 ng of active recombinant human MMP-8 (preincubated with 10 mM APMA) was added to case and control samples and allowed to mix using 5-FAM/QXL™ 520 fluorescence resonance energy transfer (FRET) peptide substrate in assay buffer using 96 well plates in quadruplicates. For the intact FRET peptide, the fluorescence of 5-FAM was quenched by QXL 520. Upon cleavage into two separate fragments by MMP-8, the fluorescence of 5-FAM was recovered and monitored at excitation/emission wavelengths (490/520 nm, respectively). After 1 h of incubation, the fluorescence signals were read by a microplate reader (Synergy HT; BioTek Instrument Inc., Winooski, VT, USA). Diluted active MMP-8 was used as positive test control; 25 μM Ilomastat was used as inhibitor control and test compound controls with no MMP were added to assess their autoimmunofluorescence.

All the data were tabulated in Microsoft Excel 2013 sheet, and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software version 17 (IBM® Company, Armonk, NY, USA) and STATA software Version 12 (StatCorp., College Station, TX, USA). Descriptive measures were estimated through frequency ratios for variables at 95% confidence intervals and 5% marginal error. Comparisons of MMP-8 activity between case and control samples were analyzed with ANOVA and/or Kruskal–Wallis test based on the data distribution. \(P < 0.05\) was considered statistically significant in the current study.

**Results**

A total of eight commercially available CHX mouthrinses from the Dominican Republic were included in the study for evaluation of antibacterial and anti-inflammatory actions and inhibitory action on MMP-8 [Tables 1 and 2]. The concentrations of CHX identified in mouthrinses were 0.12% and 0.15% containing an extract of *Coriaria caesalpinia* and hydrogen peroxide. Antibacterial action of mouthrinses was estimated by dilution plate assay, diffusion method, and short interval kill test.

Table 3 depicts the antibacterial efficacy of case and control group of mouthrinses. The results showed that sample 4 CHX rinse was significantly less effective than other tested mouthrinses. Sample 4 CHX was considered less effective due to minimal bacterial growth inhibitory action and anti-inflammatory properties. Further, all other case samples showed significant higher antibacterial efficacy than sterile saline but with the exception of sample 4, whereas samples 1, 2, 3, 5, and 6 showed higher antibacterial efficacy by displaying no colonies of bacterial growth in dilution assay. Sample 8 showed that smaller colonies of bacterial growth and colonies were nonviable when viability test was performed. The diameter of inhibition zone of antimicrobial test is usually varied, and the interpretation values for minimal inhibitory concentration considered in the present study are resistant 0–4.9 mm, intermediate 5–8.99 mm, and sensitive >9 mm. The sample 8 showed an average of 13 mm of minimum inhibitory concentration, sample 9
and positive control sample showed 12.5 + 3.48 mm, and sample 1 was with a mean of 11.79 + 3.51 mm. The samples showed that smaller halo diameter and minimal antibacterial activity are sample 4 (mean of 3.5 + 5.95 mm) and sample 7 (mean of 3.5 + 7.70 mm).

Table 4 depicts the results of *P. gingivalis* bacterial viability using killing assay method that was calculated at the end of 1st, 3rd, and 5th min intervals. The study findings showed that *P. gingivalis* as most sensitive bacteria using contact killing assay method. The bacterial viability of sample 4 was 100 µl volume of bacterial broth at the end of 1st min but not in 3 min interval.

*F. nucleatum* did not show bacterial viability in samples 1, 2, 6, 7, and 8 and positive control in all given intervals. *E. corrodens* showed bacterial viability in samples 4 and 7 in all given intervals, whereas sample 8 showed bacterial viability at 100 µl bacteria broth volume at the 1st min but not at 3rd and 5th min interval.

Table 5 depicts the results of *A. actinomycetemcomitans* bacterial viability using killing assay method that was calculated at the end of 1st, 3rd, and 5th min intervals. The study findings showed that sample 7 and 8 showed no bacterial viability at any given intervals, whereas sample 4 showed viability at 50 and 100 µl during all given intervals. Negative control was ensured that growth of bacteria was not observed at all given intervals.

Figure 3 depicts the results of MMP-8 inhibitory properties of samples that were tested in the study. All samples that were tested (i.e., samples 1–8) demonstrated higher statistical significance (*P* < 0.0001) of MMP-8 inhibition levels when compared to negative control, although sample 5 showed a minimal potential of plaque inhibition than other case samples.

### Discussion

The mouthrinses with CHX have been the most common antibacterial and anti-inflammatory agent prescribed in dental and periodontal practice. The efficiency of CHX as suitable anti-plaque agent has been proved by several studies in both *in vitro* and *in vivo* studies. However, evaluation of efficacy of commercially available CHX mouthrinses from the Dominican Republic was not studied. The purpose of the present *in vitro* study was to evaluate the antibacterial, anti-inflammatory efficacy, and inhibitory potential of MMP-8 of commercially available CHX mouthrinses from the Dominican Republic. Bacterial viability was not observed in samples 1, 2, 5 and positive control. Similar findings were reported by Herrera et al., who mentioned that both 0.12% CHX with sodium fluoride and 0.12% CHX with CPC had no viability of *F. nucleatum*.[24]

Contact kill assay was employed to demonstrate the effectiveness of CHX against *F. nucleatum*. All samples showed no *F. nucleatum* bacterial viability at all given intervals and in all bacterial broth volumes and readings.
were justified with positive control. However, the sample 7 that had hydrogen peroxide showed countless *F. nucleatum* growth. Results of the present study also showed all samples demonstrated loss of bacterial viability of *P. gingivalis* after 5 min interval and justifying efficacious antibacterial activity. Similar results were published by Afennich et al. and Dona et al., who stated loss of *P. gingivalis* viability after 5 min interval in their testing samples.[25,26]

The results of the present study demonstrated that sample 5 (0.12% CHX with sodium fluoride) showed no bacterial availability of *E. corrodens* by contact kill assay method. On the contrary, published reports of Herrera et al. stated bacterial resistance.[24] Pharmaceutical company that manufactured 0.15% CHX with hydrogen peroxide and *C. coriaria* did not state mouthrinse as an adjunctive tool for the periodontal disease treatment. Dona et al. and Grundemann et al. stated that composition of mouthrinse that contains 0.15% CHX with 2% hydrogen peroxide and 5% extract of *C. coriaria* should possess greater bacterial inhibition.[27,28] The present study expected that 0.15% CHX with other constituents such as hydrogen peroxide and *C. coriaria* must have a superior inhibitory effect on bacterial growth. However, study results depicted that positive control (i.e., 0.12% CHX with 0.05%
cetylpyridinium chloride) produced superior bacterial inhibitory potential. The discussion on this section can be summarized that significant difference in antibacterial
action was identified between concentrations of 0.12% and 0.15% mouthrinses, the result displayed 0.12% CHX was significantly more effective than mouthrinse. However, the ability to provoke anti-inflammatory effect (MMP-8) was similar in both concentrations of CHXs.

Heling et al. suggested that positive interaction occurs when CHX and hydrogen peroxide are combined in mouthrinse with associated better clinical results. However, when the mouthrinses are used separately as either CHX or hydrogen peroxide, the results are comparatively lower. However, results of the present study with regard to sample 7 (0.15% CHX with hydrogen peroxide and C. coriaria) were contrary to the previously published studies. One of the possible reasons for such result is due to the addition of C. coriaria to the combination, i.e. CHX with hydrogen peroxide, further due to addition of C. coriaria may have either inactivated or antagonized the usual effect of CHX with hydrogen peroxide. Future biochemical studies are required to explore the action of C. coriaria at varied concentrations in 0.15% CHX mouthrinses with hydrogen peroxide. Mohana et al. published an in vitro study that evaluated different solvent extracts and isolated constituents of eight different herbal agents for antibacterial activity against 11 pathogenic bacteria on human tissues using cup diffusion method. Their observations showed that C. coriaria had significant antibacterial activity against all the tested bacteria and suggested that C. coriaria as a potential herbal candidate that should be explored in medical microbiology. The authors of later-mentioned study propose that antibacterial activity of herbal agents is due to the presence of acidic acid and phenol fractions, the observations were identified using phytochemical analysis. Fragmentation of acidic action and phenol fraction resulted in the loss of antibacterial activity.

The present study indicated that sample 4 (0.12% CHX) displayed bacterial inhibition of P. gingivalis but failed to show bacterial inhibition with other three tested bacteria when employed with diffusion assays. Further, using contact kill assay, results showed the absence of bacterial inhibitory potential to E. corrodens at any all 3 time intervals. The present study results on sample 7 (CHX with hydrogen peroxide and C. coriaria) showed low bacterial inhibition potential.

Collins et al. studied tetracycline drug-resistance genes in subgingival biofilm in Dominican adult population and emphasized the importance of identifying resistance genes among Dominican population that impact on clinical relevance in managing periodontal patients. Similarly, the present study is the first attempt to study the Dominican population to estimate the effectiveness of mouthrinses over four commonly identified periodontal pathogens that have a pivotal role in the establishment of periodontal disease. The results of the study will highlight to the clinicians among Dominican Republic regarding the constituents of CHX mouthrinse and its clinical utility while managing patients with periodontal disease.

Based on our literature search, only a few studies have been reported to test efficacy of antibacterial ability of mouthrinses, and none of those published studies had reported about inhibition potential of MMP. The present study employed the MMP-8 inhibition assay on both 0.12% and 0.15% CHX mouthrinses and results denoted that both 0.12% and 0.15% CHX showed statistically significant MMP-8 inhibition potential. However, sample 5 (CHX with sodium fluoride) showed reduced MMP-8 inhibition potential effect. Although the exact mechanism on the role of CHX to inhibit MMPs was not completely established, researchers evidently believe that a chelating mechanism of MMP with metal ion for catalytic activity is linked for MMP inhibition. In addition, protein denaturation ability is another factor in MMP inhibition. However, the role of protein denaturation in MMP inhibition is dependent on CHX concentration. Gendron et al. studied the CHX on inhibition activity on MMP 2, 8, and 9 and stated that both isolated CHX mouthrinse and CHX with sodium fluoride are a clinically effective antibacterial agent for periodontitis patients. Emilson et al. explained that ionic interaction potential between these pharmaceutical compounds (i.e. CHX and sodium fluoride) may be weak and this may explain the reduced MMP inhibition action when the pharmaceutical compounds are combined. Future studies are required to address periodontal tissue and host inflammatory response to antibacterial mouthrinses.

**Future directions**

1. The results of the sample that consisted of 0.15% CHX with hydrogen peroxide and C. coriaria were not effective. This might have probably been due to the addition of C. coriaria, which may have resulted in the inactivation of the usual effects of CHX with hydrogen peroxide combination. However even though C. coriaria was reported to have potent bactericidal actions, the combination of the pharmacological compounds seems to result in unfavorable clinical effects, and this should be addressed by exploring the action of C. coriaria at varied concentrations in 0.15% CHX with hydrogen peroxide.

2. Future research design with clinical studies that focus on addressing periodontal tissue and host inflammatory responses to commercially available CHX mouthrinses for filling the gap in public health policy that motivates prevention strategies of periodontal disease.

3. Continued research studies on the present research model to validate the interpretation of the present study and strengthen the public health policies and to create public health programs on periodontal disease prevention.
Conclusion

The use of CHX mouthrinses is increasing in the clinical practice of dentistry for periodontal disease management. The use accounts for much of total drug production and is increasing worldwide, and hence newer pharmaceutical companies are emerging to manufacture similar mouthrinses. Most companies have a slight variation in the pharmaceutical compounds for antiplaque and anti-inflammatory properties. From the point of public health concerns, the present study is an attempt to report in vitro effects of eight commercially available CHX mouthrinses in the Dominican Republic to guide clinicians for appropriate management strategies on periodontal disease. The study documented that 0.12% concentration of CHX demonstrated significant plaque inhibitory action; on the contrary, 0.15% concentration was less effective. This study recommends that 0.12% CHX may be indicated as an adjunct to routine toothbrushing activity in individuals with poor plaque control and obvious symptoms of gingival inflammation.

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Conflicts of interest

There are no conflicts of interest.

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