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Original article

A pilot study on the preventative potential of alpha-cyclodextrin and hydroxytyrosol against SARS-CoV-2 transmission

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Abstract. *Background and aim of the work:* Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current pandemics of coronavirus disease. This virus is able to attack the cells of the airway epithelium by binding to the transmembrane angiotensin I converting enzyme 2 (ACE2). In the current study, we experimented a food supplement containing alpha-cyclodextrin and hydroxytyrosol as active compounds for the improvement of the defenses against the SARS-CoV-2. Hydroxytyrosol has anti-viral properties and is able to reduce the serum lipids in mice fed with high-cholesterol diet, and α-cyclodextrin has the ability to deplete sphingolipids and phospholipids from the cellular membranes. The aim of the present preliminary open non-controlled interventional study was to evaluate the efficacy of alpha-cyclodextrin and hydroxytyrosol in improving defenses against SARS-CoV-2. *Methods:* Fifty healthy volunteers at a higher risk of SARS-CoV-2 infection from Northern Cyprus and six positive individuals for SARS-CoV-2 by RT-qPCR assay were enrolled in this study. The *in silico* prediction analysis was performed using the bioinformatic tool D3DOCKING to evaluate the interactions of hydroxytyrosol and alpha-cyclodextrin with proteins involved in the biological cycle of SARS-CoV-2 in the humans. *Results:* The 50 volunteers did not become positive in 15 days for SARS-CoV-2 RT-qPCR assay after the administration of the compound for two weeks, despite they were at higher risk of infection than the general population. Interestingly, in the cohort of six positive patients, two patients were administered with the spray and became negative after five days of spray administration, despite the viral load was higher in the treated subjects than the patients that did not take the compound and that became negative after ten days. In addition, we have identified the presence of possible direct interactions among hydroxytyrosol and alpha-cyclodextrin with the viral protein Spike and the human proteins ACE2 and TMPRSS2. *Conclusions:* We preliminary reported on the results of the possible role of alpha-cyclodextrin and hydroxytyrosol in improving defenses against SARS-CoV-2. The next step would be the administration of the compound on a larger cohort in a controlled study and see whether there is a reduced infection rate of SARS-CoV-2 in the treated subjects than in the non-treated individuals. (www.actabiomedica.it)

Key words: SARS-CoV-2, ACE2, hydroxytyrosol, α-cyclodextrin

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current pandemics of coronavirus disease known as COVID-19. This virus is able to attack the cells of the airway epithelium by binding its spike protein to the angiotensin I converting enzyme 2 (ACE2) with the help of the transmembrane serine protease 2 (TMPRSS2). Both proteins, ACE2 and TMPRSS2, are localized in the cholesterol-rich lipid rafts of the cell membrane (1,2). After this binding the SARS-CoV-2 is able to enter the cells via endocytosis (3). With regard to the literature in the current knowledge, we tested whether two compounds, alpha-cyclodextrin and hydroxytyrosol, could reduce the endocytosis of the SARS-CoV-2. Hydroxytyrosol is extracted from olive leaves and fruits and has anti-viral properties and is able to reduce the serum lipids in mice fed with high-cholesterol diets, indirectly modifying the composition of the plasma membrane (4,5), in addition hydroxytyrosol directly interacts with the hydrophilic heads of the plasma membrane (6). α-cyclodextrin is able to deplete phospholipids and sphingolipids from the cell membrane. Sphingolipids, together with cholesterol, form the lipid rafts where ACE2 localizes (7). Hydroxytyrosol has a broad-spectrum antiviral activity, especially against enveloped viruses like influenza virus, HIV or coronaviruses. For instance, hydroxytyrosol is able to induce morphological changes that reduce influenza virus infectivity (8). It also enhances anti-inflammatory effects by decreasing the levels of pro-inflammatory cytokines IL-6 and TNF-α, as observed in animal models (9). Interestingly, those two cytokines rapidly increase in the most severe cases of COVID-19 (10). Whereas, α-cyclodextrin is able to deplete sphingolipids from the lipid rafts where the ACE2 receptor, specific for SARS-CoV-2, localizes (7), and to reduce serum phospholipids, which are necessary for the SARS-CoV-2 endocytosis into cells (11).

The aim of the present pilot open non-controlled interventional study was to obtain preliminary results on the role of alpha-cyclodextrin and hydroxytyrosol in improving defenses against SARS-CoV-2. Furthermore, the spray was used in two positive patients to test the effect of the spray on the viral load.

Materials and methods

Bioinformatic analysis

D3Docking (12) is able to predict the potential ligand-binding sites for each protein conformation involved in the biological cycle of SARS-CoV-2. We used this tool in order to evaluate whether hydroxytyrosol and alpha-cyclodextrin were able to interact with proteins relevant for the SARS-CoV-2 life cycle.

Spray composition

One dose of the solution (4 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-emulsifyer: glycerin (3.80%), flavoring: lemon flavor (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 50 voluntary subjects that were negative for SARS-CoV-2 and six SARS-CoV-2-positive patients detected by RT-qPCR. All voluntary subjects signed the informed consent form and the study was conducted according to the ethical principles of the "Declaration of Helsinki" (approval number: NEU/2020/83/1169). Patients' biological samples (blood and swab) were collected and analyzed to identify viral RNA by RT-qPCR and SARS-CoV-2-specific antibodies by ELISA assay. None of the subjects withdrew from the study.

Virological and serological tests

Diagnovital SARS-CoV-2 Real-Time PCR kit (A1 Life Sciences, Turkey) was used to detect SARS-CoV-2 RNA from oro-/nasopharyngeal swabs on the first and 15th days of the experiment. The viral load was evaluated according to manufacturing-based definition of quality (Cycle threshold (Ct) value at 28 is approximately equal to 2.5 x 10^4 virus number). A ve-

nous blood sample was collected from each patient to perform ELISA antibody tests (Immunoglobin M and Immunoglobin G) by Abbott COVID-19 Antibody test kit (Chicago, USA) at the first and the last days of the study.

Results

Experimentation on healthy volunteers

The clinical data of the 50 enrolled subjects are included in Table 1.

Table 1. Clinical data of the enrolled healthy subjects

	Spray users (N:50)
Mean age ± SD (Min-Max)	38.28±10.45 (26-62)
Males/Females (%Males)	29/21 (58%)
Smoker Yes/No (%Yes)	17/33 (34%)
Comorbidities Yes/No (%Yes)	9/41 (18%)
Diabetes	1
Hypercholesterolemia	1
Hypertension	2
Cardiovascular	1
Thyroid disease	2
Food allergy	1
Favism	1
Pharmacological treatments Yes/No (%Yes)	8/42 (16%)
β-blockers	1
Proton pump inhibitors	2
Hypoglycemic agents	3
Thyroid hormones analogues	1
Colchicine	1
Mean weight(kg) ± SD (Min-Max)	76.7±18.62 (50-126)
Mean height (m) ± SD (Min-Max)	1.72±0.4 (1.50-1.93)
Mean BMI (Body Mass Index) ± SD (Min-Max)	25.72±5.3 (18.8-33.8)
Type of exposure to the virus (continuous/occasional)	5/45 (10%)
Place of exposure (home/workplace)	0/50 (0%)
Withdrawal from the study	0%
Side effects	0%

The spray containing hydroxytyrosol and alpha-cyclodextrin is being used on a population (50 individuals) heterogeneous in age, sex, comorbidity and drug use without significant differences from the general population. Among these, 46 subjects were at higher risk of infection because of their job (Table 2). This study was conducted during the period when the infection in the country was most active (R_o= 2.06), from August, 16th to October, 3rd 2020. Importantly, an ELISA antibody test was conducted in order to ensure that enrolled subject had not encountered the virus before study inclusion.

All subjects used the spray four times (4 puffs per dose) a day for 15 days with a total absence of side effects. Any specific side effects were not expected as alpha-cyclodextrin and hydroxytyrosol are considered novel foods (13, 14). All individuals were negative at the beginning of the study and stayed negative so far, which were confirmed by both RT-qPCR and ELISA antibody tests. From a cohort of six patients positive for the SARS-CoV-2 infection; two symptomatic patients (high fever and backpain, respectively) had five-day recommended COVID-19 medical treatment. After seven days of their treatment they did not become negative for SARS-CoV-2, therefore the spray was administered. The patients that were administered the spray became negative to the SARS-CoV-2 test after five days, the other four were asymptomatic did not use the spray and became negative after ten days.

Table 2. Type of job of the enrolled subjects

Type of job	Number
Barber	1
Businessman	1
COVID-19 laboratory worker	12
Emergency service worker	1
Government worker	1
Hospital-based university administration	27
Hospital laboratory worker	2
Nurse	1
Physician	2
Lecturer	1
Veterinarian	1

The patients that became negative in five days had initial viral loads of $2.5x10^5$ and $2.5x10^9$, respectively. This was comparable to the patients that became negative after ten days and did not use the spray, $2.5x10^5$; $2.5x10^2$; $2.5x10^9$; $2.5x10^5$, respectively. Viral loads of these patients decreased to $2.5x10^4$; $2.5x10^2$; $2.5x10^4$; $2.5x10^2$ after five days (Table 3).

Bioinformatic analysis

The molecular docking analysis suggests a potential interaction of hydroxytyrosol and alpha-cyclodextrin for the major proteins involved in the SARS-CoV-2 endocytosis (Figure 1 and 2, Table 4 and 5). Indeed, D3Docking analysis showed good binding affinity of both hydroxytyrosol and alpha-cyclodextrin to ACE2, TMPRSS2 and S proteins.

Discussion

To date, no effective drugs or vaccines against SARS-CoV-2 have been approved. Moreover, although some treatments seem to be effective against this virus. In light of this, besides the need to find specific and safe antiviral agents or vaccines and the necessity of the social distancing and the use of facial masks, and frequent hands sanitation, it would be of crucial importance to develop remedies that could help in the fight against SARS-CoV-2 (15,16). The present preliminary study on healthy volunteers further confirm that on a heterogenous population of SARS-CoV-2-negative cohort there is an absence of side effects and interactions with other medications after two weeks of administration (17), and that none of the participants got infected with SARS-CoV-2 during the study. Interestingly, the two patients (patient 1 and 2) that used the spray in the cohort of six that were positive to the SARS-CoV-2 infection became negative after five days, whereas the remaining four patients that did not use the spray became negative after ten days. It is worth to mention that the initial viral loads of six COVID-19 positive patients were comparable, the viral loads of the two symptomatic patients that used the spray sharply decreased and became negative than non-user patients. In addition, the patients that recovered in five days had also worst symptoms like high

Table 3. Clinical, virological and serological data of the patients. Day 0 illustrates when the treatment was started to these patients. Day five represents after the fifth day of the spray administration for patients 1 and 2 (grey rows). NA = data not available.

Patient	Sex	Age	Smoker	Comorbidity	Pharmacological treatment	Weight (Kg)	Height (m)	BMI	Type of exposure	Place of exposure	SARS-CoV-2 RT-qPCR				SARS-CoV-2 ELISA		Symptoms
											Day 0	Day 5	Day 10	Day 15	Day 0		
															IgG	IgM	
1	F	47	Yes	No	No	57	1.64	21.2	Continuous	Workplace	+ ($2.5x10^5$)	-	-	-	-	+	High fever
2	M	50	No	Obesity	No	95	1.70	32.9	Continuous	Workplace	+ ($2.5x10^9$)	-	-	-	+	+	Backpain
3	M	57	No	No	No	89	1.82	26.8	Continuous	Workplace	+ ($2.5x10^5$)	+ ($2.5x10^4$)	-	-	+	+	Asymptomatic
4	M	42	No	No	No	80	1.82	24.1	Continuous	Workplace	+ ($2.5x10^9$)	+ ($2.5x10^2$)	-	-	+	+	Asymptomatic
5	F	37	No	No	No	61	1.64	22.6	Occasional	Workplace	+ ($2.5x10^9$)	+ ($2.5x10^4$)	-	-	-	+	Mild fever
6	F	36	No	No	No	58	1.62	22.1	Continuous	Workplace	+ ($2.5x10^5$)	+ ($2.5x10^2$)	-	-	NA	NA	Asymptomatic

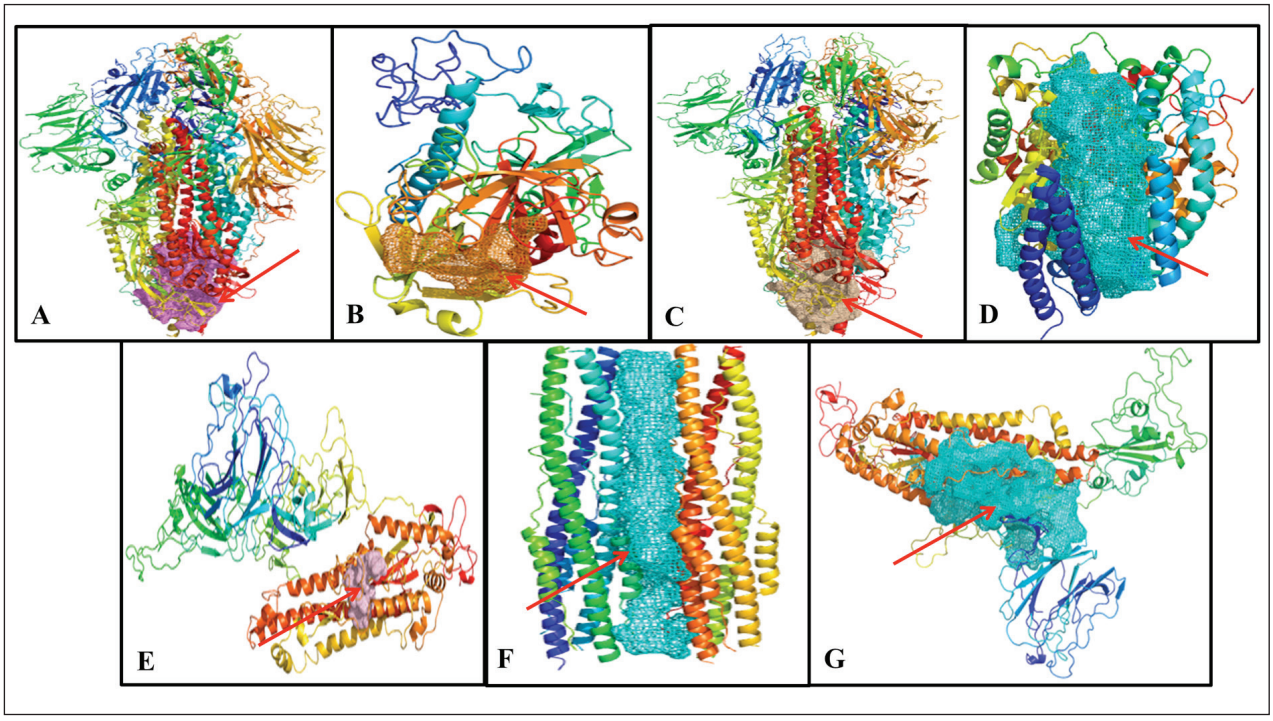

Figure 1. Site of interaction of hydroxytyrosol with the proteins Spike, TMPRSS2 and ACE2 indicated by an arrow. Legend: A, Spike protein, open conformation, pocket 2; B, transmembrane protease serine 2, C, Spike protein, closed conformation, pocket 2; D, Angiotensin converting enzyme 2; E, Spike protein, open conformation, pocket 5; F, Spike protein, S2 subunit, pocket 1; G, Spike protein, closed conformation, pocket 1.

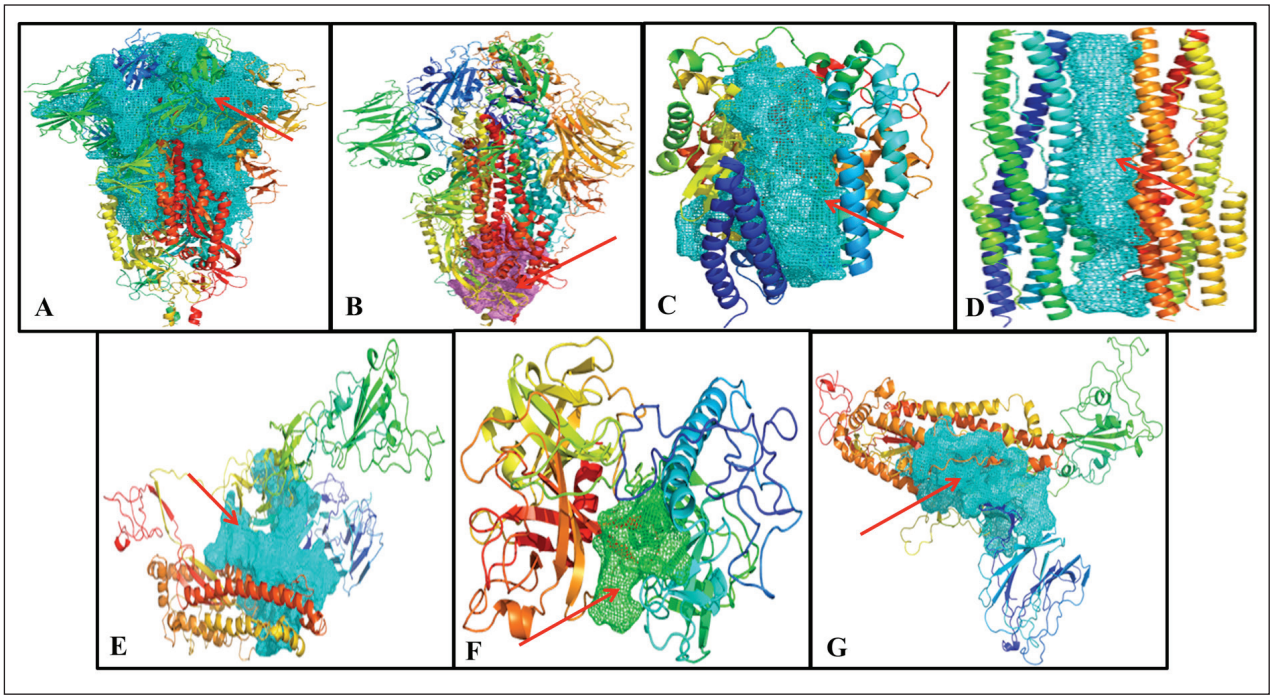

Figure 2. Site of interaction of alpha-cyclodextrin with the proteins Spike, TMPRSS2 and ACE2 indicated by the red arrow. Legend: A, Spike protein, closed conformation, pocket 1; B, Spike protein, open conformation, pocket 2; C, Angiotensin converting enzyme 2; D, Spike protein, open conformation, pocket 1; E, transmembrane protease serine 2, F, Spike protein, closed conformation, pocket 1.

Table 4. Interaction of hydroxytyrosol with ACE2, TMPRSS2 and S proteins

Target abbreviation	Target full name	Conformational state	Docking score (kcal/mol)	Uniprot ID / Protein ID	Pocket	Template PDB ID	Organism	Model Source
S protein	Spike protein	Open	-7,21	QHD43416.1	Pocket 2	6vyb	SARS-CoV-2	PDB
TMPRSS2	Transmembrane protease serine 2		-6,87	O15393	Pocket 4	-	human	Robetta
S protein	Spike protein	Closed	-6,82	QHD43416.1	Pocket 2	6vxx	SARS-CoV-2	PDB
ACE2	Angiotensin converting enzyme 2		-6,54	P59594	Pocket 1	1r42	human	PDB
S protein	Spike protein	Open	-6,5	QHD43416.1	Pocket 5	5x5b	SARS-CoV-2	Swiss-model
S protein-S2 subunit	Spike protein S2 subunit		-6,24	/	Pocket 1	6lxt	SARS-CoV-2	PDB
S protein	Spike protein	Closed	-6,18	QHD43416.1	Pocket 1	5x58	SARS-CoV-2	Swiss-model

Table 5. Interaction of alpha-cyclodextrin with ACE2, TMPRSS2 and S proteins

Target Abbreviation	Target Full Name	State	Docking score (kcal/mol)	Uniprot id / Protein Id	Pocket	Template PDB_ID	Organism	Model Source
S protein	Spike protein	Closed	-11,88	QHD43416.1	Pocket1	6vxx	SARS-CoV-2	PDB
S protein	Spike protein	Open	-11,06	QHD43416.1	Pocket2	6vyb	SARS-CoV-2	PDB
ACE2	Angiotensin converting enzyme 2		-10,92	P59594	Pocket1	1r42	human	PDB
S protein	Spike protein	Open	-9,71	QHD43416.1	Pocket1	5x5b	SARS-CoV-2	Swiss-model
TMPRSS2	Transmembrane protease serine 2		-9,42	O15393	Pocket2	-	human	Robetta
S protein	Spike protein	Closed	-8,88	QHD43416.1	Pocket1	5x58	SARS-CoV-2	Swiss-model

fever or backpain, whereas the patients that recovered in ten days were asymptomatic. Overall, the current study showed that when the infection was most active in Cyprus, the antibody and RT-qPCR tests were found negative in 50 subjects at the end of two weeks although they were at higher risk of infection because of their jobs and their exposition to COVID-19-positiveindividuals. Six patients positive for SARS-CoV-2 were included in the study, two of them used the spray and reduced viral load within five days and became negative for RT-qPCR test. The final results show that the recommended treatment with spray combination might reduce the virus load and shorten the duration of the treatment.

Furthermore, preliminary bioinformatic docking studies found that hydroxytyrosol and alpha-cyclodextrin may interact with viral and human proteins relevant for the SARS-CoV-2 entry into the cells. Therefore, besides the known effects of alpha cyclodextrin and hydroxytyrosol on the phospholipids homeostasis and the anti-viral and anti-inflammatory effects of hydroxytyrosol (4-7), alpha-cyclodextrin and hydroxyty-

rosol may exert their activity also by direct attachment to the viral particles through interaction with Spike or by interaction with the receptor ACE2 or with TMPRSS2. Therefore, we cannot exclude additional mode of action of these two molecules. More in-depth bioinformatic studies are needed to confirm these preliminary findings on the molecular interactions and test what might be the effects of these interactions. The absence of a control group and the small sample size related with the prevalence of SARS-COV-2 infections in the regional area are the main limitations of this pilot study.

It should be noted that these are promising preliminary results, however this study has some limitations. Above all there is not a control group that did not use the spray during the experimentation. The next step of our experimentation will be the administration of the spray to a wider range of people and include a control group to see whether there is a reduced infection rate of SARS-CoV-2 in the treated subjects than in the non-treated individuals. Moreover further *in vitro* study need to be performed in order to verify the mechanism of action of these two compounds.

Conflict of interest: MCE, SP, EM, AD, KD, KA, GC, HKS, MS, GT, NS, MF, GMT, MD, GF, ISG, MB, and TS are co-inventors of a food supplement, containing the two active compounds experimented in the present study, submitted to the Italian Patent Office (date: 13/10/2020, number 102020000024118).

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