*Article*

# A Throat Lozenge with Fixed Combination of Cetylpyridinium Chloride and Benzydamine Hydrochloride Has Direct Virucidal Effect on SARS-CoV-2

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**Abstract:** Viruses are the most common causative agents of inflammation in the oral cavity and throat region. Most respiratory tract infections are self-limiting and require no specific treatment. However, patients often use different self-medication therapies that can treat both the symptoms and the cause. Throat lozenges with a fixed combination of benzydamine hydrochloride and cetypiridinium chloride (BH/CPC) have been shown to provide effective symptomatic relief for sore throat, but their effect on viruses has not been investigated to date. The antiseptic, cetylpyridinium chloride (CPC), has already been described as a successful bactericide. In addition, there are some studies suggesting its efficacy against certain enveloped viruses. Thus, the aim of our study was to examine the virucidal activity of CPC and a combination of BH/CPC as a free active substance or as lozenge on SARS-CoV-2 in vitro. Under in-laboratory simulated conditions of lozenge administration, we incubated SARS-CoV-2 with three different concentrations of each of the active substances, CPC, free BH/CPC or BH/CPC, as a lozenge suspension for 1 min, 5 min and 15 min of contact time. Infective viral particles were detected in cell cultures and the viral titre was calculated accordingly. Our results show that all active substances in high-concentration suspensions, as well as a medium concentration of the BH/CPC combination, exhibited a 4-log reduction in viral titre. Additionally, the highest concentration of BH/CPC as a lozenge had a faster virucidal effect compared to CPC as a free active substance alone, since a contact time as short as 1 min reduced the initial virus concentration by more than 4-log. This study demonstrates the effective strong virucidal effect of the lozenge, with the possibility of viral load reduction in the oral cavity and, consequently, reduced risk of viral transmission.

**Keywords:** cetylpyridinium chloride; benzydamine hydrochloride; SARS-CoV-2; respiratory tract infections

## 1. Introduction

Respiratory tract infections (RTIs) are among the most common diseases that affect humans, with typical symptoms, such as rhinitis, cough, fever, and sore throat.

Among the microbes, viruses are the most common causative agents of inflammation in the oral cavity and throat area. Bacterial respiratory tract infections (RTIs) are less common, and often develop after a viral infection. The most common viruses associated with RTIs are picornaviruses (human rhinoviruses (HRV)), coronaviruses (common human coronavirus (hCoV) OC43, 229E, NL63, HKU1), ortho- and paramyxoviruses (influenza viruses, parainfluenza viruses (PIV1-4)), and respiratory syncytial virus), respiratory adenoviruses and others [1].

Many of the circulating human pathogenic viruses are enveloped with a lipid bilayer and infect their target cells by inducing fusion of the viral envelope with the cell membrane. Many classes of enveloped viruses are human pathogens, including respiratory viruses such as influenza viruses (four genera in the *Orthomyxoviridae* family: influenza A, B, C and D), the respiratory syncytial virus (RSV, *Pneumoviridae* family), coronaviruses (*Coronaviridae* family), and others [2].

Human coronaviruses are commonly circulating viruses, and they were considered the second most common viral agent responsible for 10–15% of all upper respiratory tract infections (URTIs) in humans [3]. They replicate in the nasopharynx and generally cause mild, self-limited URTIs with short incubation periods, although lower tract respiratory infections and pneumonia have occasionally been described [4].

After the emergence of more virulent coronaviruses, such as severe acute respiratory syndrome (SARS)-CoV-1 in 2002, and Middle East respiratory syndrome (MERS)-CoV in 2012, it was recognised that coronaviruses can also cause severe pneumonias with longer incubation periods and often fatal outcome [5]. As of late 2019, Coronavirus Disease 2019 (COVID-19), caused by a novel corona virus, SARS-CoV-2, has resulted in a global pandemic with more than 144 million people infected and over three million deaths globally [6]. Symptoms of COVID-19 may vary from no symptoms being present to fever, cough, and severe illness, with difficulty breathing. Furthermore, the disease can cause serious medical conditions and can lead to death [7,8].

Respiratory viruses are mainly transmitted via respiratory secretions during exhalation in the form of droplets, but indirect contact with contaminated fomites has also been shown to spread infection. A reduction in the viral load on the site of infection reduces the risk of transmission via both routes, while simultaneously reducing the symptoms of the patients and the potential spread of infection to the lower respiratory tract [1].

Cetylpyridinium chloride (CPC), a quaternary ammonium compound (QAC), is an antiseptic with the actions and uses typical of cationic surface-active agents (surfactants). In addition to its emulsifying and detergent properties, quaternary ammonium compounds show bactericidal activity against Gram-positive and, in higher concentrations, some Gram-negative bacteria. They also have variable antifungal activity and are effective against some viruses [9,10]. Their virucidal activities are not widely reported, although some reports against enveloped viruses were made in the literature relating to surface disinfection [11]. Among this group of compounds, CPC has recently been shown to be active against influenza, both in vitro and in vivo, through direct attack on the viral envelope, with an in vitro EC50 of 5–20 μg/mL [12]. CPC is usually used in medicated oral rinses, throat lozenges and sprays.

This study investigated the in vitro virucidal activity of CPC as a free active substance, the combination of benzydamine hydrochloride (BH) and CPC as a free active substance, and the combination of BH and CPC as a throat lozenge.

To our knowledge, this is the first study to examine the virucidal effect of a throat lozenge containing a fixed combination of BH and CPC on SARS-CoV-2.

## 2. Materials and Methods

### 2.1. Cell Preparation

Seven-day-old Vero E6 cells (passage 13–16) were trypsinized and resuspended in the Dulbecco's Minimal Essential Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), to obtain a final concentration of $1 \times 10^5$ cells/mL. A total of 100 μL of prepared cell suspension was then transferred in each well of the 96-well cell culture plate and incubated overnight at 37 °C and 5% $CO_2$ to reach the 80% cell confluence required for quantitative suspension tests.

### 2.2. Virus Isolation and Quantification

SARS-CoV-2 was isolated from a throat swab of a patient diagnosed with COVID-19 in Vero E6 cells cultivated with DMEM, supplemented with 10% FBS. The virus was passaged 5 times on Vero E6, aliquoted and stored at −80 °C until further use.

The virus concentration was determined with the endpoint dilution assay and expressed as fifty-percent tissue culture infectious dose/mL ($TCID_{50}$/mL), calculated with the Spearman and Kärber method. The stock concentration used in all experiments was $1.17 \times 10^9$ $TCID_{50}$/mL.

## 2.3. Preparation of Reagents

All the test products used in our experiments were provided by Krka, d. d., Novo mesto, Slovenia, whereas the other reagents used were prepared at the Institute of Microbiology and Immunology, Ljubljana, Slovenia. The interfering substance was prepared by first dissolving 3 g of bovine albumin in 97 mL of water. Next, 97 mL of the prepared and filtrated bovine albumin solution was mixed with 3 mL of sheep erythrocytes (BioSap SEA, BioGnost, Zagreb, Croatia) to achieve the final composition of interfering substance used in our experiments. Hard water was freshly prepared under aseptic conditions on the day of the experiment according to SIST EN 14476:2013 + A2:2019 "Chemical Disinfectants and Antiseptics—Quantitative Suspension Test for the Evaluation of Virucidal Activity in the Medical Area" (standard) and was used within 12 h [13].

For each active substance, three different concentrations were prepared by dissolving the tested substance in either 4 mL (high concentration), 20 mL (medium concentration) or 30 mL (low concentration) of hard water. All active substance compositions and experimental conditions are presented in Table 1.

**Table 1.** Test product and test conditions considered.

| Test Product | Test Temperature (°C) | Interfering Substance 2 | Contact Time |
|---|---|---|---|
| BH [1]/CPC [2] lozenge Septolete total lozenge, containing 3 mg BH and 1 mg CPC dissolved in 4 mL, 20 mL and 30 mL of hard water Lozenge ingredients: BH, CPC, eucalyptus oil, levomenthol, anhydrous citric acid (E330), sucralose (E955), isomalt (E953), brilliant blue FCF (E133) | 37 | 3.0 g/L bovine albumin solution and 3.0 mL/L erythrocytes | 1, 5 and 15 min |
| Placebo lozenge for BH/CPC experiments Placebo lozenge dissolved in 4 mL, 20 mL and 30 mL of hard water lozenge ingredients: eucalyptus oil, levomenthol, anhydrous citric acid (E330), sucralose (E955), isomalt (E953), brilliant blue FCF (E133) | 37 | 3.0 g/L bovine albumin solution and 3.0 mL/L erythrocytes | 1, 5 and 15 min |
| CPC as free active substance 1 mg of CPC dissolved in 4 mL, 20 mL and 30 mL of hard water | 37 | 3.0 g/L bovine albumin solution and 3.0 mL/L erythrocytes | 1, 5 and 15 min |
| BH/CPC as active free substances 3 mg of BH and 1 mg of CPC dissolved in 4 mL, 20 mL and 30 mL of hard-water | 37 | 3.0 g/L bovine albumin solution and 3.0 mL/L erythrocytes | 1, 5 and 15 min |

[1] Benzydamine hydrochloride; [2] cetylpyridinium chloride.

## 2.4. Quantitative Suspension Test for the Evaluation of Virucidal Activity

To mimic the physico-chemical properties of the human oral cavity, several factors, such as temperature, saliva composition (interfering substance) and the amount of saliva produced during lozenges dissolution the dynamics of lozenges dissolution (different virus–substance contact time), were taken into consideration. Experimental procedures were carried out, in accordance with the standard requirements. In brief, an active substance was first dissolved in hard water and then added to a suspension of 1:10 ratio of SARS-CoV-2 and an interfering substance. The mixture was maintained at 37 °C for different incubation periods (1 min, 5 min and 15 min). At the end of each contact time, an aliquot of incubated suspension was diluted in an ice-cold cell maintenance medium DMEM with 2% FBS to immediately suppress the virucidal action of the active substance used. The

prepared dilutions, from 1:10 to $1:10^8$ of the initial virus-active substance suspension, were transferred onto Vero E6 cell monolayer in 96-well microtitre plates and incubated for 7 days at 37 °C and 5% $CO_2$. After incubation, the plates were screened for CPE and the titres of infectivity were calculated according to Spearman and Kärber [14].

For every SARS-CoV-2 aliquot used, a virus titration test was performed immediately after thawing, and 15 min later to evaluate the effect of thawing on virus concentration and to determine the exact working virus concentrations for each experiment.

### 2.5. Control Experiments

In addition to experiments evaluating the virucidal effect of active substances on SARS-CoV-2, control experiments were also carried out in accordance with the standard.

Each experiment included untreated cells that served as a negative control, providing information on cell viability throughout whole incubation period.

Virus viability test was performed for each quantitative suspension test to examine the stability of the virus in the maintenance medium and to evaluate the virus infectivity under experimental conditions during all contact times. A mixture of 0.1 mL of viral suspension, 0.1 mL of interfering substance and 0.8 mL of hard water was incubated at 37 °C for 15 min. A volume of 0.1 mL was sampled at the beginning and after 15 min of incubation. Ten-fold serial dilutions were prepared in DMEM supplemented with 1% FBS, and 100 µL of prepared serial dilutions were added per well in eight replicas.

To avoid false positive results due to the morphological changes caused by the cytotoxic effect of the active substance on Vero E6 cells under experimental conditions, we determined the highest concentration of the tested substance that causes no visible morphological changes in the cells. First, 10-fold dilutions of the tested substance were prepared in the maintenance medium and 100 µL of each dilution was transferred to the cell monolayer in eight replicates. Cells were incubated for 48 h and screened for any morphological changes.

The inhibiting effect of ice-cold media on the virucidal activity of the tested product was also determined. A suspension of 0.1 mL of interfering substance, 0.1 mL of maintenance medium and 0.8 mL of test product was prepared. Then, 0.1 mL of prepared mixture was transferred to 0.8 mL of ice-cold maintenance medium and placed at 4 °C. Next, 0.1 mL of viral suspension was added to the ice-cold suspension and incubated on ice for 15 min. After incubation, serial dilutions were prepared and transferred onto cell culture in eight replicas.

To determine the impact of the highest non-cytotoxic concentration of the active substance on the cell susceptibility to the virus, we prepared 10-fold dilutions of the tested substances in maintenance medium, removed the culture medium and added 100 µL of the prepared active substance dilution onto cell monolayers in eight replicates. After 1 h of incubation at 37 °C and 5% $CO_2$, supernatants were removed, and cells were washed with maintenance medium and inoculated with prepared 10-fold serial dilutions of SARS-CoV-2.

All the control experiments were carried out at the same time and under the same conditions as the quantitative suspension tests.

### 2.6. Electron Microscopy

The effect of CPC as a free active substance, and of BH/CPC as a lozenge, on virus morphology was additionally evaluated with electron microscopy. Virus particles were visualized under the transmission electron microscope after exposure to CPC and a BH/CPC lozenge in high-concentration suspensions. An 800-µL test-product suspension (or maintenance medium for negative control) was prepared, and 100 µL of the interfering substance and 100 µL of the virus suspension were added. After a contact time of 15 min at 37 °C, samples were prepared for electron microscopy. Electron-microscopy grids (400-mesh copper grids, coated with Formvar and carbon-supported) were prepared with ultracentrifugation in the Airfuge system. The prepared grids with concentrated viral particles were negatively

contrasted with 2% phosphotungstic acid and examined under the transmission electron microscope at from 30,000- to 100,000-fold magnification.

### 2.7. Statistical Analysis

To evaluate the effect of active substances on SARS-CoV-2 concentration and infectivity, we compared the calculated virus concentrations after all three incubation periods with different active substances using an independent *t*-test in the SPSS Statistics V26 software tool.

## 3. Results

### 3.1. Results of Control Procedures

#### 3.1.1. Cells and Virus Control

Cell and virus control were performed continuously for each test. All controls passed the criteria.

#### 3.1.2. Virus Control for the Total Contact Time of the Test

Virus test suspension was incubated for 15 min, which is the total incubation time in the test procedure. It was performed in parallel with each virucidal test. In the observed time period, there was no significant reduction in the infective virus concentration. When testing the low concentration for a combination of BH and CPC as a free active substance and placebo lozenge, a critical decrease in the concentration of infectious viruses (value 1 of the logarithmic level) was achieved. Results are shown in Table 2.

**Table 2.** Stability test of infective viruses during the incubation period in the test procedure.

| Test Virus | Test Product and Concentration | Titre at the Test Start (T = 0) $\log_{10}(c)$ (TCID$_{50}$/mL) $\pm$ 95% CI | Titre at the Test End (T = 15) $\log_{10}(c)$ (TCID$_{50}$/mL) $\pm$ 95% CI | Titre Difference |
|---|---|---|---|---|
| SARS-CoV-2 strain 751/20 | Virucidal activity 0.25 mg/mL CPC and one BH/CPC lozenge/4 mL | 8.94 ± 0.89 | 8.57 ± 0.62 | <1 |
| | Virucidal activity 0.05 mg/mL CPC and one BH/CPC lozenge /20 mL | 8.50 ± 0.68 | 8.31 ± 0.69 | <1 |
| | Virucidal activity 0.033 mg/mL CPC and one BH/CPC lozenge/30 mL | 9.76 ± 0.79 | 9.00 ± 0.80 | <1 |
| | Virucidal activity 0.75 mg/mL + 0.25 mg/mL BH/CPC and one placebo lozenge/4 mL | 8.99 ± 0.67 | 9.12 ± 0.71 | <1 |
| | Virucidal activity 0.15 mg/mL + 0.05 mg/mL BH/CPC and one placebo lozenge/20 mL | 9.68 ± 0.67 | 9.78 ± 0.77 | <1 |
| | Virucidal activity 0.099 mg/mL + 0.033 mg/mL BH/CPC and one placebo lozenge/30 mL | 9.95 ± 0.67 | 8.93 ± 0.65 | 1.02 |

CPC—cetylpyridinium chloride; BH—benzydamine hydrochloride; CI—confidence interval; TCID$_{50}$—50% tissue culture infectious dose.

#### 3.1.3. Test Product Activity Suppression in Ice-Cold Media

The test was performed for a high concentration of the test products. Results are shown in Table 3. The inhibitory effect of the ice-cold media on the action of the test product, used in a high concentration against viral infectivity, was confirmed. The virus

titre before and after exposure did not differ significantly, nor did it approach the difference of 1-log concentration value given as the cut-off value by the standard guidelines.

**Table 3.** Suppression efficiency of test product's activity.

| Test Virus | Test Product Concentration | Titre at the Test Start (T = 0) $\log_{10}$(c) (TCID$_{50}$/mL) $\pm$ 95% CI | Titre at the Test End (T = 15) $\log_{10}$(c) (TCID$_{50}$/mL) $\pm$ 95% CI | Titre Difference |
|---|---|---|---|---|
| SARS-CoV-2 strain 751/20 | 0.25 mg/mL CPC as free active substance | 9.76 $\pm$ 0.79 | 9.75 $\pm$ 0.63 | <1 |
| | 1 BH/CPC lozenge/4 mL | 9.76 $\pm$ 0.79 | 9.38 $\pm$ 0.76 | <1 |
| | 0.75 mg/mL BH + 0.25 mg/mL CPC as free active substance | 9.95 $\pm$ 0.67 | 10.25 $\pm$ 0.74 | <1 |
| | 1 placebo lozenge/4 mL | 10.46 $\pm$ 0.75 | 10.13 $\pm$ 0.85 | <1 |

CPC—cetylpyridinium chloride; BH—benzydamine hydrochloride; CI—confidence interval; TCID$_{50}$—50% tissue culture infectious dose.

### 3.1.4. Interference Control—Control of Cell Susceptibility

Interference control was performed in two separate procedures: first for CPC as a free active substance and a BH/CPC lozenge, and then for BH/CPC as a free active substance and a placebo lozenge. Virus concentration in the exposed cells was slightly lower compared to virus concentration in non-exposed cells. However, the difference was still acceptable and within the 1-log difference. The results indicated that the test products had no major impact on cell susceptibility to virus infection.

### 3.1.5. Cytotoxic Effect

Dilutions 10-1 to 10-11 of the initial concentration were tested, and the cytotoxic effect (deformation of cells and destruction of cell monolayer) was observed in dilutions 10-1 and 10-2. The virus propagation effect was checked only in the cells inoculated with test product dilutions ranging from 10-3 to 10-11. This observation was considered in the interpretation of results (cytotoxic level). The level of cytotoxicity with respect to virus dilution was at 4.5 $\log_{10}$(c) (TCID50/mL) for undiluted BH/CPC as a free active substance, the BH/CPC lozenge, and the placebo lozenge. For all other test products in medium and at a low concentration, and CPC as a free active substance in a high concentration, the level of cytotoxicity was 3.5 $\log_{10}$(c) (TCID50/mL).

### 3.2. Results of Test Product Effectiveness on Virus Inactivation

The virus was exposed to each of the three tested substance concentrations for contact times of 1, 5 and 15 min. Each test was performed in duplicate on different days. The graphical results of SARS-CoV-2 exposure to the test product suspension at a high concentration and for long contact times are presented in Figure 1, showing the virucidal effects of a specific test product.

Numerical results and reductions are presented as the average of both tests in Tables 4 and S1.
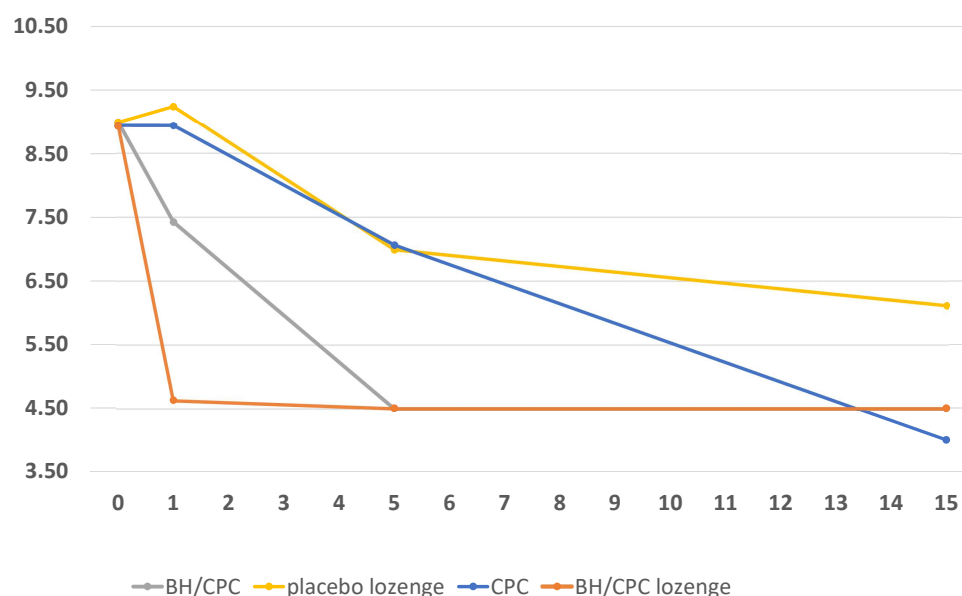
**Figure 1.** SARS-CoV-2 exposure to the test product suspension in high concentration and contact times; CPC—cetylpyridinium chloride; BH—benzydamine hydrochloride; $TCID_{50}$—50% tissue culture infectious dose.

**Table 4.** Test product concentrations with pH values and log reduction of infective virus concentration for each of the contact times.

| | CPC as Free Active Substance | CPC/BH as Free Active Substance | BH/CPC Lozenge | Placebo Lozenge |
|---|---|---|---|---|
| High concentration (disolved in 4 mL) | | | | |
| pH value | 7.61 | 7.80 | 2.85 | 2.99 |
| Time (min) | $\Delta\log_{10}(c)$ ($TCID_{50}$/mL) $\pm$ 95% CI | | | |
| 1 | $-0.00 \pm 1.06$ | $-1.57 \pm 0.86$ * | $-4.32 \pm 0.82$ * | $+0.25 \pm 1.01$ |
| 5 | $-1.88 \pm 0.96$ * | $-4.49 \pm 0.67$ * | $-4.44 \pm 0.75$ * | $-2.00 \pm 0.92$ * |
| 15 | $-4.94 \pm 0.75$ * | $-4.49 \pm 0.67$ * | $-4.44 \pm 0.75$ * | $-2.88 \pm 0.83$ * |
| Medium concentration (disolved in 20 mL) | | | | |
| pH value | 7.66 | 7.73 | 4.2 | 4.45 |
| Time (min) | $\Delta\log_{10}(c)$ ($TCID_{50}$/mL) $\pm$ 95% CI | | | |
| 1 | $-0.06 \pm 0.99$ | $-1.96 \pm 0.95$ * | $-1.69 \pm 0.96$ * | $-0.81 \pm 0.92$ |
| 5 | $-0.75 \pm 0.98$ | $-3.30 \pm 0.96$ * | $-3.56 \pm 0.86$ * | $-1.06 \pm 0.98$ |
| 15 | $-2.44 \pm 0.79$ * | $-5.36 \pm 0.77$ * | $-5.00 \pm 0.68$ * | $-0.65 \pm 1.05$ |
| Low concentration (disolved in 30 mL) | | | | |
| pH value | 7.67 | 7.80 | 4.64 | 5.25 |
| Time (min) | $\Delta\log_{10}(c)$ ($TCID_{50}$/mL) $\pm$ 95% CI | | | |
| 1 | $-0.32 \pm 1.08$ | $-0.89 \pm 1.03$ | $-0.88 \pm 1.11$ | $+0.13 \pm 1.06$ |
| 5 | $-0.70 \pm 1.04$ | $-1.58 \pm 0.98$ * | $-1.19 \pm 1.03$ | $-0.46 \pm 0.98$ |
| 15 | $-1.69 \pm 1.03$ * | $-2.44 \pm 0.94$ * | $-3.01 \pm 1.05$ * | $-1.46 \pm 0.97$ |

CPC—cetylpyridinium chloride; BH—benzydamine hydrochloride; CI—confidence interval; * statistical significant reduction of virus titre ($p < 0.05$).

### 3.2.1. Effects of Test Products in High Concentration

Free active substance: CPC exhibited a mean $\log_{10}$ reduction in viral titre of $0.00 \pm 1.06$ at 1 min, $1.88 \pm 0.96$ at 5 min and $4.94 \pm 0.75$ log10 at 15 min of exposure. BH/CPC exhibited results with a mean log10 reduction of $1.57 \pm 0.86$, $\geq 4.49 \pm 0.67$ and $\geq 4.49 \pm 0.67$ at 1, 5 and 15 min of exposure, respectively.

Lozenge: BH/CPC exhibited a mean $\log_{10}$ reduction in viral titre of $4.32 \pm 0.82$ as early as at 1 min of exposure; at 5 and 15 min of exposure, the reductions were $\geq 4.44 \pm 0.75$.

As expected, the placebo lozenge exhibited considerably smaller reductions. At 1 min of exposure, an increase in viral titre was observed, with values $0.25 \pm 1.01$, followed by the reduction of $2.00 \pm 0.92$ and $2.88 \pm 0.83$ at 5 and 15 min, respectively.

The negative control did not exhibit any negative effect on virus titre.

### 3.2.2. Effects of Test Products in Medium Concentration

Free active substance: With CPC as a free active substance, a mean $\log_{10}$ reduction of $0.06 \pm 0.99$, $0.75 \pm 0.98$ and $2.44 \pm 0.79$ was observed at 1, 5 and 15 min of exposure, respectively. With the BH/CPC, values were $1.96 \pm 0.95$, $3.30 \pm 0.96$ and $5.63 \pm 0.77$ at 1, 5 and 15 min of exposure, respectively.

Lozenge: BH/CPC exhibited a mean $\log_{10}$ reduction of $1.69 \pm 0.96$, $3.56 \pm 0.86$ and $5.00 \pm 0.68$ at 1, 5 and 15 min of exposure, respectively. The placebo lozenge exhibited reductions of $0.81 \pm 0.92$, $1.06 \pm 0.98$ and $0.65 \pm 1.05$ at 1, 5 and 15 min of exposure, respectively. The negative control did not exhibit any negative effect on virus titre.

### 3.2.3. Effects of Test Products in Low Concentration

Free active substance: CPC exhibited $\log_{10}$ reductions of $0.32 \pm 1.08$, $0.70 \pm 1.04$ and $1.69 \pm 1.03$ at 1, 5 and 15 min of exposure, respectively. With the BH/CPC combination, reductions were $0.89 \pm 1.03$, $1.58 \pm 0.98$ and $2.44 \pm 0.94$.

Lozenge: With of the BH/CPC combination, a mean $\log_{10}$ reduction of $0.88 \pm 1.11$, $1.19 \pm 1.03$ and $3.01 \pm 1.05$ was observed at 1, 5 and 15 min of exposure, respectively. The placebo lozenge exhibited an increase in viral titre of $0.13 \pm 1.06$, at 1 min of exposure, and reduction of $0.46 \pm 0.98$ and $1.46 \pm 0.97$ at 5 and 15 min, respectively. The negative control did not exhibit any negative effect on the virus titre.

### 3.3. Electron Microscopy Analysis

The negative control samples observed under the electron microscope contained intact virus particles with no visible morphological changes or damage; clearly expressed peplomers of the S protein (corona) were present, and an intact viral envelope was recorded (Figure 2A,B). On the contrary, in samples exposed to high concentration of test products, CPC (as a free active substance) (Figure 2C,D) or a BH/CPC lozenge (Figure 2E,F) a destruction of the viral envelope is visible, with unclear peplomers formation or even without peplomers and envelope. Virus exposure to the test products severely damaged the outer layer of the viral envelope, which is the most likely the reason for loss of viral infectivity. In some damaged viruses, the internal nucleocapsid is exposed and visible. The viral envelope destruction is also noted, with a contrast accumulation in the virus internal site (black virus centre).
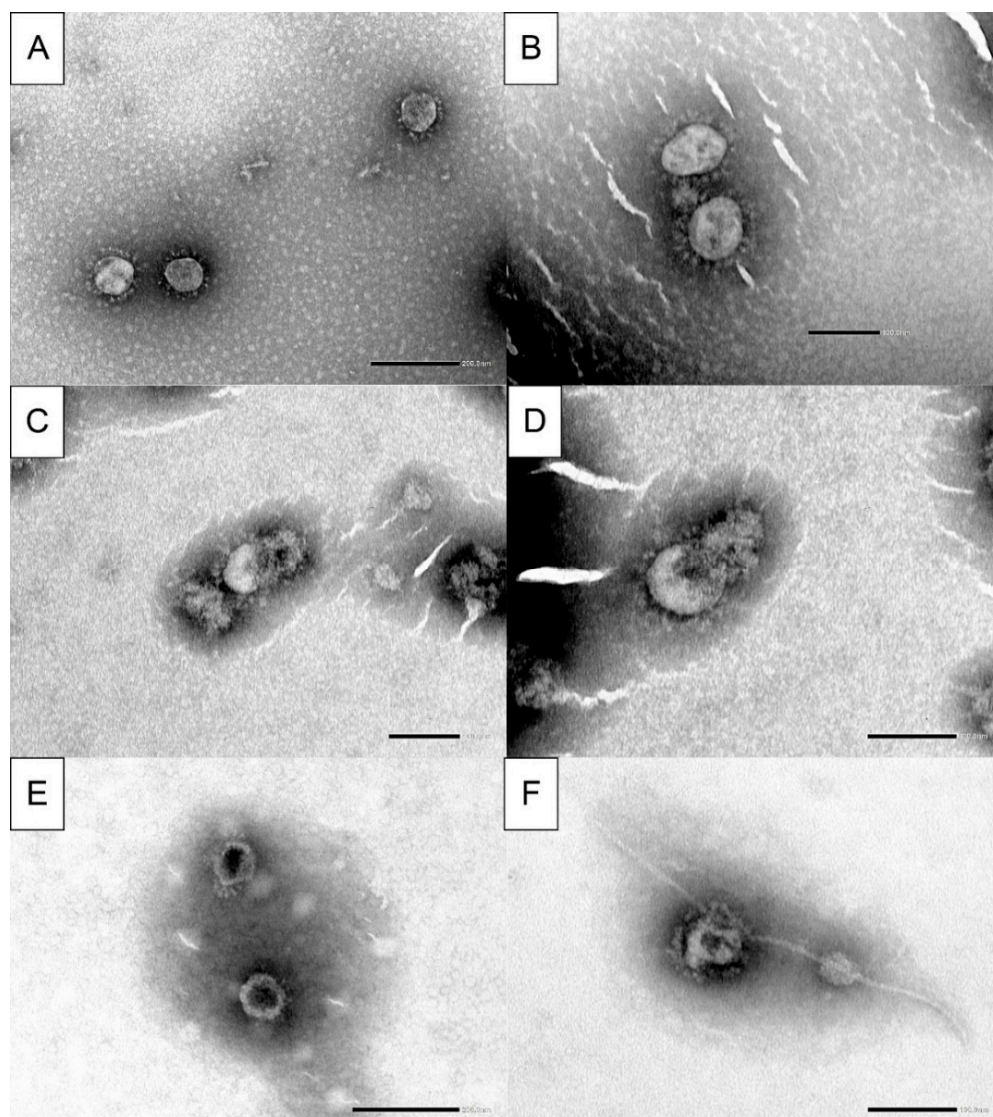
**Figure 2.** Electron micrographs of viral particles after ultracentrifugation and negative contrast. (**A**,**B**)—viral particles in maintenance media, not exposed to test product; 50,000-fold (**A**) and 80,000-fold (**B**) magnification; intact SARS-CoV-2 particles with peplomers (corona). (**C**,**D**)—15 min exposure to high CPC concentration (0.25 mg/mL); 80,000-fold (**C**) and 100,000-fold (**D**) magnification; damaged viral particles, rarely visible peplomers, disrupted envelope and exposed internal nucleocapsid. (**E**,**F**)—15 min exposure to high concentration of a BH/CPC lozenge (1 lozenge in 4 mL); 60,000-fold (**E**) and 100,000-fold (**F**) magnification; outer-layer damage and accumulation of negative contrast agent inside the nucleocapsid (black virus centre), rare peplomers, destruction of outer layer and nucleocapsid (**F**).

## 4. Discussion

The virucidal effect of CPC against some enveloped viruses has been investigated and confirmed [12]. This in vitro study confirmed a virucidal effect of CPC, and the combination of BH and CPC as a free active substance and as a lozenge on SARS-CoV-2.

The virucidal test, conducted in controlled laboratory conditions for suspensions of test products in a high concentration, showed a 10,000-fold reduction in infective virus concentration (99.99% reduction), i.e., a 4-log reduction, which is the reduction required by the standard for virucidal activity.

The speed of the reduction in infectious viruses decreases with a decreasing concentration of the test product. A significant difference between the virucidal activity of the

active BH/CPC lozenge and the placebo lozenge was also demonstrated, especially in high and medium concentrations of the test product suspensions.

The combination of BH and CPC as a lozenge showed a faster virucidal effect compared to CPC as a free active substance, as the sufficient, 4-log reduction in virus concentration was exhibited after one minute of contact in a high concentration. A similar reduction was noted only after 15 min of contact for CPC as a free active substance and between 5 and 15 min of contact with the BH/CPC lozenge in medium concentration (lozenge dissolved in 20 mL), which is a concentration that can theoretically be achieved in the actual use of a throat lozenge.

If taking the whole saliva flow rate of approximately 4.0–5.0 mL/min into account during eating, chewing and other stimulating activities (e.g., in lozenge application) [15] and considering the time needed to dissolve the lozenge (on average, 5 min) and the constant release of active substances from the lozenge, this medium concentration of test products (test products suspended in 20 mL) could hypothetically be achieved in the actual use of a throat lozenge in the mouth.

However, the lozenge suspended in 30 mL (low concentration) did not achieve a 4-log reduction within 15 min. With CPC as a free active substance, we were also not able to see a 4-log reduction after 15 min contact time for medium- and low-concentration suspensions. However, there a clear trend of virus reduction was observed in all test-product concentrations (except for the placebo lozenge) throughout the 15 min contact time.

An important and additional finding of this study is the highly effective virucidal activity of the BH/CPC combination, either as lozenge or free active substance, which has a more rapid effect compared to CPC alone. Our assumption is that BH, i.e., the additional component, has an effect on the destabilization and loss of infectivity of viral particles. Another parameter in the lozenge suspension that influences the virucidal dynamics could be extreme acidic conditions. In the free active substance suspensions of CPC and BH/CPC, the pH value in all tested concentrations was, on average, 7.65 and 7.78, which is near neutral. However, for the BH/CPC lozenge, the pH value was, on average, 3.90. It seems that the low pH value of the BH/CPC lozenge suspension in our study might be an additional factor, with a synergistic effect on the very rapid loss of virus infectivity. Studies also confirmed that the pH value has an important influence on virus stability and infectivity in the environment [16]. In both extreme acidic and extreme alkaline environments, viral proteins on the surface were irreversibly denatured, causing a loss of infectivity.

The virucidal effect of CPC and the BH/CPC lozenge was additionally seen in the morphology analysis of virus particles. After virus incubation in suspensions of CPC as a free active substance and of BH/CPC as a lozenge, a similar effect was noted, i.e., destruction of the virus envelope. With the BH/CPC lozenge, we observed a destabilization of the nucleocapsid, which might be due to the low pH environment that viruses were exposed to [17].

It should be emphasized that the action site of a throat lozenge is oral and pharyngeal mucosa, which is difficult to simulate in an in vitro laboratory environment. An additional point that needs to be considered is that the potential in vivo activity of a throat lozenge is only its effect on viruses released from infected cells and viruses in saliva. It was shown in previous studies that the concentration of viruses released in the saliva, is in the range of 4–6 $\log_{10}$ genome copies per millilitre [18–20], with highest concentration occurring five to six days after symptom onset. In this study procedure, the virus concentration was much higher, between 8.00 and 10.00 $\log_{10}$ TCID$_{50}$/mL.

The route of SARS-CoV-2 infection is currently considered to be via respiratory droplets, and the virus particle is viable in aerosols for up to three hours [21,22]. Preventive measures such as hand hygiene, facial masks and social distancing have been the primary means of public infection control. To date, the relationship between the lung and throat viral load in terms of disease severity is unclear, nor is it known how the reduction in the viral load in the throat may affect the resulting lung disease or viral transmission. If it is

expected that higher concentrations of the virus in the throat might increase the possibility of infecting others, then strategies to reduce the number of infective virus particles in mucous membranes could help reduce the risk of transmission. Therefore, when assuming that the throat is the major site of replication in early stages, the use of topical agents that could damage or destroy the lipid envelope of the virus has the potential to reduce the viral load in the oropharynx.

Several case reports have also confirmed the efficacy of mouth rinses in reducing the SARS-CoV-2 load in the saliva [23–25].

Further investigation into the mechanism of virucidal activity and the clinical effects of the BH/CPC lozenge for control of RTIs is warranted.

## 5. Conclusions

The results presented in this study are based on a controlled and defined laboratory environment, adapted to mimic the physiological conditions of the oral cavity. All active test products, i.e., CPC, BH/CPC as a free active substance and BH/CPC as a lozenge exhibit important virucidal activity in in vitro conditions.

The study additionally confirmed a significant difference between the active BH/CPC lozenge and the placebo lozenge, as well as a greater effect of the combination of BH CPC, either as a lozenge or as a free active substance, in comparison to CPC alone.

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