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The pharmacokinetics of hexylresorcinol-containing lozenges and their antimicrobial efficacy against oral and respiratory microorganisms

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ABSTRACT

Background: Hexylresorcinol (HR) lozenges provide symptomatic relief for sore throats. Despite its recognised anaesthetic and antiseptic properties, evidence of HR bactericidal activity in these formulations is limited, being only recently described in planktonic bacteria. We defined antimicrobial/antiviral activity in planktonic and biofilm models and characterised the pharmacokinetics of HR release from lozenges.

Methods: Antimicrobial activity (purified or released from lozenges) was determined against oropharyngeal pathogens using minimum inhibitory concentration (MIC) and Log_{10} reduction assays. Antiviral activity was determined by suspension test (EN14476). Antibiofilm effects employed minimum biofilm eradication concentration assays and confocal laser scanning microscopy. HR release from lozenges was studied in vitro and in vivo using HPLC.

Results: HR exhibited MICs ≤ 16 µg/mL against 19/25 strains including: Streptococcus, Staphylococcus and Candida spp. Marked bactericidal activity (>3_{log10}; >99.9% reduction) occurred within 10 minutes. Significant anti-biofilm activity was evident in streptococcal and candidal biofilms ($p < 0.05$). Log_{10} reduction in virucidal infectivity of HR in lozenges ranged from 1-log_{10} to 3.5-log_{10}. In vivo, HR exhibited rapid release (within 1 minute) from lozenges into saliva.

Conclusion: Rapid release and antimicrobial activity of HR against oropharyngeal pathogens was evident, occurring at concentrations ≥ 2-fold lower than present in saliva, highlighting the potential application of HR in the treatment of oropharyngeal infections.

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Introduction

Antiseptic agents (e.g. alcohols, biguanides, peroxides, surfactants and essential oils) are commonly employed in over-the-counter topical formulations in the treatment of a range of dental and oropharyngeal diseases (e.g. periodontal disease, recurrent oral ulceration and pharyngitis). The COVID-19 pandemic has led to a re-evaluation of active pharmaceutical ingredients as effective antiviral and antimicrobial agents [1], heightened by the recent finding that a 30 second mouthwash eliminated the live viral load of SARS CoV-2 in hospitalised patients for up to 1 hour [2]. In comparison to mouthwashes, lozenge formulations afford added benefits, including higher doses and controlled release of active agents within the oral cavity [3], highlighting the theoretical possibility of achieving higher sustained concentrations of active antimicrobial/antiviral delivery [4]. Despite this, and the variety of antimicrobial lozenge formulations available (e.g. cetylpyridinium chloride, 2.4-dichlorobenzyl alcohol, amylmetacresol and hexylresorcinol), the antimicrobial and antiviral activity of lozenge formulations has, to date, received relatively little attention.

Oral delivery of biologics is perhaps the least invasive and therefore most convenient route for the administration of small-molecule drugs. However, with macromolecules (proteins and peptides) this route is far less effective, partly hampered by low intestinal permeability and stomach acid. Increased interest exists in developing more routes for non-invasive systemic delivery of biologics, with lozenges representing a useful route for targeted delivery of drugs to specific (e.g. oral) tissues.

A range of essential oils (e.g. eucalyptus, juniper, lemon balm, pine turpentine, sage and tea tree) have all been shown to possess antimicrobial properties [5], with several (e.g. eucalyptus, lemon and tea tree oils) currently used in dentistry as antimicrobial

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agents [6]. Hexylresorcinol (HR) is an organic phenol (dihydroxybenzene) that is naturally produced in higher plants [7]. Organic phenols, such as HR, are employed as disinfectants due to their ability to interact with and disrupt the bacterial cell membrane, increasing membrane permeability and interfering with bacterial metabolism. Active HR (an alkylresorcinol) acts as a topical anaesthetic to relieve sore throats and clinical studies have demonstrated its analgesic effects with the onset of relief occurring within 5 minutes and for a duration of up to 2 hours [8]. Structurally, HR ($C_{12}H_{18}O_2$) consists of two hydroxyl groups (–OH) with its local anaesthetic properties likely due to its ability to block neuronal sodium channels, thereby preventing initiation and conduction of nerve impulses [9,10].

HR is currently employed in mouthwashes, lozenges creams/lotions, and is utilised as a food-additive [11]. The antimicrobial activity of HR has been described *in vitro* against a range of pathogens including *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Streptococcus pyogenes* using log reduction assays and planktonic growth conditions [12]. *In vivo*, however, (in both health and disease) bacteria exist predominantly in complex multi-species biofilm communities, effectively encased in an extracellular polymeric matrix (EPS). EPS is an entangled polymer network of polysaccharides, extracellular DNA (eDNA), proteins and lipids [13]. The biofilm state confers protection from mechanical shear forces and increased tolerance to antimicrobials. The inherent ability of biofilms to resist antibiotics, in part occurs through reduced metabolic activity, persister cell formation and reduced antibiotic/small molecule diffusion through the EPS polymeric network via charge-interactions [14,15]. Although HR has been in use for many years, few studies have examined its bactericidal and antibiofilm effectiveness in lozenges, or assessed its antiviral activity. In addition, to our knowledge, no *in vivo* studies have been performed.

Three important properties are required for a chemical agent to effectively target EPS-embedded microbes namely: *substantivity* (the ability to bind to oral/oropharyngeal tissues and be released at therapeutic doses); *penetrability* (of the EPS polymeric network) and *selectivity* (the ability to affect specific bacteria found within a mixed population) [16]. Neutrally-charged phenolic essential oils have demonstrated efficacy in inducing significant plaque reduction [17]. The report of HR antimicrobial activity against *S. pyogenes* ATCC 19615 biofilms [18] highlights the possibility that HR may afford a selective advantage in biofilm disruption compared to other plant-based phenolic antimicrobials (e.g. thymol and menthol). The mechanism of action of phenols such as hexylresorcinol involves cell-wall disruption [19]. *In vitro* studies have also, interestingly, demonstrated the antiviral effect of HR against cytomegalovirus and parainfluenza virus [20]. Whilst these results appear promising, the pharmacokinetics of HR release from lozenge formulations in the oral cavity *in vivo* are unknown, and the effective available concentrations of HR within the oral cavity have yet to be elucidated. We hypothesised that HR lozenges have antiviral and antimicrobial activity (including against bacterial biofilms) and that we can observe HR release at clinically significant concentrations *in vivo* in humans.

In this study, we sought to increase our understanding of the antimicrobial effectiveness of topical HR in lozenge formulations, by firstly determining the *in vitro* activity of HR at clinically relevant concentrations against a range of reference and clinical isolates from oro-pharyngeal infections in planktonic (free-floating) and biofilm (sessile) cell models and, its antiviral activity against enveloped and non-enveloped viruses. Secondly, we sought to characterise the pharmacokinetics of HR release *in vivo* in human volunteers.

Materials and methods

Test materials

Strepsils® triple action Blackcurrant (BC) and Honey-Lemon (HL) flavoured lozenges (containing 2.4 mg hexylresorcinol per lozenge) were used in this study (Reckitt Health Ltd, UK). Hexylresorcinol (HR, powdered form) and menthol (crystalline form) were provided by Reckitt Health Ltd, UK. (Slough, UK). HR and menthol were freshly prepared in ethanol and water, respectively, prior to use.

Microbial strains used and growth conditions

The oral and respiratory microorganisms used in this study are included in Table 1 and included Gram-positives, Gram-negatives, strict anaerobes and fungi. The aerobic bacteria were maintained on blood agar (BA; Blood agar base no. 2, Neogen, Paisley, UK) supplemented with 5% (v/v) defibrinated horse blood (Fisher Scientific, Loughborough, UK) at 37°C and overnight cultures were grown in brain heart infusion (BHI; Oxoid, Basingstoke, UK) broth and cation-adjusted Mueller Hinton broth (MH; Mueller Hinton broth 2, Sigma-Aldrich Ltd, UK) was used for susceptibility testing. The anaerobic bacteria were maintained on fastidious anaerobe agar (FAA; Neogen) supplemented with 5% (v/v) defibrinated horse blood and were grown anaerobically at 37°C and overnight cultures and susceptibility testing were grown in fastidious anaerobe broth (FAB; Neogen, UK; without blood) and of anaerobic bacteria. The

Table 1. MIC (μg/mL), MBEC (μg/mL) and FICI determinations for (purified) hexylresorcinol and menthol against oral and respiratory pathogens including Gram-positives, Gram-negatives, strict anaerobes and fungi.

	MIC (μg/mL)			MBEC (μg/mL)	FICI	Source
Test microorganisms	HR	Menthol	EtOH	HR	HR-Menthol	
Staphylococcus aureus 37862	16	8192	>1024	128	1.02 (Additive)	BAL (UHW)
S. aureus 37861	32	>1024	>1024			BAL (UHW)
S. aureus 38622	32	8192	>1024		1.02 (Additive)	Sputum (UHW)
S. aureus (MRSA) 1004A	32	>8192	>1024	256	<1.12 (Additive)	[21]
Streptococcus dysgalactiae 33323	16	>8192	>1024	512	<3 (Indifferent)	Sputum (UHW)
Streptococcus mutans DSM 20523	32	>8192	>1024	128	0.62 (Additive)	DSM
Streptococcus pneumoniae 7874	16	>8192	>1024	32		(UHW)
S. pneumoniae NCTC 12977	16	>8192	>1024			NCTC
Streptococcus pyogenes 21966	8	>1024	>1024			Throat swab (UHW)
S. pyogenes 38387	8	>2048	>1024	64	<1.03 (Additive)	Throat swab (UHW)
Aggregatibacter actinomycetemcomitans DSM 8324	2	2048	>1024	256	<0.9 (Additive)	DSM
A. actinomycetemcomitans ATCC 33384	32	>8192	>1024	256	<1.12 (Additive)	ATCC
Moraxella catarrhalis 38305	16	8192	>1024	128	3.07 (Indifferent)	Sputum (UHW)
M. catarrhalis 38296	16	>8192	>1024		<1.5 (Additive)	Sputum (UHW)
M. catarrhalis 38618	16	>1024	>1024			Sputum (UHW)
Fusobacterium nucleatum ATCC 49256	64	>4096	>1024	64		ATCC
Porphyromonas gingivalis NCTC 11834	8	4096	>1024	64		NCTC
P. gingivalis (W50) ATCC 53978	8	4096	>1024	64		ATCC
Prevotella intermedia	16	4096	>1024	64		Oral (COMDL)
Prevotella nigrescens	8	4096	>1024	64		Oral (COMDL)
Candida albicans GBJ	8	1024	>1024	>512	3 (Indifferent)	[22]
C. albicans ATCC 90028	16	>1024	>1024	>512		ATCC
C. albicans 8367	16	>1024	>1024			BAL (UHW)
C. albicans 8368	16	>1024	>1024			BAL (UHW)
C. albicans 8373	16	>1024	>1024			BAL (UHW)

MRSA, methicillin-resistant *Staphylococcus aureus*; DSM, German collection of microorganisms and cell cultures; NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; BAL, bronchoalveolar lavage; UHW, University Hospital of Wales, Cardiff; COMDL, Clinical Oral Microbiology Diagnostic Laboratory, Cardiff; EtOH, ethanol equivalents.

yeasts were maintained on Sabouraud dextrose (SDA; Neogen) agar at 30°C and grown in Sabouraud dextrose broth (SDB; Neogen) for overnight cultures and RPMI 1640 medium (made according to manufacturer's protocol) was used for the susceptibility testing assays. The streptococci and *Aggregatibacter actinomycetemcomitans* (*A.a*) strains were grown microaerophilically in 5% CO_2 for 48 h, and anaerobes grown anaerobically in 10% CO_2, 10% H_2, 80% N_2 for 96 h.

Determination of minimum inhibitory concentration (MIC)

Menthol and three different forms of HR were tested for antimicrobial activity; HR in its purified form, as well as in two varieties of hard-boiled lozenges (BC and HL). Stock solutions of HR (10 mg/mL for bacteria and 1 mg/mL for *Candida* species) were made up freshly in ethanol prior to use. Alongside this, the HR containing lozenges were dissolved in 4.8 mL of either MH broth, RPMI or artificial saliva [23] to give a final HR concentration of 0.5 mg/mL (the lozenges are insoluble in ethanol). Ethanol was also tested as a control (at equivalent concentrations to those in the HR MIC dilutions) for all the experiments using purified HR.

MICs were performed by broth microdilution method according to Jorgensen et al. [24] following standard CLSI guidelines [25]. Overnight microbial cultures were for 24 h, with the exception of *Streptococcus* sp. and *Enterococcus faecalis* (48 h) and anaerobic bacteria (72 h). Cultures were adjusted in PBS to an OD_{600} of 0.8 (anaerobic bacteria) and between 0.08 and 0.10 for other strains equivalent to $\approx 10^8$ colony forming units (CFU)/mL (0.5 McFarland standard). Two-fold serial dilutions of HR (0.5–512 μg/mL) and menthol (1–8192 μg/mL) were prepared in MH broth, FAB (anaerobic bacteria) or RPMI 1640 medium (*C. albicans*) alongside ethanol equivalent controls (v/v) in sterile, flat-bottom 96-well microtiter plates ($n = 3$). The adjusted microbial cultures were diluted 10-fold and 5 μL of culture was added to the microtiter plate containing the serial dilutions of the test compounds to give a final concentration of 5×10^5 CFU/mL. The plates were incubated at 37°C for 16–20 h, with the exception of *Streptococcus* sp. (48 h) and anaerobes (96 h). Growth in the plates was then assessed visually and confirmed by adding 30 μL resazurin dye solution per well (0.01% in dH_2O). Ethanol-equivalent and negative (untreated) controls were also tested. MIC is defined as the lowest concentration of test compound that showed no visible growth after 16–20 h incubation. Results were expressed as mode ($n = 3$). Growth controls (no test compounds) and sterility controls (no bacteria) were also performed.

Checkerboard assays with HR and menthol

Checkerboard broth dilution assays were performed as previously described [26,27] to study the combined effect of HR and menthol against a range of test microorganisms. Menthol (100 μL)

was placed in the wells of row 1, then serially diluted along the ordinate with MH broth, FAB (anaerobic bacteria) or RPMI 1640 medium (*C. albicans*). Serially diluted HR (50 µL) was then added to the wells in decreasing concentration along the abscissa. Each microtiter well was inoculated with the test organism (5×10^5 CFU/mL) and incubated at 37°C for 16–96 h at 37°C as described above. Growth was interpreted by adding 30 µL resazurin dye solution per well. Results were expressed as mode (n=3). Fractional Inhibitory Concentration Index (FICI) was interpreted as synergistic (FICI ≤ 0.5), additive (0.5 < FICI < 2), indifferent (2 ≤ FICI < 4) and antagonistic (FICI ≥ 4) based on the equation below.

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = \overset{A}{FIC} + \overset{B}{FIC} = FICI$$

Determination of minimum biofilm eradication concentration (MBEC)

For the MBEC assay [28], overnight microbial cultures were adjusted 10^8 CFU/mL and 100 µL of adjusted culture added to the wells of a 96-well microtiter plate. Plates were sealed with parafilm to avoid dehydration and biofilms were grown for 24 h at 30 rpm for aerobes, or statically for 48 h (*Streptococcus* spp. and *Enterococcus faecalis*) and 72 h (*A.a*) with 5% CO_2 and 96 h for anaerobic bacteria at 37°C. The spent medium was replaced with 100 µL fresh FAB after 48 h to assist biofilm formation by the anaerobic bacteria. After incubation, non-adherent cells were removed by washing with phosphate buffered saline (PBS). The biofilms were then exposed to HR or ethanol equivalent controls (v/v). Two-fold serial dilutions were prepared in the required medium ($n = 3$) and added to the biofilm with a starting concentration of 512 µg/mL. The plates were again incubated statically or shaking (as above) at 37°C for 24 h. After incubation, the supernatants were removed from the biofilms and 100 µL fresh medium was added to each well. Plates were re-incubated for a further 24 h at 37°C to look for regrowth of the biofilm. Microbial biofilm formation was assessed visually and by adding 30 µL resazurin dye solution per well. Growth controls (no test compounds) and sterility controls (no microorganism) were also performed.

Microbicidal activity of HR lozenges in artificial saliva using a Log_{10} reduction assay

HR lozenges (BC and HL) containing 2.4 mg per lozenge were dissolved separately at 45 ± 1°C in 4.8 mL of artificial saliva. The bactericidal assay was performed in artificial saliva by broth microdilution method [23]. Overnight microbial cultures ($n = 3$) were adjusted to an OD_{600} of 0.08–0.10 in PBS (0.5 McFarland standard, ~10^8 CFU/mL). Lozenges in artificial saliva were inoculated with 0.1 mL of the adjusted microbial culture. The sample was vortexed thoroughly and then tested after 1, 5, 10 and 30 minutes; 10 minutes being consistent with the time a lozenge takes to dissolve in the mouth. After the required time points, 1 mL of the sample mixture was removed and transferred into 9 mL of neutralizing diluent (peptone water, 1 g; sodium chloride, 9 g; lecithin, 3 g; polysorbate 80, 10 mL; distilled water, 990 mL; pH 6.6 ± 0.2). Neutralization validation was performed against all the test organisms. Solutions were serially diluted to 10^{-4}, plated onto the appropriate agar medium and incubated for a minimum of 4 days at 37°C. The number of colonies in each dilution were counted and the mean log reduction in CFUs/mL was calculated from the three test replicates. As the positive control, 0.1 mL of test inoculum for each microorganism in 4.9 mL artificial saliva (with no lozenges) was tested at the same time points. For this study, a less than 1 log reduction was considered to be low antimicrobial activity, a 1 to 3 log reduction to be moderate activity, and a greater than a 3-log reduction to be high antimicrobial activity.

Confocal laser scanning microscopy (CLSM) biofilm formation and disruption assays

For the biofilm formation assay, overnight cultures ($n = 3$) of *C. albicans* ATCC 90028 were adjusted to ~10^7 CFU/mL (OD_{600} 0.37) using RPMI 1640 medium in glass-bottom optical 96-well plates and the adjusted cultures were diluted 1:10 in RPMI medium. The plates were incubated on a rocker (20 rpm) for 45 minutes at 37°C to assist biofilm formation. The biofilms were then gently washed (3x) in prewarmed RPMI medium to remove non-adherent cells. For the biofilm disruption assay, *S. pyogenes* 38387 (24 h cultures) were diluted to OD_{600} of 0.05, in BHI. The adjusted cultures were diluted 1:10 and the glass-bottom optical 96-well plates were incubated for 24 h microaerophilically in 5% CO_2, statically at 37°C.

All the biofilms were then exposed to HR at a concentration equivalent to MBEC, ¼MBEC, and MIC along with the MBEC ethanol equivalent control (v/v). The plates were further incubated for 24 h at 37°C (with 20 rpm shaking for *C. albicans* ATCC 90028). After 24 h, the supernatants were gently removed without disturbing the biofilms and the biofilms were stained with 7 µL LIVE/DEAD Baclight stain (BacLight Bacterial Viability Kit Invitrogen, Paisley, UK; 2 µL SYTO 9 component A and 2 µL propidium iodide component B in 1 mL PBS). Plates were incubated for 2–3 minutes and wrapped in foil to avoid light exposure. A further

43 µL of PBS was then added to each well to keep the biofilms hydrated before visualisation by CLSM. Five CLSM Z-stack images were taken for each well using an inverted Leica SP5 confocal microscope [LIVE/DEAD staining depicts LIVE (SYTO 9: $\lambda_{ex}/\lambda_{em}$ maximum, 480/500 nm) and DEAD cells (propidium iodide: $\lambda_{ex}/\lambda_{em}$ maximum, 490/635 nm)] as green and red, respectively, under × 63 lens (under oil).

Cells and viruses

Vero E6 (ATCC # CRL-1586), Vero E6 expressing ACE2 and TMPRSS2 (Vero A/T), RD cells (Culture Collections, UKHSA # 85111502) and human foetal foreskin fibroblasts (HFFF) were maintained at 37°C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Merck) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Merck; DMEM-10). MDCK cells (ATCC # CCL-34) were maintained at 37°C, 5% CO_2 in DMEM supplemented with 10% FBS and 1X non-essential amino acids (Merck). The England-2 strain of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2; Public Health England) and Parainfluenza virus type 5 (PIV-5), were grown in Vero E6 cells in the presence of 2% FBS. Influenza A virus (IAV; strain A/X-31 H3N2) was grown in MDCK cells in serum-free medium in the presence of 1 µg/mL TPCK trypsin (Merck). Enterovirus 71 (EV-A71; Culture Collections, UKHSA, # 0812214 v) was grown in RD cells in the presence of 2% FBS. Cytomegalovirus (CMV) expressing GFP was grown in HFFFs as described previously [29].

For plaque assays, Vero A/T (SARS-CoV-2), Vero E6 (PIV-5), MDCK (IAV), or RD (EV-A71) cells were seeded at $1x10^5$/well in 12-well plates 18 h prior to use, to give a monolayer of ~ 70% confluency. Samples were serially diluted 10-fold in serum-free DMEM and cells infected for 1 h at 37°C, 5% CO_2. Virus inoculum was removed and cells overlayed with a 1:1 mix of 2.4% Avicel® and 2X MEM (20% 10X MEM; 2% L-Glutamine; 4% FBS; 5.4% sodium bicarbonate [7.5% soln.]) for SARS-CoV-2, PIV-5 and EV-A71, or a 1:3 mix of 2.4% Avicel® and overlay media (15% 10X MEM; 4% BSA [7.5% soln.]; 1.5% L-Glutamine; 3% sodium bicarbonate [7.5% soln.]; 1.5% 1 M HEPES; 0.75% Dextran [1% soln.]) for IAV. Cells were incubated for 72 (SARS-CoV-2, IAV) or 96 h (PIV-5, EV-A71) at 37°C, 5% CO_2 at which point overlay was removed, cells were washed once with phosphate buffered saline (PBS) and fixed with 1 mL/well methanol for 5 minutes. Following the removal of methanol, cells were stained with 0.1% (w/v) crystal violet, and plaques counted.

For CMV titrations, HFFFs were seeded at $1x10^4$/well in 96-well plates 18 hours prior to use, to give a monolayer of ~ 70% confluency. Samples were serially diluted 10-fold in DMEM-10, media removed from cells, and samples added at 100 µl/well in duplicate. At 24 hours the number of GFP positive cells was determined using an Incucyte® Live Cell Analysis System (Sartorius; Version 2022B Rev2), with each GFP positive cell equating to 1 plaque forming unit (PFU). All SARS-CoV-2 work was carried out in an approved Category 3 facility.

Virucidal Log_{10} reduction assays

BC and HL lozenges were dissolved in 4.8 mL/lozenge of an artificial saliva formulation used previously [20], while purified HR was reconstituted in ethanol and added to artificial saliva to both give a final HR concentration of 0.5 mg/mL. Then, 50 µL of virus, containing approx. 10^6 PFU, was added to 450 µl aliquots of dissolved lozenge, purified HR, or artificial saliva for controls, vortexed to mix, and incubated at room temperature for 1, 5, 10, or 20 minutes (lozenge and artificial saliva), or for 1 or 5 minutes (purified HR; HR-1 and HR-5 samples). Samples were neutralised by the addition of 500 µl DMEM + 2% FBS and serially diluted for titration by plaque assay, as above.

In vitro HR release from lozenges

To assess release of HR from lozenges over time, BC and HL lozenges were dissolved and stirred continuously in PBS or artificial saliva (AS) (10 mL, 1 tube per timepoint/flavour) in an aerobic environment at 37°C. Then, at pre-determined timepoints (1, 5, 10 and 30 minutes), samples (1 mL, $n = 3$) were taken from the respective tubes and immediately frozen at −20°C until analysis by high performance liquid chromatography (HPLC), as described below. Samples (diluted 1 in 1000) and HR standards were prepared in mobile phase. Samples were evaluated in duplicate, then expressed as mean HR concentration ± SD ($n = 3$).

In vivo HR release from lozenges

Ethical approval was obtained from Cardiff University School of Dentistry Research Ethics Committee (Reference: 2310). Informed written consent was obtained from 10 study (volunteer) participants. Inclusion criteria included being >18 with no history of infectious illness within the 2 weeks prior to the test. The washout period for throat sprays, mouthwash, chewing gum or throat lozenges was 12 h and participants did not ingest food or water in the 1 h prior to the scheduled dosing time. Participants received a single (2.4 mg) HR dose in the form of a lozenge of either BC (5 subjects) or HL (5 subjects).

Participants were instructed to dissolve the lozenge in the mouth for 60 minutes. After 1, 5, 15, 30, 45 and 60 minutes, participants gave a saliva sample while holding the lozenge in the buccal sulcus. Samples were immediately frozen at −80°C, prior to analysis by HPLC.

The samples were prepared according to a modified method of [30]. Briefly, samples and HR standards (prepared in 50 µL human saliva) were mixed with an equal volume of Milli-Q water in a 2-mL tube, then acetonitrile (900 µL, 90% v/v) was added to precipitate proteins. The solution was vortex mixed at room temperature for 3 minutes, then centrifuged twice at 13,500 rpm at 4°C for 10 minutes (transferring the supernatant to a new 2 mL tube each time). The final supernatant was transferred to an amber glass vial for HPLC analysis, as described below. Samples were evaluated in duplicate, then expressed as mean HR ± SEM ($n = 5$).

Quantification of HR

The HR detection method was adapted from [31]. Briefly, high-pressure liquid chromatography with fluorescence detection (HPLC-FLD) was performed using a Dionex ICS-3000 ion chromatography system (Thermo Scientific, Gloucester, UK) equipped with a Dionex AS autosampler (maintained at 4°C). Data was collected and processed using Chromeleon 6.80 software. Chromatographic separation was achieved using a Gemini® 5 mm C18 110 Å column (4.6 × 30 mm, Phenomenex, Macclesfield, UK) connected to a SecurityGuard Gemini® C18 cartridge (4 ×3.0 mm, Phenomenex, Macclesfield, UK) inside a column oven at 30°C. The mobile phase of 0.01 M KH_2PO_4 (pH of 3.0 with 25% H_3PO_4)/acetonitrile (60:40 v/v) was eluted with isocratic conditions at 1.0 mL/min and the detector was set for an excitation wavelength of 280 nm and emission wavelength of 310 nm. An injection volume of 20 µL and run time of 15 minutes were used. The retention time of HR was 5 minutes. To quantify the HR content, calibration curves were constructed by preparing a series of concentrations of HR (0.016–80 µg/mL) in the relevant solvent for the in vitro and in vivo experiments. All solvents were of general reagent grade (unless stated) and were from Fisher Scientific (Loughborough, UK).

Statistical analysis

MIC and MBEC values are presented as the mode of three biological repeats. Other data values represent either means ±SD or means ±SEM. For each test, $p < 0.05$ was considered significant. Normality assessment of the data (Shapiro–Wilk analysis) was performed to assess whether parametric or nonparametric statistical testing was appropriate. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism 10.4.1.

Results

Minimum inhibitory concentration (MIC) determination for hexylresorcinol and menthol

The results of the antimicrobial susceptibility testing of HR and menthol against oral and respiratory microorganisms (25 strains) are presented in Table 1. HR exhibited an MIC range from 2 to 64 µg/mL, with the lowest MICs obtained against Gram-positive *S. pyogenes* 21966 and 38387 (8 µg/mL), Gram-negative *A.a* DSM 8324 (2 µg/mL) and anaerobic bacteria *P. gingivalis* NCTC 11834, *P. gingivalis* W50 and *P. nigrescens* (8 µg/mL). HR also demonstrated antimicrobial efficacy against *C. albicans* exhibiting an MIC range between 8 and 16 µg/mL. In contrast, menthol did not show any antimicrobial activity below 1024 µg/mL against any of the organisms tested. The ethanol vehicle controls showed no inhibitory effects against the test organisms at equivalent concentrations to those used for HR and menthol.

Minimum biofilm eradication concentration (MBEC) determinations for hexylresorcinol

Results of the MBEC assays for 16 strains are shown in Table 1. It was observed that the MBEC values for HR increased by 1- (*Streptococcus pneumoniae* 7874) to 7-fold (*A. actinomycetemcomitans* DSM 8324) compared to the MIC, except for *F. nucleatum* ATCC 49256 whose MIC and MBEC values were both the same (64 µg/mL). The lowest MBEC value (32 µg/mL) was recorded against *S. pneumoniae* 7874, with the highest (512 µg/mL) for *C. albicans* GBJ and ATCC 90028. Hence, HR was most effective against biofilms of anaerobes. The ethanol equivalents were ineffective against the test organisms at the highest concentration tested (1024 µg/mL).

Combination effects with HR and menthol in checkerboard assays

Results of the microdilution checkboard assay to study the combined effect of HR and menthol against the test organisms, (determined as FIC Index), are presented in Table 1. Amongst the eleven test organisms selected for the study, eight organisms exhibited additivity (FICI = 0.625 to < 1.5) while three organisms exhibited indifference. Additivity indicated that

the HR-menthol combination had no increase in antimicrobial activity (except from the additive effect of both compounds combined) while indifferent activity indicated that HR-menthol together showed no increase in antimicrobial activity. The ethanol equivalents did not inhibit growth of the test organisms within the range tested.

Comparison of the antimicrobial efficacy of the two different HR lozenges tested

The BC and HL lozenges gave virtually the same MICs for all the bacteria tested compared to purified HR, the only discrepancies being within a one-fold difference of each other, (i.e. within acceptable pipetting error). *S. pyogenes* was the exception to this where both lozenges showed much higher MICs than for the purified form HR (>256 vs 64; and 64 vs 16 µg/mL respectively). This was the case regardless of which medium (MH broth or artificial saliva) the lozenge was dissolved in (Table 2a).

Effect of dissolution of the lozenges in artificial saliva on MIC determinations

Comparing the MICs determined for lozenges dissolved in artificial saliva with those dissolved in MH broth again generally gave similar results. The exception (Table 2b) was *S. pyogenes* (>256 vs 64 µg/mL) when tested against both lozenges, and *S. pyogenes* (64 vs 16 µg/mL) when tested against purified HR (in ethanol). The MICs in these cases were ≥ 2-fold greater than for HR dissolved in ethanol, which could not be considered to be solely due to pipetting error.

Microbicidal activity of HR lozenges in artificial saliva

Evidence of the microbicidal activity of the BC and HL lozenges was observed for all the test microorganisms at the 1-minute time point of the Log_{10} reduction assay as shown in Figure 1 (Tables S1 and S2); with the majority (8/9 strains) reaching > 3 log reduction (high; >99.9%) by the 10-minute time point.

Effect of HR on biofilm formation and disruption

In the biofilm disruption assay, CLSM revealed 24 h treatment of the *S. pyogenes* 38387 biofilms with HR induced concentration-dependent biofilm disruption at 64 µg/mL (MBEC), reflected by significant decreases in cell viability (increased DEAD:LIVE cell ratio) (Figure 2a; $p < 0.05$). In the biofilm formation assay, HR-induced disruption of *C. albicans* ATCC 90028 biofilms was evident at 128 µg/mL (¼MBEC), with a concomitant significant increase in DEAD/LIVE cell ratio (Figure 2b; $p < 0.05$).

Virucidal Log_{10} reduction assays

The virucidal activity of purified HR and HR in dissolved lozenges was determined against enveloped respiratory viruses SARS-CoV-2, Parainfluenza type 5 (PIV-5), and Influenza A virus (IAV); the enveloped human herpesvirus, Cytomegalovirus (CMV); as well as non-enveloped enterovirus type 71 (EV-A71), a surrogate for rhinovirus also transmitted via respiratory secretions. Testing of SARS-CoV-2 against doubling dilutions of purified HR demonstrated that HR suffered almost complete loss of potency at concentrations lower than the lozenge concentration (Figure 3a) . Following 1 minute incubation with purified HR, we were unable to detect

Table 2. Bactericidal activity (minimum inhibitory concentration determinations; MICs; µg/mL) of purified hexylresorcinol (dissolved in ethanol) and hexylresorcinol lozenges (in MH broth or artificial saliva) for a range of oral and respiratory bacteria.

Strains	HR	Ethanol	HL	BC
(a) Lozenges dissolved in Mueller-Hinton (MH) Broth				
Moraxella catarrhalis 38305	16	>256	16	16
Staphylococcus aureus 38622	64	>256	64	64
Streptococcus dysgalactiae 33323	32	>256	32	64
Streptococcus pyogenes 21966	16	>256	64	64
Candida albicans ATCC 90028	16	>64	32	32
Candida albicans GBJ	16	>64	32	32
Candida albicans 8367	32	>64	32	64
(b) Lozenges dissolved in artificial saliva (AS)				
Moraxella catarrhalis 38305	64	>256	64	64
Staphylococcus aureus 38622	64	>256	64	128
Streptococcus dysgalactiae 33323	64	>256	64	64
Streptococcus pyogenes 21966	64	>256	>256	>256
Candida albicans ATCC 90028	16	64	32	64
Candida albicans GBJ	16	64	32	32
Candida albicans 8367	16	64	32	64

As the HR lozenges were dissolved directly into MH broth or artificial saliva, the ethanol controls are only relevant for MICs using purified HR.

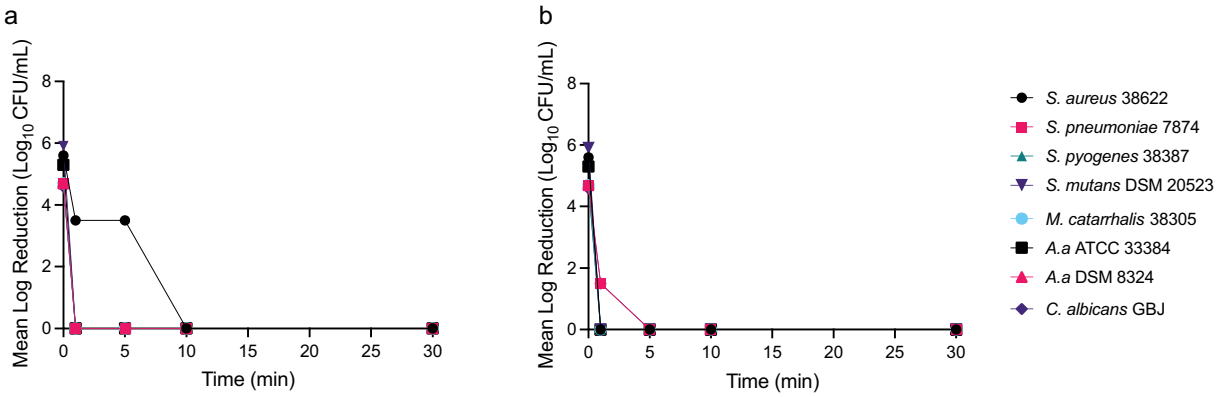

Figure 1. Microbicidal activity (mean Log_{10} reduction) of HR lozenges (a) BC and (b) HL in artificial saliva against a range of oropharyngeal bacterial and fungal isolates using a 30 minute Log_{10} reduction assay (n = 3).

Antimicrobial activity was defined as: < 1 log reduction (low; <90%), 1 to 3 log reduction (moderate; 90–99.9%); > 3 log reduction (high; >99.9%).

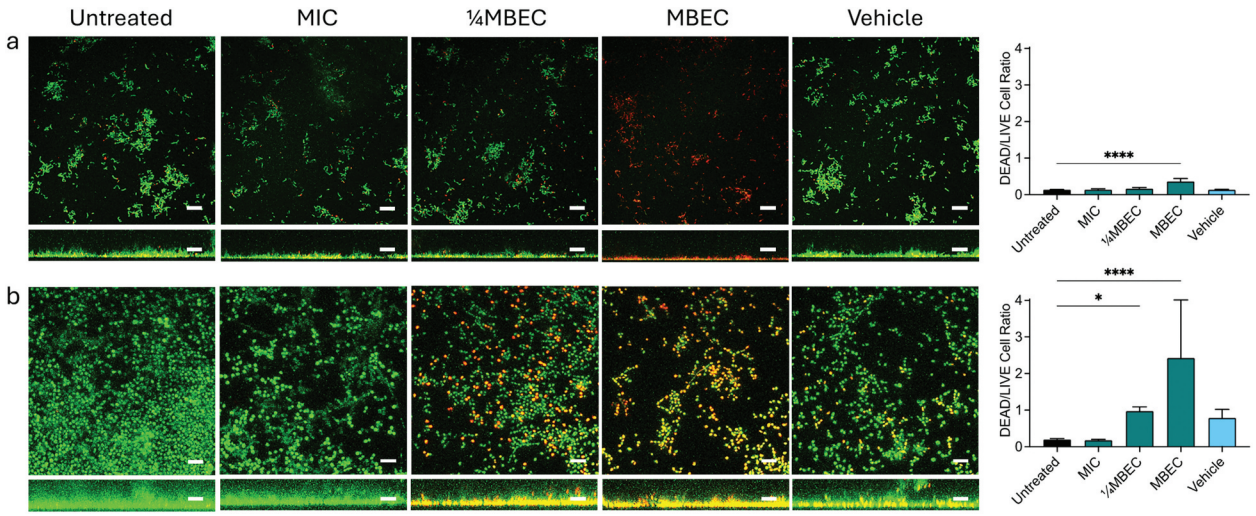

Figure 2. CLSM showing the antibiofilm effects of hexylresorcinol (HR) on (a) *S. pyogenes* 38387 biofilm disruption tested at MIC (8 µg/mL), ¼MBEC (16 µg/mL) and MBEC (64 µg/mL), (b) *C. albicans* ATCC 90028 biofilm formation tested at MIC (16 µg/mL), ¼MBEC (128 µg/mL) and MBEC (512 µg/mL) with associated DEAD/LIVE cell ratios from COMSTAT image analysis.

Statistical significance defined as ****, $p < 0.0001$; *, $p < 0.05$. Scale bars; 20 µm.

viable virus for either SARS-CoV-2, PIV-5 or CMV, with log_{10} reductions of > 5, > 4, and > 5, respectively, as shown in Figure 3b (Table S3). In contrast, against IAV we observed a 2.7 log_{10} reduction in viable virus after 1 minute, with virus undetectable (log_{10} reduction of > 4) after 5 minutes. Against the non-enveloped EV-A71, we observed no reduction in virus infectivity even after extending the incubation time to 20 minutes.

Against enveloped viruses, HR in dissolved lozenges resulted in time-dependent decreases in viable virus but, was overall less effective than purified HR against all viruses (Figure 3c). Against SARS-COV-2 the BC lozenge outperformed the HL lozenge with log_{10} reductions of 3.5 and 2 respectively after 20 minutes. Similar differences in activity were also noted for the two lozenges against PIV-5 at early time points. However, by 20 minutes log_{10} reductions had equalised to 2.8 and 2.9 for BC and HL lozenges respectively. Lozenge HR activity was weakest against IAV, mirroring the observations with purified HR, with log_{10} reductions of 1.7 and 1.4 after 20 minutes with BC and HL respectively. Surprisingly, against the non-enveloped EV-A71 we observed higher activity of the lozenges compared to purified HR. However, the overall activity was far lower than when compared to enveloped viruses, with log_{10} reductions of ≤ 1, and these did not increase with time.

As HR released from lozenges demonstrated lower virucidal activity than purified HR we undertook a component analysis of the lozenge formulations to determine if any components were responsible for inhibiting HR activity. Components were tested at lozenge concentrations (confidential information) against SARS-CoV-2 in artificial saliva for 10 minutes individually, or in combination, ± HR (Table S4). No components, neither individually nor in combination, showed virucidal activity in the absence of HR. In the

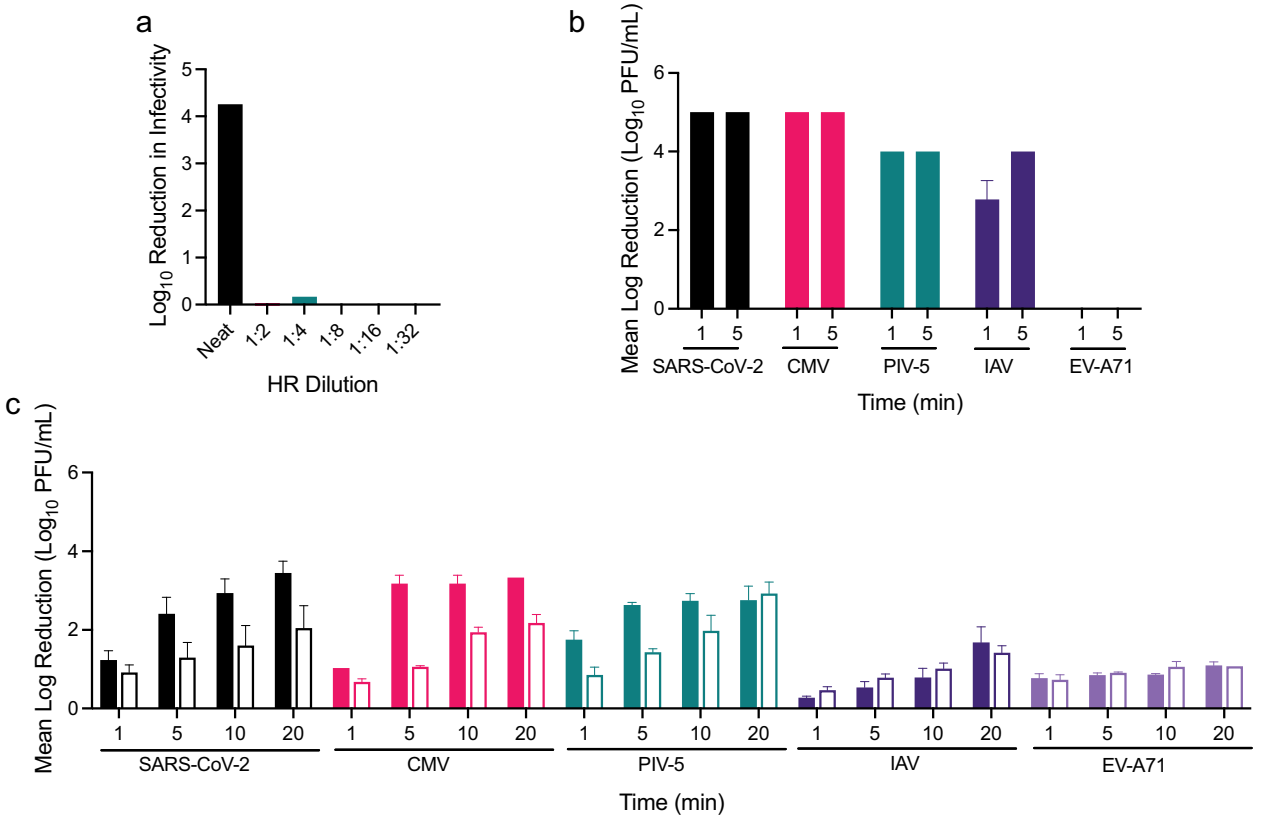

Figure 3. Virucidal activity of purified HR, or HR in dissolved lozenges, assessed against enveloped (SARS-CoV-2, PIV-5, IAV and CMV) and non-enveloped (EV-A71) viruses. (a) The activity of purified HR (undiluted and at doubling dilutions) was determined against SARS-CoV-2 and (b) undiluted at 1 and 5 minutes. (c) The activity of HR in lozenges was determined at 1, 5, 10, and 20 minutes (solid bars, BC lozenge; open bars, HL lozenge). Virucidal activity was determined as the $\log_{10}$ reduction in virus titre compared to an artificial saliva control. Error bars represent the standard error of the mean (SEM) from ($n \geq 2$ replicates).

presence of HR, all component combinations resulted in a $\log_{10}$ reduction in viable virus of > 5, similar to that of purified HR alone. With no inhibition of HR activity visible by any component, it remained unclear why the virucidal activity of purified HR was greater than that of HR in the dissolved lozenges.

In vitro HR release from lozenges in artificial saliva

HR release from the two lozenge formulations was similar in both PBS and AS. *In vitro* drug release mostly occurred during the first 10 minutes of dissolution (85.3–141.4%); the release being slower from 10 to 30 minutes (21.5–51.0%) (Figure 4, Table 3).

In vivo HR release from lozenges

Salivary concentrations of HR were estimated by HPLC, showing considerable inter-subject variability in all pharmacokinetic parameters measured. The time taken to reach C_{max} (82.5 ± 24.0 and 60.3 ± 13.0 µg/mL for BC and HL lozenges, respectively) varied from 1 to 5 minutes (Figure 5, Table 4, Figure S1, Table S5). Mean AUCs for BC and HL lozenges were not significantly different

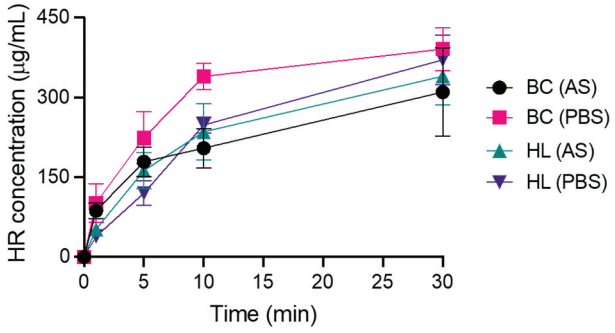

Figure 4. *In vitro* quantification of HR release from BC and HL lozenges using HPLC-fluorescence detection following dissolution in artificial saliva or PBS for up to 30 minutes (n = 3, mean ± SD).

AS, artificial saliva; PBS, phosphate-buffered saline.

Table 3. *In vitro* release of HR from BC and HL lozenges as a percentage of total HR available (2.4 mg per lozenge) in artificial saliva or PBS.

Time (minutes)	% Theoretical Drug Release			
	BC (AS)	BC (PBS)	HL (AS)	HL (PBS)
1	36.1 ± 6.2	42.3 ± 15.2	21.7 ± 2.5	16.0 ± 1.2
5	74.7 ± 11.4	93.4 ± 20.3	67.6 ± 14.1	50.0 ± 9.7
10	85.3 ± 15.4	141.4 ± 10.3	98.1 ± 22.1	103.4 ± 6.5
30	129.1 ± 34.6	162.9 ± 16.8	141.7 ± 22.7	154.4 ± 19.4

AS, artificial saliva; PBS, phosphate-buffered saline.

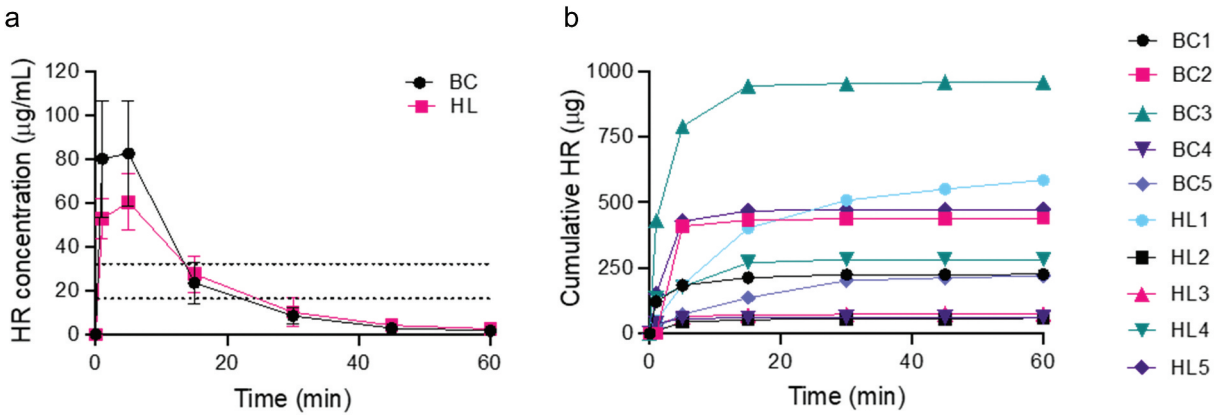

Figure 5.HPLC-FLD analysis of *in vivo* saliva sample HR content. (a) Mean saliva HR concentration–time curve during *in vivo* dissolution of BC (n = 5, mean ± SEM) and HL (n = 5, mean ± SEM) HR lozenges. Horizontal dotted lines show 16 and 32 µg/mL levels. (b) Cumulative HR release profiles of BC and HL HR lozenges as a function of time for each individual participant (n=2). Where error bars are not visible, they are within size of data points.

Table 4. *In vivo* mean pharmacokinetic parameters calculated from the release profiles of HR from human participant samples over time.

Lozenge	AUC ± SEM (µg/mL.min)	t > MIC (min)	C_{max} ± SEM (µg/mL)	t_{max} (min)
BC	1256 ± 374.8	22.5	82.5 ± 24.0	5
HL	1133 ± 286.4	25.0	60.3 ± 13.0	5

AUC, area under the curve calculated using GraphPad Prism v10.3.0; t > MIC (min), the time where HR levels exceed an MIC value of 16 µg/mL; C_{max}, the maximum salivary concentration of HR; t_{max}, the time taken to reach $C_{max.}$ SEM, standard error of the mean.

(1256 ± 374.8 and 1133 ± 286.4 µg/mL.min ± SEM, respectively). Mean salivary HR concentration was above the maximum recorded MIC value (64 µg/mL) for 8.1 minutes in the BC group. Although the mean C_{max} for HL lozenges was just below the maximum MIC value, 3 out of 5 participants in this group had a C_{max} > 64 µg/mL and at > MIC of 7–10.4 minutes. Cumulative salivary HR during sampling ranged from 58.6 to 958.7 µg (out of a total HR content of 2.4 mg per lozenge) (Figure 5b).

Discussion

HR has an excellent safety profile, having been employed in lozenge formulations in the treatment of pharyngitis for over 60 years, with the prolonged analgesic effects of HR being well-documented [8]. HR is widely employed, not only for its anaesthetic effects but also in the food industry to prevent spoilage in fruit and shellfish [32–34]. HR also occurs naturally at high concentrations in grains and cereals [7].

In nature, phytochemicals (e.g. terpenoids, alkaloids and phenolics) such as HR have important roles in preventing bacterial and viral predation and have been employed in a range of consumer healthcare products (e.g. toothpastes, mouthwashes and disinfectants) with a range of different applications [35]. These products (at least *in vitro*) demonstrate direct and indirect antimicrobial effects including: reducing bacterial biofilm and dental plaque production and, inhibiting bacterial adhesion to the oral mucosa and hard tissues [36,37]. In practice, whilst topical mouthwash formulations have been reported to be effective in the literature *in vitro* [38], very few topical antimicrobial agents have proved to be effective in the oral environment. Problems which have limited their use include toxicity and, effective dose restriction/inactivation within the oral environment, resulting in decreased efficacy and poor substantivity. Data on the bioavailability of essential oils is limited and is based almost solely on animal studies. However, most essential oil components (e.g. phenols such as thymol, carvacrol, and eugenol) are rapidly metabolised and excreted by the kidneys as polar, water-soluble compounds or expelled as CO_2 from the lungs [39].

Whilst HR has been proposed as an effective antimicrobial agent based on *in vitro* studies, the pharmacokinetics and C_{max} of HR are critically important in determining likely *in vivo* effectiveness. Hence, both the pharmacokinetics of HR release in a static *in vitro* environment (of constant volume) and in a dynamic *in vivo* environment where the concentrations of the agent would be directly affected by saliva production and swallowing were determined. The

in vitro model predicted a rapid release of HR from the lozenge in the initial 10 minutes of dissolution in both PBS and AS; the latter being employed to more accurately mimic the *in vivo* dissolution of the lozenge and HR release [23]. This rapid release was predicted to continue (albeit more slowly) for the duration of the experiment. Interestingly, drug release measured by HPLC in the *in vitro* experiments exceeded the theoretical maximum in both PBS and AS. This finding may reflect the large intra-sample variability at longer incubation times (standard deviations ranging from 16.8% to 34.6% at 30 minutes). It could also have arisen due to the HR standards being diluted directly in the mobile phase, while the HR samples were solubilised in AS or PBS, which may have directly affected the calibration.

Here, we defined the antimicrobial activity of HR against a wide range of oropharyngeal pathogens ($n = 25$) including Gram-positives, Gram-negatives, anaerobes and fungi, using standardised MIC and MBEC assays in planktonic and biofilm culture, which revealed its broad antimicrobial activity as also reported by others [12,18,40]. All strains (save *F. nucleatum*) exhibited MICs ≤ 32 μg/mL (i.e. ≥4-fold below the observed C_{max} of HR in saliva). It was also evident that the time taken to reach C_{max} (i.e. t_{max}) was very rapid, being within 5 minutes for both lozenge formulations. The MIC results with *C. albicans* (8–16 μg/mL) were perhaps the most surprising as the chitin/polysaccharide-based cell-wall of fungi is thought to provide increased resistance to antimicrobial disruption [40].

Oral biofilms are a contributing feature in many oral and systemic diseases [41,42] and form rapidly intraorally on mineralised teeth and/or prosthodontic materials (e.g. acrylic and titanium) via adherence to a susceptible surface [43]. Most existing antibiotics are only effective against planktonic, actively dividing bacterial cells and hence fail to eradicate biofilm infections where cells often have low metabolic rates or dormancy and therefore, significantly reduced susceptibility [28,44,45]. The MBEC and biofilm formation models employed in this study have been extensively employed to screen antibiofilm activity [14] and as expected, the MBEC values exceeded those of the MICs; these decreases in effectiveness being attributable to the biophysical and biomechanical environment of the biofilm [21,46]. However, the initial MBEC results (with 13/16 strains having values ≤256 μg/mL) were encouraging as the cumulative HR release profiles demonstrated that these concentrations were attainable in the current oral formulations *in vivo*.

To better understand the potential effects of these factors, we modelled the *in vitro* microbicidal activity of HR released via dissolution of the lozenges in artificial saliva over 30 minutes in a Log_{10} reduction assay. In these experiments, marked microbicidal activity against all the test microorganisms (including both bacterial and fungal pathogens) was evident within 10 minutes, with high antimicrobial activity (>3 log reduction) observed for both lozenge formulations. The time where HR levels exceeded an MIC value of 16 μg/mL [i.e. $t >$ MIC (min)] was 22.5–25 minutes for the standard lozenges when dissolved intraorally, well over the 10 minutes required for high antimicrobial activity (>3 log reduction) to be achieved.

To our knowledge, *in vivo* real-time release of HR from lozenges has been measured here for the first time using this small-scale feasibility study. These studies employed HR concentrations which assumed a dilution factor of 1.5 to 2 mL of saliva/minute [47]. The finding that the *in vivo* dissolution profile of HR into saliva demonstrated marked inter-subject variability in the individual-release profiles and their corresponding calculated pharmacokinetic parameters had previously been reported for drug release from lozenges [48,49]. This variation is presumed to reflect individual variation in salivary-flow, swallowing rates and levels of hydration, which was clearly evident in our study, where the total saliva collected from participants over the 60-minute study period varied from 2 to 22.2 mL (Table S6). The *in vivo* studies demonstrated that salivary concentrations of HR exceeded 16 μg/mL for the first 15 minutes in the majority of subjects (26/30 test samples).

Previous studies have demonstrated how agents within a formulation may affect and indeed direct antiviral activity [2]. In the case of HR, it was evident that the antiviral activity was unrelated to the excipients in the lozenge formulation. Furthermore, antimicrobial activity was absent in the plant-derived menthol flavouring ($C_{10}H_{20}O$) and in the ethanol solvent controls included in these experiments (both with MICs ≥1024 μg/mL). Whilst menthol alone was not antibacterial, it did demonstrate an additive effect (for 8/11 of the strains tested) when administered with HR. Synergistic effects such as inhibition of multidrug efflux pump activity in *Staphylococcus aureus* have been described by phytochemicals such as terpenoids and phenolics [50]. In addition, potentiation of various classes of antibiotic by HR in pathogenic Gram-negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* has also recently been demonstrated [40]. HR is believed to have a multimodal mode of action exemplified by increased membrane viscosity/disruption (particularly in Gram-negative bacteria) and decreased enzymatic activity [51].

Employing the biofilm model with confocal imaging, we sought to demonstrate the potential antibiofilm activity of HR at concentrations achievable in the oral cavity. Here, we employed two pathogens (*S. pyogenes* and *C. albicans*) which are important in oropharyngeal disease and where topical agents may

be employed. In both assays, HR showed efficacy as witnessed by increased cell death and decreased biofilm thickness in the confocal imaging at MBEC concentrations against *S. pyogenes* (at 64 µg/mL) and *C. albicans* (at 512 µg/mL). Interestingly, these concentrations, at least in the case of *S. pyogenes*, were below those observed intraorally in the current HR lozenge formulations. The activity of HR against *C. albicans* (16 µg/mL) observed here was clearly unrelated to a direct antimicrobial activity as the MIC values were similar to those observed in *S. pyogenes* (8 µg/mL), but reflected the previously reported anti-fungal activity of 4-hexylresorcinol e.g. in the topical veterinary fungicide Acrisorcin. Whilst the mechanism of these antibiofilm effects is uncertain, it has been demonstrated that phenols such as HR, exhibit *in vitro* bactericidal effects by prevention of bacterial adherence and modification of the bacterial cell membrane with alteration of cell surface charge and permeability [12,18]. Moreover, alkylresorcinols have also been shown to function as bacterial signalling molecules (in place of acylated homoserine lactones) being recognised by PauR (a LuxR receptor homolog) in quorum-dependent regulatory systems [52]; demonstrating significant dose-dependent inhibition of both bacterial growth [53] and spore formation [54].

Although topical antimicrobials against SARS CoV-2 have been the subject of recent intense research, this has principally involved mouthwash preparations [1]. Paradoxically, little research has been undertaken on the antiviral activity of lozenges, especially HR. As previously shown, increased antiviral activity against enveloped viruses, when compared to non-enveloped viruses was evident in our studies [55], reflecting the importance of lipid membrane disruption in mediating the efficacy of topical antiviral agents [2]. In a clinical setting, patients with PCR-confirmed COVID-19 infections were found to have SARS-CoV-2 base levels ranging from approximately $1x10^2$ to $1x10^7$ pfu/mL in saliva [2]. Therefore, the level of antiviral activity observed against SARS-CoV-2 with the BC lozenges (if extrapolated to these clinical saliva samples) would represent a > 99% reduction in virus levels, equating to potentially undetectable virus levels after 20 minutes in patients presenting with low base-level virus loads. In keeping with their higher antimicrobial activity, the virucidal activity of BC lozenges against SARS-CoV-2 and PIV-5 was more rapid, although equal efficacy was achieved after 20 minutes against PIV-5 for both lozenge formulations. These results were encouraging as, we have previously demonstrated how a single mouthwash application can effectively eliminate carriage of live SARS CoV-2 in saliva for up to 1 hour [2].

The finding that the *in vitro* antimicrobial activity of pure HR compared to the dissolved lozenges was markedly different was unsurprising as the contrasting pharmacokinetics of solid lozenges and mouthwashes has been previously described [56]. Interestingly, the effect of the formulations on virucidal activity was clear, with the antiviral activity against enveloped viruses of HR alone being significantly greater than that observed for HR in lozenge form. Whilst component analysis of the lozenges failed to identify any inhibitory effects of the excipients, the effects of the flavourings/dyes were not tested, due to their being classed as 'commercial proprietary information'. These excipients may alter pH levels, and therefore lipid membrane integrity [57], although it was not possible to test this during the course of this study. Previous studies have shown that pH is highly influential in modulating antimicrobial activity in lozenge formulations [58] and therefore even small changes in pH afforded by different flavourings/dyes, may explain the virucidal differences observed between the different lozenge types containing the same concentration of HR. The antiviral mechanism of action of HR is unknown but, likely involves disruption of the lipid membranes of the viral envelope as described in oral rinses [2,59]. It has also been hypothesized to involve alterations in viral protein–lipid interactions [55], impediment of DNA or RNA binding leading to viral resistance to replication [60], and impairment of viral entry [61].

Whilst the *in vitro* and *in vivo* studies demonstrated that HR could attain antimicrobial levels in both artificial and human saliva following lozenge administration, they do not prove clinical efficacy. Possible future directions for *in vivo* work would be to first study the partitioning of HR within the oral biofilm and saliva, and secondly to study the effectiveness of HR on bacterial carriage within the mouth. With this data in place, the effectiveness of the lozenges could be compared in a suitably powered, randomised, controlled trial (employing placebo lozenges) in human subjects to demonstrate clinical efficacy.

This study demonstrates that HR, particularly in lozenge form which affords the opportunity for high dosing and rapid bioavailability, may be of unrecognised use in the management of oropharyngeal biofilm-related infections and viral transmissible diseases. The results here demonstrate that these antimicrobial effects were significant against a range of oropharyngeal bacteria (in doses readily achievable in saliva). HR also demonstrated prolonged activity against both bacterial and fungal biofilms. The application of HR in lozenge formulation as an alternative to the traditional topical polyene and azole-derived anti-fungal medications in the treatment and prevention oral candidal mucosal disease [62], is particularly interesting and the subject of ongoing investigation.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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