In vitro and clinical studies on the efficacy of α-cyclodextrin and hydroxytyrosol against SARS-CoV-2 infection

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Abstract. – **OBJECTIVE:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new coronavirus responsible for the current pandemic of coronavirus disease 2019 (COVID-19). This virus attacks cells of the airway epithelium by binding transmembrane angiotensin-converting enzyme 2 (ACE2). Hydroxytyrosol has anti-viral properties. Alpha-cyclodextrin can deplete sphingolipids and phospholipids from cell membranes. The aim of the present experimental study was to evaluate the efficacy of α-cyclodextrin and hydroxytyrosol in improving defenses against SARS-CoV-2 infection in *in vitro* cell models and humans.

PATIENTS AND METHODS: For *in vitro* experiments on Vero E6 cells, RNA for RT-qPCR analysis was extracted from Caco2 and human fibroblast cell lines. For study in humans, the treatment group consisted of 149 healthy volunteers in Northern Cyprus, considered at higher risk of SARS-CoV-2 infection than the general population. The volunteers used nasal spray containing α-cyclodextrin and hydroxytyrosol for 4 weeks. The control group consisted of 76 healthy volunteers who did not use the spray.

RESULTS: RT-qPCR experiments on targeted genes involved in endocytosis showed a reduction in gene expression, whereas cytotoxicity and cytoprotective tests showed that the compounds exerted a protective effect against SARS-CoV-2 infection at non-cytotoxic concentrations. None of the volunteers became positive to SARS-CoV-2 RT-qPCR assay during the 30 days of treatment.

CONCLUSIONS: Treatment with α-cyclodextrin and hydroxytyrosol nasal spray improved defenses against SARS-CoV-2 infection and reduced synthesis of viral particles.

Key Words:
    SARS-CoV-2, ACE2, Hydroxytyrosol, α-cyclodextrin.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a new coronavirus that causes the coronavirus disease, known as COVID-19. SARS-CoV-2 binds and invades oral and nasopharyngeal epithelia. Its spike protein binds angiotensin-converting enzyme-2 (ACE2) after cleavage by the accessory protease furin. The virus then enters the cell by endocytosis[4]. Viral spike protein binding of ACE2 may also occur with the help of another enzyme, transmembrane serine protease 2 (TMPRSS2). ACE2, furin and TMPRSS2 are all localized in cholesterol-rich lipid rafts of the cell membrane[1-3]. Following indications in the literature, we tested whether two compounds, alpha-cyclodextrin and hydroxytyrosol, could reduce endocytosis of SARS-CoV-2. Hydroxytyrosol can be extracted from olive leaves and fruits. It is known to have anti-viral properties and to reduce serum lipids in mice fed high-cholesterol diets by indirectly modifying plasma membrane composition[5,6]. Hydroxytyrosol also interacts directly with the hydrophilic heads of plasma membrane phospholipids[7]. Alpha-cyclodextrin depletes cell membrane phospholipids and sphingolipids. Sphingolipids and cholesterol form the lipid rafts that host ACE2 receptors[8]. Hydroxytyrosol has broad-spectrum antiviral activity, especially against enveloped viruses, like influenza virus, HIV and coronaviruses. For instance, it may induce morphological changes that reduce influenza virus infectivity[9]. The exact mechanism of action is unclear but seems to require the presence of the envelope. The second compound, α-cyclodextrin, depletes sphingolipids from lipid rafts which bear the ACE2 receptor, specific for SARS-CoV-2[8]. Alpha-cyclodextrin may reduce sphingolipids by binding their hydrophobic hole. This may reduce the number of sphingolipids necessary to make the lipid rafts[8]. The spray was designed to act locally, possibly decreasing the number of ACE2 receptors available for the virus to enter oro-pharyngeal epithelial cells.

The aim of the present study was to obtain data on the efficacy of alpha-cyclodextrin and hydroxytyrosol against SARS-CoV-2 endocytosis and infection in *in vitro* cell models and in human subjects.

Materials and Methods

Test Compounds

Alpha-cyclodextrin (Cavamax W6) was obtained from Wacker Chemie AG (Munich, Germany) and hydroxytyrosol (MOMAST plus 30) was acquired from Bioenutra [Ginosa (TA), Italy]. Stock solution for α-cyclodextrin was made with DMSO (10 mM). Compound dilutions were prepared freshly for each experiment. Remdesivir (Cat. No. HY-104077 - MedChemExpress, NJ, USA) was used as control.

Mammalian Cell Lines

The cytotoxicity and antiviral activity of the compounds were studied in Vero E6 cells (*Cercopithecus aethiops*, kidney, ATCC CRL-1586). These cells were chosen because they express ACE2 and are therefore highly susceptible to SARS-CoV-2 infection. The cell line was routinely maintained in DMEM supplemented with 1% glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum, FBS (complete medium).

The human Caco2 cell line is derived from a colorectal adeno-carcinoma specimen from a Caucasian male. Cell lines were obtained from ATCC and cultured as suggested in EMEM, 10% FBS, 1% non-essential amino acids, 2 mM glutamine. All experiments were performed between passages 15 and 20. The human fibroblast cell line GM09607, obtained from Coriell Institute (New Jersey, USA) was cultured in DMEM 10% FBS and tested between passage 22-30.

Cells were treated in duplicate with 10 mM α-cyclodextrin and 10 μM hydroxytyrosol for 6 hours and then pelleted for RNA extraction. Compound dilutions were prepared freshly for each experiment and discarded after use.

Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from 1 million Caco2 cells or primary fibroblasts using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocols. The SuperScript VILO cDNA Synthesis Kit was used to generate first strand complementary DNA (cDNA). Quantitative Real-Time Polymerase ChainReaction (RT-qPCR) was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) on a QuantStudio 3 Real-Time PCR System. The primers used in the qPCR experiments are listed in **Supplementary Table I**.

MTS Cell Viability Assay

A cytotoxicity experiment was performed in parallel with the antiviral assay, using cells from the same passage. Exponentially growing

Vero E6 cells were seeded into a 96-well plate at their optimal density in complete medium, for 24 hours. Later, cells were exposed to different concentrations of the two compounds, alone and in combination (six concentration points, 5-fold dilutions, 100-20-4-0.8-0.16-0.032 μM), in complete medium (2% FBS) for 72 hours. Compound dilutions were performed in culture medium. Remdesivir was included as comparator drug. The experiment was performed in duplicate. Cytotoxic effect was evaluated by MTS colorimetric assay (Promega, Madison, WI, USA) and confirmed by microscope observation of the cell monolayer. Cytotoxic concentration 50% (CC_{50}) was calculated by interpolation of the dose-response curves generated by Magellan™ software.

Antiviral Activity Assay

Exponentially growing Vero E6 cells were seeded into a 96-well plate at their optimal density in complete medium; 24 hours later cells were exposed to different concentrations of compounds (same as above), then, infected with SARS-CoV-2 at 0.01 $TCID_{50}$/cell (50% tissue culture infectious dose) multiplicity of infection and cultured for 72 hours. Compound dilutions were performed in culture medium. Two replicates for each concentration point were examined. At the end of the incubation period, antiviral activity was examined by ELISA assay (quantifying SARS-CoV-2 nucleoprotein, NP) and by microscope observation of cytopathic effect. Inhibitory concentration 50% (IC_{50}) and selectivity index ($SI_{50}=CC_{50}/IC_{50}$) were calculated to define the therapeutic window.

Spray Composition

One dose of the solution (8 sprays = 0.5 ml, density = 1.1 g/ml) contains the following ingredients: water (52.57%), active compounds: hydroxytyrosol (3.80%), α-cyclodextrin (0.20%), co-emulsifier: glycerin (3.80%), flavoring: lemon aroma (0.98%), acidifier: citric acid (0.30%), preservatives: sodium benzoate (0.10%), potassium sorbate (0.10%), viscosity control: xanthan gum (0.05%), sweeteners: fructose (38.06%), steviol glycosides (0.02%), sucralose (0.02%).

Subject Selection

Near East University (Nicosia, Cyprus) enrolled 225 human volunteers who were RT-qPCR and antibody (Immunoglobin M and Immunoglobin G) negative for SARS-CoV-2. Seven additional subjects, members of the Near East University Hospital staff, who were initially offered the spray, refused to use it and were later found positive to the SARS-CoV-2 RT-qPCR test. Twelve volunteers who had already used the spray were in close contact with these seven members of the Near East University Hospital staff. The remaining 213 volunteers were at a high risk of SARS-CoV-2 infection by virtue of their occupation. During this study, 149 volunteers used the spray, and 76 did not and therefore acted as controls. All volunteers signed the informed consent form, and the study was conducted according to the ethical principles of the Declaration of Helsinki. The Internal Review Board of the Near East University (Nicosia, Cyprus) approved the study (approval number: NEU/2020/83/1169). Biological samples (blood and swab) were collected from all subjects and analyzed for viral RNA by RT-qPCR and for SARS-CoV-2-specific antibodies by ELISA assay. None of the 225 subjects withdrew from the study.

Virological and Serological Tests

Diagnovital SARS-CoV-2 Real-Time PCR kit (A1 Life Sciences, Gebze/Kocaeli, Turkey) was used to detect SARS-CoV-2 RNA in oro-naso-pharyngeal swabs on the first, 15th and 30th (last) day of the experiment. The viral load was evaluated according to manufacturing-based definition of quality [Cycle threshold (C_t) = 28 is approximately equivalent to 2.5×10^4 viruses]. A venous blood sample was collected from each patient to perform ELISA antibody tests (Immunoglobin M and Immunoglobin G) using the Abbott COVID-19 Antibody test kit (Chicago, IL, USA) on the first and last day of the study.

Results

Molecular Analysis

RT-qPCR revealed a small reduction in expression of genes involved in cholesterol homeostasis and lipid raft composition in Caco2 cells and fibroblasts (GM09607) (Figure 1). Specifically, when the two cell lines were treated with α-cyclodextrin and hydroxytyrosol, we observed a decreasing trend in *ERLIN1*, *ERLIN2* and *RFTN1* expression compared to control. Genes involved in endocytosis (*CAV1*, *CTLB*, *SGMS*) were also downregulated in fibroblasts.
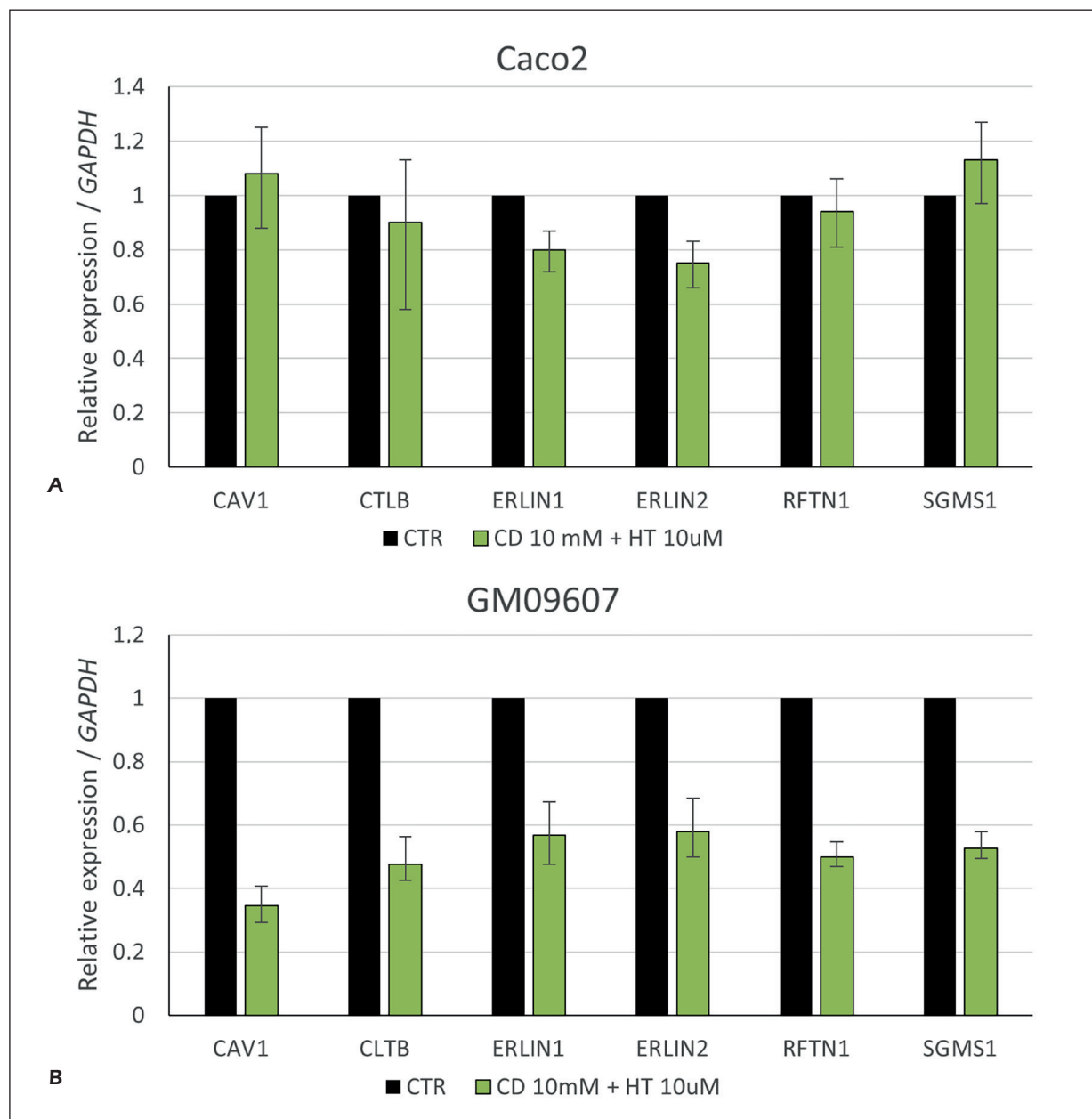
Figure 1. Gene expression profile under α-cyclodextrin (CD) and hydroxytyrosol (HT) treatment. **A,** Expression levels of genes involved in cholesterol homeostasis and lipid raft composition in Caco2 cell lines compared to calibrator (CTR, Relative expression =1). **B,** Expression levels of endocytosis genes in GM09607 (fibroblast) cell lines. (CTR: control; GAPDH: housekeeping gene in RT-qPCR analysis).

Antiviral Activity

Experiments in Vero E6 cells revealed that the combination of α-cyclodextrin and hydroxytyrosol effectively reduced viral replication at non-cytotoxic concentrations. In fact, at 0.8 μM α-cyclodextrin + hydroxytyrosol there was a 32% reduction in viral replication calculated by ELI-SA assay of SARS-CoV-2 nucleoprotein levels with respect to control after 72-hour treatment (Table I).

Clinical Testing

The clinical data of the 225 volunteers (heterogeneous in age, sex, comorbidity and drug use without

Table I. Viability and antiviral activity data of test compounds at the different concentrations, compared to untreated control (=100%).

	Alpha cyclodextrin (CD) + Hydroxytyrosol (HT)									
	Concentration, μM	Viability, % of control		CC$_{50}$ (μM)	Concentration, μM	Viral replication (NP, ng/ml)			IC$_{50}$ (μM)	SI$_{50}$
		Mean	SD			Mean	SD	% of control		
	Control	100.0	0.0		Control	1140	103	100		
CD + HT	100	11.5	0.0	1.7	100	Toxic	–	–	1	1.7
	20	18.4	0.5		20	Toxic	–	–		
	4	7.5	0.5		4	Toxic	–	–		
	0.8	92.9	17.6		0.8	772	18	68		
	0.16	87.7	21.9		0.16	1056	149	93		
	0.032	84.4	21.8		0.032	856	95	75		
	Remdesivir (REM)									
	Concentration, μM	Viability, % of control		CC$_{50}$ (μM)	Concentration, μM	Viral replication (NP, ng/ml)			IC$_{50}$ (μM)	SI$_{50}$
		Mean	SD			Mean	SD	% of control		
	Control	100.0	0.0		Control	1140	103	100		
REM	100	80.6	100	> 100	10	47	0	4	4.9	>20
	10	92.1	10		2	876	160	77		
	1	98.1	1		0.4	916	215	80		
	0.1	97.6	0.1		0.08	–	–	–		

Cytotoxicity data (i.e., viability expressed as % of control, mean and standard deviation) was obtained by MTS assay and CC$_{50}$ values for the test compounds were calculated. Viral replication data (i.e., levels of SARS-CoV-2 nucleoprotein, NP, expressed in ng/mL, mean and standard deviation, and as % of control) obtained by ELISA assay, with calculated IC$_{50}$ values. Calculated SI50 values (CC50/IC50). Infection in control wells was in line with previous experiments (mean and standard deviation of SARS-CoV-2 nucleoprotein 1140 ± 103 ng/mL).

significant differences with respect to the general population) who used the spray for 30 days and those who did not use it is shown in Tables II and III. All participants were followed up for 30 days until the last spray administration. Although all participants were at a higher risk of SARS-CoV-2 infection due to their occupation, and at least twelve of them were in close contact with seven hospital colleagues who turned out to be positive and shared the same working environment, none of them was infected while using the spray.

Discussion

Lipid rafts are plasma membrane micro-domains involved in many cell functions. In-ternalization of ligands and receptors by these domains occurs by a ubiquitous process defined as raft-dependent endocytosis. Numerous virus-es, e.g., HIV and influenza virus, use lipid rafts and endocytosis mechanisms to enter the host[10]. Gene expression or genomic variations related to endocytosis may play a role in modulating cell physiology. In our model, expression of these genes is reduced when endothelial Caco2 cells and fibroblasts are treated with natural com-pounds, such as α-cyclodextrin and hydroxy-tyrosol. We used these cell lines because they were available in our laboratory and endocytosis is a ubiquitous process. In line with Wittkowski et al[11], our results indicate that treatment with a derivative of alpha-cyclodextrin may be useful to downregulate endocytosis[11]. Genes, such as

Table II. Clinical data of subjects who used the spray.

Characteristic	Value
Participants, n	149
Mean age ± SD (Range)	37±11.45 (20-76)
Male/Female (% Male/% Female)	74/75 (49.7%/ 50.3%)
Smoker Yes/No (% Yes)	63/86 (42.3%/ 57.7%)
Comorbidity, n (%)	22/127 (14.8%)
Diabetes	4 (2.7%)
Hypercholesterolemia	4 (2.7%)
Hypertension	9 (6.0%)
Cardiovascular	2 (1.7%)
Thyroid disease	3 (2.6%)
Food allergy	1 (0.8%)
Favism	1 (0.8%)
Arrhythmia	1 (0.8%)
Obesity	2 (1.7%)
Dyslipidemia	3 (2.0%)
Ulcerative colitis	1 (0.8%)
Crohn's disease	1 (0.8%)
Hypoglycemia	1 (0.8%)
Pharmacological treatment, n (%)	15/134 (10%/90%)
β-blocker	1 (0.8%)
Proton pump inhibitor	2 (1.7%)
Hypoglycemic agent	4 (3.5%)
Synthetic thyroid hormone	2 (1.7%)
Colchicine	1 (0.8%)
Angiotensin II receptor blocker	3 (2.6 %)
α-blocker	1 (0.8%)
Generic anti-hypertensive agent	1 (0.8%)
Diuretic agent	1 (0.8%)
Anti-vertigo agent	1 (0.8%)
Immunosuppressant	1 (0.8%)
Mean weight(kg) ± SD (Range)	75 ± 16.67 (48-126)
Mean height (m) ± SD (Range)	1.70 ± 0.1 (1.50-1.96)
Mean body mass index ± SD (Range)	26.0 ± 7.24 (17.6-37.9)
Type of exposure to the virus (continuous/occasional)	149/0
Place of exposure (home/workplace)	0/149
Withdrawal from the study	0/149
Side effects	0/149
Acquired SARS-CoV-2 infection	0/149

Table III. Clinical data of subjects who did not use the spray.

Characteristic	Value
Participants, n	76
Mean age ± SD (Range)	31.7 ± 11.45 (20-76)
Male/Female (% Male/ % Female)	39/37 (51.3%/ 48.7%)
Smoker Yes/No (% Yes)	28/48 (36.8%/ 63.2%)
Comorbidity, n (%)	15/61 (19.7%)
Diabetes	2 (2.6%)
Hypertension	3 (3.9%)
Cardiovascular	1 (1.3%)
Thyroid disease	2 (2.6%)
Obesity	4 (5.3%)
Ulcerative colitis	2 (2.6%)
Chronic migraine	1 (1.3%)
Vertigo	1 (1.3%)
Chronic pharyngitis	1 (1.3%)
Pharmacological treatment, n (%)	6/70 (7.9%)
Synthetic thyroid hormone	2 (2.6%)
Angiotensin II receptor blocker	1 (1.3%)
Anti-vertigo agent	1 (1.3%)
Anti-inflammatory drug	2 (2.6%)
Pain killer	1 (1.3%)
Anticoagulant	1 (1.3%)
Mean weight(kg) ± SD (Range)	72.9 ± 16.67 (48-126)
Mean height (m) ± SD (Range)	1.70 ± 0.1 (1.50-1.96)
Mean body mass index ± SD (Range)	24.9 ± 7.24 (17.6-37.9)
Type of exposure to the virus (continuous/occasional)	76/0
Place of exposure (home/workplace)	0/76
Withdrawal from the study	0/76
Side effects	0/76
Acquired SARS-CoV-2 infection	0/76

ERLIN2 (endoplasmic reticulum lipid raft-associated protein 2) have been found over-expressed in cancer[12], while *ERLIN1* and *RFTN1* are over-expressed in infections[13-15]. *ERLIN2* is a player in the regulation of cytosolic lipid content in cells[16]. A reduction in expression of this gene could therefore explain reduced endocytosis. Raftlin (RFTN1) is a protein localized exclusively in lipid rafts. Knockout or over-expression of the *RFTN1* gene in the DT40 B-cell line resulted in altered lipid raft composition[17], suggesting that also in our system, where this gene is downregulated in two cell lines, there could be a modification in lipid raft composition, and therefore in the capacity of SARS-CoV-2 to enter the cells. Moreover, *ERLIN1* is a regulator of cell cholesterol homeostasis; its silencing *in vitro* led to decreased hepatitis C virus infection[13].

The *in vitro* experiments performed in Vero E6 cells showed that the combination of α-cyclodextrin and hydroxytyrosol effectively reduces viral replication at non-cytotoxic concentrations. In fact, after 72-hour treatment at 0.8 μM α-cyclodextrin + hydroxytyrosol, we recorded a ~32% reduction in viral replication, calculated on the basis of SARS-CoV-2 nucleoprotein levels measured by ELISA assay, with respect to control cells treated with Remdesivir.

These results further sustain our preliminary studies that suggested reduced risk of contracting the infection and reduced duration of symptoms, probably due to fewer membrane lipid rafts containing ACE2 associated with α-cyclodextrin treatment, while in addition hydroxytyrosol may have direct antiviral effects[9,18,19].

A total of 225 healthy volunteers participated in the study, all at higher risk of exposure to the virus than the general population because of their profession. The spray was tested by 149 of them. None of the volunteers reported any side effects or discomfort related to spray use. The 76 participants who did not use the spray were included as controls. Molecular virology and serology tests showed that none of the volunteers were infected by the virus during the study. Among spray users, at least 12 were in close contact with seven COVID-19-positive co-workers who did not use the spray. Although the volunteers were

exposed to the virus and worked in the same environment, viral replication and infection did not occur in spray users, which strengthens our *in vitro* findings.

Conclusions

Overall, our *in vitro* and clinical studies showed that both α-cyclodextrin and hydroxytyrosol improve defenses against SARS-CoV-2 infection and reduce the expression of genes involved in the endocytosis and synthesis of viral particles. We tested our model and investigated how the spray altered the model to decrease viral infection. We verified our model with *in vitro* studies, demonstrating mRNA changes in relevant cell lines, antiviral activity in viral assays at appropriate non-toxic levels of the drugs, and finally the spray seemed to protect healthcare workers in contact with COVID-19 patients against contracting COVID-19. This is interesting in view of the fact that the infection rate in Health Care workers is estimated to be around 10% in a clinical setting[20].

Conflict of Interest
The Authors declare that they have no conflict of interests.

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