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

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Abstract

**Background:** 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.

**Methods:** Mouthrinse formulations were Chlorhexidine (0.2%), Citrox (1%; Periplus™)/Hyaluronic acid (0.2%)<sup>®</sup>, Chlorhexidine (0.2%)/Citrox (1%; Periplus™), Chlorhexidine (0.2%)/Phenoxytol (0.1%<sup>®</sup>) and Citrox (1%; Oralcleans)<sup>®</sup> (Oraldent Ltd; UK). The test microorganisms were the bacteria, Actinomyces viscosus ATCC 1598; Actinomyces odontolyticus NCTC 9935, Clostridium difficile R8651, Prevotella intermedia NCTC 13070<sup>®</sup>, Prevotella denticola R20771, Porphyromonas gingivalis NCTC 11834<sup>®</sup>, Streptococcus gordonii ATCC 10558<sup>®</sup>, 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.

**Results:** Planktonic cells of aerobic microorganisms were inhibited by all mouthrinses at concentrations ≤2% (v/v) of the stock preparation. Chlorhexidine (0.2%)/Citrox (1%)<sup>®</sup> had the highest antimicrobial activity, followed by Citrox (1%)<sup>®</sup>, 0.2% Chlorhexidine, Chlorhexidine (0.2%)/Phenoxytol (0.1%<sup>®</sup>) and Citrox (1%)/Hyaluronic acid (0.2%)<sup>®</sup>. 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.

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

**Keywords:** Periodontal diseases, dental caries, oral candidosis planktomic 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 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%)⁶. 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 study were Actinomyces viscosus (ATCC 1598), Actinomyces odontolyticus (NCTC 9935), Clostridium difficile (R865, clinical isolate), Prevotella intermedia (NCTC 13070⁷), Prevotella denticola (R20771), Porphyromonas gingivalis (NCTC 11834⁸), Streptococcus gordonii (ATCC 10558⁹), 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, Actinomyces odontolyticus NCTC 9935, Clostridium difficile R8651, Prevotella intermedia NCTC 13070⁷, Prevotella denticola R20771 and Porphyromonas gingivalis NCTC 11834⁸ 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 h at 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 620ₙₘ. 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-bottomed microtitre 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 620ₙₘ. Following a further incubation period of 6 h, the absorbance at 620ₙₘ 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 were recorded as the lowest concentration of
Table 1a. Minimum inhibitory concentration % (v/v) of mouthrinses against planktonic microorganisms.

| Microorganism | A   | B   | C   | D   | E   |
|---------------|-----|-----|-----|-----|-----|
| 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. parapalis  | 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%)².
Sample C: Chlorhexidine (0.2%)/Citrox (1%); Perioplus™.
Sample D: Chlorhexidine (0.2%)/Phenoxetol (0.1%); Oralclens™.
Sample E: Citrox (1%)/Oralclens™.

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

| Microorganism | A   | B   | C   | D   | E   |
|---------------|-----|-----|-----|-----|-----|
| 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. parapalis  | >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 | 0.25 | 0.063 | 0.125 | 0.125 |

Sample A: Chlorhexidine Control (0.2%).
Sample B: Citrox (1%)/Hyaluronic acid (0.2%)².
Sample C: Chlorhexidine (0.2%)/Citrox (1%); Perioplus™.
Sample D: Chlorhexidine (0.2%)/Phenoxetol (0.1%); Oralclens™.
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%); Oralclens™. For anaerobic microorganisms, Actinomyces odontolyticus, Clostridium difficile and Prevotella intermedia had relatively high MICs against the 5 mouthwashes (≥4% (v/v)). Actinomyces viscous, 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.
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 anti-
microbial 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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