Antimicrobial activity of novel mouthrinses against planktonic cells and biofilms of pathogenic microorganisms

Sladjana Malic[1*], Charlotte Emanuel[2†], Michael AO Lewis[2†] and David W Williams[2†]

*Correspondence: S.malic@mmu.ac.uk

†These authors contributed equally to this work.

[1]School of Healthcare Science, Manchester Metropolitan University, Manchester M1 5GD, UK.

[2]School of Dentistry, College of Biomedical and Life Sciences, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK.

Abstract

<u>Background</u>: Oral diseases pose major public health problems on a global scale. Such diseases have considerable impact on individuals and communities by causing pain and suffering, impairment of function and reduced quality of life. The objective of this study was to evaluate the antimicrobial activity of five mouthrinses against a variety of microorganisms associated with infections of the oral cavity and other body sites.

<u>Methods</u>: Mouthrinse formulations were Chlorhexidine (0.2%), Citrox (1%; Perioplus™)/Hyaluronic acid (0.2%)®, Chlorhexidine (0.2%)/Citrox (1%; Perioplus™), Chlorhexidine (0.2%)/Phenoxetol (0.1%)® and Citrox (1%; Oralclens)™ (Oraldent Ltd; UK). The test microorganisms were the bacteria, Actinomyces viscosus ATCC 1598; Actinomyces odontolyticus NCTC 9935, Clostridium difficile R8651, Prevotella intermedia NCTC 13070[T], Prevotella denticola R20771, Porphyromonas gingivalis NCTC 11834[T], Streptococcus gordonii ATCC 10558[T], Streptococcus sanguinis NCTC 7863, and the fungi, Candida albicans ATCC 90028, Candida dubliniensis CD36, Candida krusei ATCC 6258, Candida glabrata ATCC 2001, Candida tropicalis ATCC 750 and Candida parapsilosis ATCC 22019. Determination of mouthwash antifungal and antibacterial properties was done using a microtitre plate assay. *In vitro* biofilms were constructed using 96-well plates and exposed to a range of mouthrinse concentrations. The minimum biofilm eradication concentration (MBEC) was established by examining subsequent re-growth of biofilm cells. Results were compared with the minimum inhibitory concentrations (MICs) obtained for planktonic cells cultured in 96-wells plates in various mouthrinse concentrations.

<u>Results</u>: Planktonic cells of aerobic microorganisms were inhibited by all mouthrinses at concentrations ≤2% (v/v) of the stock preparation. Chlorhexidine (0.2%)/Citrox (1%)™ had the highest antimicrobial activity, followed by Citrox (1%)™, 0.2% Chlorhexidine, Chlorhexidine (0.2%)/Phenoxetol (0.1%)® and Citrox (1%)/Hyaluronic acid (0.2%)®. Some anaerobic bacteria (Actinomyces odontolyticus, Clostridium difficile, Prevotella intermedia) exhibited higher MICs for all 5 mouthwashes. There was a noticeable increase (up to 16-fold) in tolerance to the mouthwashes by the majority of aerobic microorganisms when the minimum biofilm eradication concentration was compared to the minimum inhibitory concentration.

<u>Conclusion</u>: The results highlight enhanced antimicrobial activity using a combined preparation of Chlorhexidine/Citrox compared with Chlorhexidine alone.

<u>Keywords</u>: Periodontal diseases, dental caries, oral candidosis planktonic microorganisms, biofilm, antimicrobial activity, Citrox

Introduction

Dental caries and periodontal diseases are significant health problems of humans [1], and arise irrespective of socioeconomic class, although they are more prevalent in deprived populations [2]. Dental caries affects 60-90% of children in industrialised countries [3] and approximately 10-15% of adults are affected by severe periodontal disease. These plaque-mediated diseases lead to premature dental exfoliation and have a significant impact on the quality of life [4,5]. Periodontal disease has been also implicated with systemic chronic diseases such as cardiovascular disease [6].

An important factor in both caries and periodontal disease is the oral microflora and it is the biofilms produced by these organisms that are at the centre of disease pathogenesis. Thus, biofilm control by mechanical debridement and use of adjunctive antimicrobials is of utmost importance in prevention of plaque-mediated diseases. Typical adjuncts to oral hygiene and prevention of the aforementioned diseases have been the use of mouthwashes and toothpastes containing antimicrobial components such as triclosan and Chlorhexidine. Both these components are classed as biocides as they target multiple sites to achieve antimicrobial effects. Triclosan (5-Chloro-2(2,4-dichlorophenoxy)phenol) is a halogenated phenol, and a non-ionic broad spectrum antimicrobial that has multiple uses including being an active component in detergents, clothing, toothpastes, and mouthwashes [7]. Concerns regarding triclosan use in 'everyday products' has been raised, as over exposure of microorganisms to this agent might propagate resistance and indeed there has been evidence of low-level resistance in *Staphylococcus aureus* [1,8]. Triclosan has also been

detected in sediment, soils, and aquatic species [7] and recent environmental concerns have been raised leading to some calls for its use to be limited [7-11]. Chlorhexidine has also been associated with adverse effects including mucositis, altered taste, and staining of dental tissues and restorations [1,12]. Such undesirable reactions to Chlorhexidine have resulted in calls for modified clinical practice [13,14] and along with patient preferences have been the drivers for research into alternative mouthwashes/remedies for oral care.

For centuries, plant products have been used as treatments for diseases, and more recently in the development of new drugs [13]. Previous studies have indicated the effective and efficient use of natural antimicrobials in inhibiting the oral microflora [13,14]. Citrox, is a soluble formulation of bioflavonoids, derived from citrus fruits, and this agent has antimicrobial activity against bacteria, fungi and viruses [15-18].

This present study investigated potential potentiation of antimicrobial activity through use of combinations of Citrox (1%) with Chlorhexidine (0.2%; Perioplus™), and Citrox (1%)/Hyaluronic acid (0.2%)®. To this end, reducing Chlorhexidine concentration may result in reduced side effects, whilst maintaining antimicrobial properties. Hyaluronic acid is a major component of collagen, and an important factor in wound healing, and thus could be significant in the treatment of periodontal disease [19]. Small molecular weight hyaluronic acid has also been found to induce expression of toll-like receptors, which are important components of the innate immune system [20]. Thus, the combination of Citrox with hyaluronic acid may provide both bactericidal and wound healing properties *in vivo*. Often, *in vitro* assessment of antimicrobial activity is performed only against planktonic cells, and these tend to be much more susceptible to antimicrobials than their biofilm counterparts. In this present study, we evaluated the antimicrobial activity of 5 different mouthrinses against 14 test strains including bacteria and *Candida* species, cultured both planktonically and as biofilms.

Materials and methods

Microorganisms investigated in this studywere *Actinomyces viscosus* (ATCC 1598), *Actinomyces odontolyticus* (NCTC 9935), *Clostridium difficile* (R865, clinical isolate), *Prevotella intermedia* (NCTC 13070^T), *Prevotella denticola* (R20771), *Porphyromonasgingivalis* (NCTC 11834^T), *Streptococcus gordonii* (ATCC 10558^T), *Streptococcus sanguinis* (NCTC 7863), *Candida albicans* (ATCC 90028), *Candida dubliniensis* (CD36 [21]), *Candida krusei* (ATCC 6258), *Candida glabrata* (ATCC 2001) and *Candida tropicalis* (ATCC 750).

The bacteria *Actinomyces viscosus* ATCC 1598, *Actinomycesodontolyticus* NCTC 9935, *Clostridium difficile* R8651, *Prevotella intermedia* NCTC 13070^T, *Prevotella denticola* R20771 and *Porphyromonas gingivalis* NCTC 11834^T were cultured under anaerobic conditions at 37˚C on Fastidious Anaerobe Agar (FAA) and in Fastidious Anaerobe Broth (FAB). *Streptococcus* species were cultured aerobically at 37˚C using Blood Agar

(BA) and Brain Heart Infusion Broth (BHI). *Candida* species were cultured aerobically at 37˚C using Sabouraud Dextrose Agar (SDA) and Sabouraud Broth (SAB). All media was obtained from Lab M (International Diagnostics Group plc, Bury, UK) and prepared as per the manufacturer's instructions. Broths were not adjusted for pH or glucose content. All mouthrinses were provided courtesy of Oraldent Limited, UK.

Minimum inhibitory concentration (MIC) determination for planktonic cells

Microbial preparations of bacteria and *Candida* species were generated by overnight incubation, and adjusted to a turbidity equivalent to a MacFarland standard of 3.0. Serial dilutions of the test mouthrinses in the respective culture medium were prepared, resulting in a concentration range of 0.007% to 8% (v/v) of the parent mouthrinses. A 100-µl volume of each test dilution was then combined with 100 µl of microbial suspension. Negative controls of broth and bacterial suspensions without antimicrobial were also included. To the wells of 96-well microtitre plates, 200 µl of the preparations were added and incubated for 24 hat 37˚C, under the appropriate atmospheric conditions. Following incubation, the relative growth of the microbial species was estimated by recording the turbidity of wells using spectrophotometric absorbance at 620_{nm}. Absorbance readings were standardised using 'microbial-free' control mouthwash dilutions. The MIC was recorded as the lowest concentration of mouthwash that showed ≥80% reduction in absorbance compared to the controls without mouthrinses.

Minimum Biofilm Eradication Concentrations(MBEC) for biofilms

Suspensions of each microorganism (MacFarland standard 3.0) were incubated in the wells of a flat-bottomedmicrotitre plate for 24 h at 37˚C. BHI was used as the culture medium for bacteria, FAB was used for culture of anaerobic bacteria and SAB for *Candida*; incubation was without agitation to allow formation of a biofilm. The medium was then removed by gentle aspiration and the biofilm washed with 100 µl of phosphate buffered saline (PBS) to remove planktonic cells. Fresh medium containing test mouthrinse concentrations or negative control broth was added to the biofilms. Biofilms were then incubated for a further 24 h under the described conditions. The medium was subsequently removed by gentle aspiration and the biofilm washed with PBS. Fresh broth (200 µl) was added and the biofilms were disrupted by repeated pipetting and agitation. The turbidity of the resuspended biofilm was observed by measuring the absorbance at 620_{nm}. Following a further incubation period of 6 h, the absorbance at 620_{nm} was once again recorded. The relative growth of the microorganisms was determined by the difference in absorbance over this 6 h period. The mean value was calculated from quadruplicate samples in each well and the MBEC recorded as the lowest concentration of

Table 1a. Minimum inhibitory concentration % (v/v) of mouthrinses against planktonic microorganisms.

Mouthrinse	Minimum inhibitory concentration % (v/v)				
	A	B	C	D	E
Microorganism					
C. albicans	1	2	0.5	2	0.5
C. dubliniensis	0.5	1	0.25	2	0.25
C. krusei	0.5	0.5	0.125	0.25	0.125
C. glabrata	0.5	1	0.25	0.5	0.5
C. parapsilosis	0.5	1	0.125	1	0.25
C. tropicalis	0.25	1	0.125	0.25	0.25
S. gordonii	0.5	2	0.25	0.5	1
S. sanguinis	0.5	2	0.25	1	0.5
A. odontolyticus	>4	>4	>4	>4	>4
A. viscosus	0.25	0.5	0.125	0.25	0.25
C. difficile	>4	>4	>4	>4	>4
P. denticola	4	0.5	0.5	0.5	0.5
P. gingivalis	0.5	1	0.25	0.5	0.5
P. intermedia	>4	>4	2	>4	2

Sample A: Chlorhexidine Control (0.2%).
Sample B: Citrox (1%)/Hyaluronic acid (0.2%)®.
Sample C: Chlorhexidine (0.2%)/Citrox (1%)–Perioplus™.
Sample D: Chlorhexidine (0.2%)/Phenoxetol (0.1%)®.
Sample E: Citrox (1%)–Oralclens™.

Table 1b. Minimum biofilm eradication concentration % (v/v) of mouthrinses against microorganisms grown in biofilms.

Mouthrinse	Minimum Biofilm Eradication Concentration % (v/v)				
	A	B	C	D	E
Microorganism					
C. albicans	4	>4	4	>4	2
C. dubliniensis	2	4	2	>4	4
C. krusei	2	1	0.5	2	0.5
C. glabrata	2	4	2	4	4
C. parapsilosis	>4	4	1	>4	1
C. tropicalis	2	4	2	4	4
S. gordonii	0.5	2	0.5	1	1
S. sanguinis	0.5	2	0.5	1	1
A. odontolyticus	0.25	1	0.125	0.5	0.25
A. viscosus	0.125	0.25	0.125	0.25	0.125
C. difficile	0.5	4	1	4	1
P. denticola	0.125	1	0.5	0.5	0.5
P. gingivalis	0.125	2	0.25	1	1
P. intermedia	0.25	4	0.063	0.125	0.125

Sample A: Chlorhexidine Control (0.2%).
Sample B: Citrox (1%)/Hyaluronic acid (0.2%)®.
Sample C: Chlorhexidine (0.2%) / Citrox (1%)–Perioplus™.
Sample D: Chlorhexidine (0.2%)/Phenoxetol (0.1%)®.
Sample E: Citrox (1%)–Oralclens™.

the test mouthwash that demonstrated a ≥80% reduction in absorbance compared to the control. All experiments were performed on 3 separate occasions.

Results

Minimum inhibitory concentrations for planktonic growth of the mouthrinses for the 14 test microorganisms are shown in **Table 1a**. Inhibition of planktonic cells of all aerobic microorganisms occurred with the 5 mouthrinses at concentrations ≤2% (v/v). Chlorhexidine (0.2%)/Citrox (1%; Perioplus™) demonstrated highest antimicrobial activity against aerobic microorganisms, followed by Citrox (1%; Oralclens)™, 0.2% Chlorhexidine, Chlorhexidine (0.2%)/Phenoxetol (0.1%)® and Citrox (1%)/Hyaluronic acid (0.2%)®. For anaerobic microorganisms, *Actinomyces odontolyticus. Clostridium difficile* and *Prevotella intermedia* had relatively high MICs against the 5 mouthwashes (≥4% (v/v)). *Actinomyces viscosus, Prevotella denticola* and *Porphyromonas gingivalis* had MIC ranging between 0.125%-1% (v/v).

Minimum Biofilm Eradication Concentrations(MBEC) for biofilms

The recorded MBECs in the biofilm assay are shown in **Table 1b**. In the case of aerobic species biofilms, there was a noticeable increased tolerance (MBEC values of up to 16-fold of the previous MIC values) for the majority of the tested microorganisms against all 5 mouthrinses formulations. The MBECs for anaerobic species were generally lower than for the aerobic species. Interestingly, for *A. odontolyticus, C. difficile* and *P. intermedia* the MBEC was lower than the MIC planktonic growth phase, a finding that could indicate relatively poor biofilm development.

Discussion

Oral disease is a worldwide health concern having significant impact on patient quality of life and function. Of further concern is that it is expected that the incidence of dental caries and periodontal disease will continue to increase as a result of the growing intake of sugars in the diet, tobacco use, and inadequate exposure to fluorides, and lack of access to dental care [22].

Increasing antibiotic resistance has promoted interest in the therapeutic use of non-conventional or alternative medicines and plant extracts [13,23-26]. It has been shown that naturally occurring biocides are effective in inactivating a variety of microorganisms by affecting multiple target sites and are therefore less prone to development of resistance compared to conventional antibiotics targeting specific bacterial sites [27,28]. There is great potential for the development of novel bioactive compounds, as there are approximately 500,000 plant species worldwide, of which only 1% have been phytochemically investigated [29].

Hooper et al., [13] previously tested two Citrox formulations against a range of oral microorganisms and reported MICs of 1% (v/v) in planktonic and biofilm assays. Extracts from *Citrus sudachi* have also been observed to elicit antimicrobial

activity against bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Helicobacter pylori* [16]. The relatively high inoculum used in this present study was employed to mimic the *in vivo* challenge of having to combat an established high level of microorganisms, as encountered in dental plaque, and was based on previous evaluations and thus would allow comparisons [13].

The results of this present study demonstrated the antimicrobial effects of 5 mouthrinse formulations on a range of oral microorganisms and *C. difficile*. Four of these mouthwash formulations contained Citrox, which is a combination of natural bioflavonoids. Three of these Citrox mouthrinses were supplemented with Chlorhexidine, hyaluronic acid or phenoxetol.

All test mouthrinses had substantial antimicrobial activity against aerobic planktonic microorganisms at concentrations ≤2% (v/v) with a naerobic species generally less susceptible. This activity against planktonic cells is clearly important, as free-living microorganisms are abundant in saliva and provide the source of bacteria for colonization and subsequent biofilm formation on oral surfaces [30,31]. The inhibition of biofilm growth is widely recognised as being more problematic than that of planktonic cells. The reasons for this are multifactorial and complex. It has been suggested that the extracellular polymeric substance (EPS) that encases biofilm cells is a key component in limiting access of antimicrobial agents to the biofilm cells, possibly through charge mediated sequestration [32]. Alternatively, the biofilm cells themselves may exhibit different phenotypes, and some, may have reduced activity due to nutrient or gaseous limitations [33]. Since cells with reduced growth rates can be more resistant to many antimicrobials, this too could be a reason for enhanced tolerance. It was perhaps not surprising therefore to find that the antibiofilm activity of the mouthrinses against some species was on occasion up to 16-fold higher their planktonic counterparts. Surprisingly however, the MBECs of anaerobic biofilms were frequently lower compared with the MICs for the planktonic counterparts. One caveat to consider was that the biofilm growth for anaerobic bacteria was relatively limited compared to that of the aerobes and this could explain the lower susceptibility compared to their planktonic equivalents. Anaerobic species often require nutrient rich media (e.g. FAB) for growth compared to aerobes and the former also exhibit much slower growth. It may be that the 24 h incubation used for biofilm formation was not sufficient for a robust biofilm growth of the anaerobes, rendering them more susceptible to the mouthrinses. It was also possible that the biofilms generated by the anaerobes could also have been less stable, which may have led to a loss of biofilm cells during the washing steps. To overcome this problem, future studies could incorporate longer incubation periods to allow biofilm formation and in addition, to incorporate artificial saliva into the model system to increase biofilm attachment, as well as utilising a more nutrient rich culture medium.

Conclusions

In conclusion, the results suggest that Chlorhexidine (0.2%)/Citrox (1%; Perioplus™) had highest antimicrobial activity and use of this combination formulation was more effective than either of its constituent antimicrobial components used alone (Citrox or Chlorhexidine). The reason for this finding may be indicative of different targets of these antimicrobial components leading to an enhanced and possible synergistic effect. The mouthrinse, Oralclens™ (1% Citrox) demonstrated a slightly higher antimicrobial activity than mouthrinse Citrox (1%)/Hyaluronic acid (0.2%)®. Importantly however, previous studies have demonstrated that 0.2% hyaluronan-containing gel provides benefit as an adjunct to scaling and root planning (SRP) in chronic periodontitis patients leading to a significant improvement in gingival parameters [34].

Given the reported problems of several over-the-counter mouthrinses, such as staining of enamel, burning sensation, alterations in taste, and the presence of an alcohol component [30,35,36], there is a need for continual development of effective mouthrinses to aid oral hygiene regimes. As a result, natural compounds such as those incorporated into Citrox may be suitable alternatives. Further more, by combining Citrox with other supplements as shown in this preliminary study, enhanced antimicrobial effects against common oral pathogens can be obtained. These findings support further investigation into Citrox as a potential future preparation for oral care products and potentially other clinical areas where biofilms need to be prevented.

List of abbreviations
MBEC: Minimum biofilm eradication concentration
MIC: Minimum inhibitory concentration
FAA: Fastidious anaerobe agar
FAB: Fastidious anaerobe broth
BA: Blood agar
BHI: Brain heart infusion broth
SDA: Sabouraud Dextrose Agar
SAB: Sabouraud Broth
PBS: Phosphate buffered saline
MRSA: Methicillin-resistant *Staphylococcus aureus*

Competing interests
The authors declare that they have no competing interests.

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