European Review for Medical and Pharmacological Sciences 2021; 25 (1 Suppl): 90-100

Scoping review on the role and interactions of hydroxytyrosol and alpha-cyclodextrin in lipid-raft-mediated endocytosis of SARS-CoV-2 and bioinformatic molecular docking studies

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Abstract. – OBJECTIVE: The aim of the study was to show the effect that two naturally occurring compounds, a cyclodextrin and hydroxytyrosol, can have on the entry of SARS-CoV-2 into human cells.

MATERIALS AND METHODS: The PubMed database was searched to retrieve studies published from 2000 to 2020, satisfying the inclusion criteria. The search keywords were: SARS-CoV, SARS-CoV-2, coronavirus, lipid raft, endocytosis, hydroxytyrosol, cyclodextrin. Modeling of alpha-cyclodextrin and hydroxytyrosol were done using UCSF Chimera 1.14.

RESULTS: The search results indicated that cyclodextrins can reduce the efficiency of viral endocytosis and that hydroxytyrosol has antiviral properties. Bioinformatic docking studies showed that alpha-cyclodextrin and hydroxytyrosol, alone or in combination, interact with the viral spike protein and its host cell receptor ACE2, thereby potentially influencing the endocytosis process.

CONCLUSIONS: Hydroxytyrosol and alpha-cyclodextrin can be useful against the spread of SARS-CoV-2.

Key Words:
Hydroxytyrosol, Cyclodextrin, SARS-CoV-2, Endocytosis, Lipid raft.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus responsible for the new coronavirus disease (COVID-19) that has spread worldwide from Wuhan, China, since December 2019. The current COVID-19 outbreak is characterized by human-to-human transmission. The virus resides in the mucous membranes and is transmitted through coughing, sneezing, droplet inhalation and direct contact of contaminated hands with mouth, nose and eyes[1]. SARS-CoV-2 may persist for two days on respiratory mucous membranes in macaques before it spreads to the lower respiratory tract[2]. This two-day period provides a window for preventive and therapeutic approaches[3]. The

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virus has an average incubation of 6.4 days and a base reproduction number of 2.24-3.58[3]. Coronaviruses are enveloped viruses with a positive single-stranded RNA genome. Three viral proteins (M, E and S) are incorporated in the viral membrane. Cell infection by enveloped viruses proceeds *via* i) interaction of viral surface proteins with cell surface receptors to attach the virus to the cell surface and ii) membrane fusion triggered by a conformational change in a fusion protein[4]. SARS-CoV-2 uses the spike protein on its pericapsid to bind the host cell receptor ACE2 in lipid rafts and enter the cell by endocytosis[5]. SARS-CoV-2 has almost 80% genomic sequence homology with SARS-CoV (the coronavirus responsible for the SARS epidemic of 2003-2004)[6,7]. The aim of this study was to show the potential effects of two naturally occurring compounds, alpha-cyclodextrin and hydroxytyrosol, on SARS-CoV-2 entry into human cells.

Materials and Methods

Literature Scoping Review

The scoping review section followed PRISMA guidelines for scoping reviews.

Eligibility Criteria

Since SARS-CoV-2 and SARS-CoV are very similar, in order to understand the entry mechanism of SARS-CoV-2 and how it could be exploited to find a treatment using natural compounds, our search included original and review articles focusing on the biological cycle of coronaviruses, especially SARS-CoV-2 and SARS-CoV, and the effects of hydroxytyrosol and alpha-cyclodextrin on their infectivity. The articles had to be written in English and published in the period 2000-2020. Congress abstracts and papers not written in English and studies not relevant to the topic of the present manuscript were excluded.

Literature Sarch

The PubMed database was searched to retrieve articles published from 2000 to 2020 satisfying the inclusion criteria. The search keywords were: (((SARS-CoV) OR (SARS-CoV-2)) OR (coronavirus)) AND (lipid raft); (((SARS-CoV) OR (SARS-CoV-2)) OR (coronavirus)) AND (endocytosis); (((SARS-CoV) OR (SARS-CoV-2)) OR (coronavirus)) AND (hydroxytyrosol); (((SARS-CoV) OR (SARS-CoV-2)) OR (coronavirus)) AND (cyclodextrin). In addition, the reference lists of the papers found were scanned manually to find further relevant papers.

Study Selection

All the resulting articles were assessed independently for eligibility by authors SP and MB, who evaluated titles and abstracts according to the above inclusion criteria. Any disagreement was resolved by discussion. Papers mentioning the entry mechanism of SARS-CoV-2 or SARS-CoV and the role of hydroxytyrosol and alpha-cyclodextrin in the viral biological cycle were included in the review. Once a paper was found eligible, its references were screened to find new papers.

Bioinformatics Study

Modeling of Alpha-Cyclodextrin and Hydroxytyrosol

Modeling of alpha-cyclodextrin and hydroxytyrosol were done with the help of UCSF Chimera 1.14 to determine and visualize the interactive forces between them.

Molecular Docking and Visualization

Alpha-cyclodextrin, hydroxytyrosol and the alpha-cyclodextrin + hydroxytyrosol complex (henceforth "the complex") were docked with the spike protein (PDB ID: 6VXX) and ACE2 (PDB ID: 6M0J) using AutodockVina-based YASARA software[8]. To complete the molecular docking study, prepared receptor and ligand files were used to set target and play macros. Receptor was prepared with the help of the Prepare protein protocol of Discovery studio 4.5. Docking was performed with the specific binding site, using the active site prediction parameter of Discovery studio. Macro file dockrun_mcr was used to calculate the interaction energy between receptor and selected ligands independently. Twenty-five VINA docking runs of the ligand object 2 to the receptor object 1 was done using YASARA. Further docked complexes were saved in PDB file format for 2D-3D interactive visualization study with Discovery studio client. The resulting log files were sorted on the basis of binding energy [kcal/mol] and dissociation constant [pM]. A compound with more positive binding energies has stronger binding, and negative energies indicate no binding[8].

Results

Literature Review

Based on the search criteria, 199 papers published in English from 2000 to 2020 were included, 27 of which were duplicates. Of the remaining 172 articles, only 84 pertained to the topic of the current review and were read completely. After reading, 10 papers were included in the present review. Another 38 papers were included after reading the references of the 10 articles, as shown in the flow diagram (Figure 1).

Mechanism of Viral Entry

We now describe the biological cycle of SARS-CoV in relation to SARS-CoV-2. Since SARS-CoV appeared earlier, more studies have been performed on it, and therefore, more is known about it. In addition, given its high genomic similarity to SARS-CoV-2, SARS-CoV life cycle may overlap with that of SARS-CoV-2.

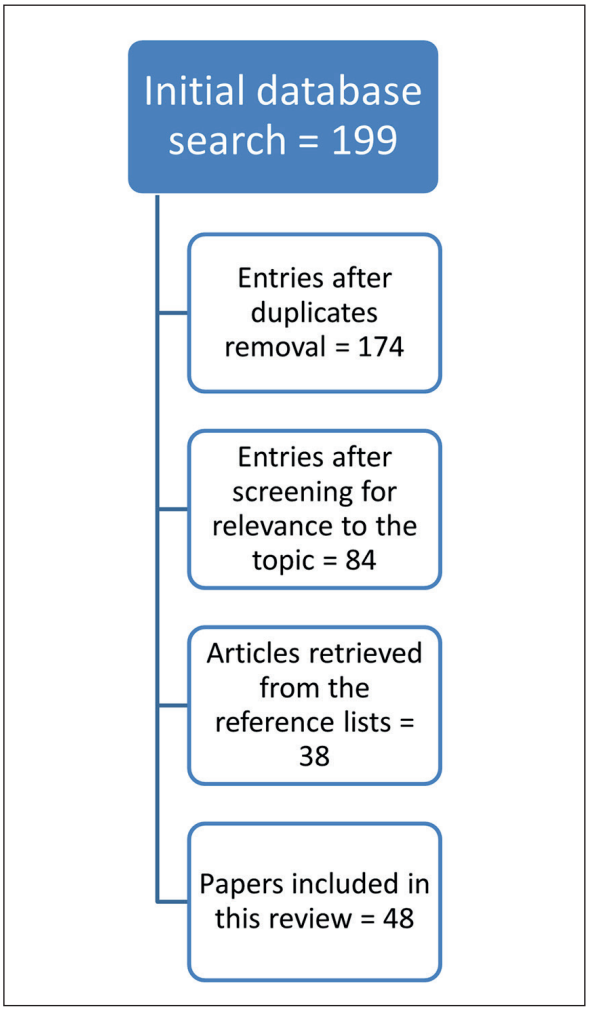

Figure 1. Flow diagram of literature search.

Entry of SARS-CoV into permissive cells is mediated by binding of its spike protein to a cell receptor. In most cases, ACE2 is the major receptor contributing to entry of SARS-CoV. ACE2 is expressed abundantly on the surface of lung and intestinal epithelial cells. It also localizes predominately where lipid rafts are more concentrated. In the specific case of epithelial cells, lipid rafts are located in the apical region[9]. The importance of lipid rafts was confirmed in a study in Vero E6 cells that showed that lipid rafts are involved in the entry of SARS-CoV[9]. An infectivity assay also showed that integrity of lipid rafts is required for productive infection by pseudo-typed SARS-CoV[10]. SARS-CoV can enter host cells by endosomal or non-endosomal pathways, depending on the presence of proteases. In either case, only when the spike protein is bound to its receptor and cleaved into S1 and S2 by proteases, fusion between viral and cell membranes takes place[9,11]. Consistently with the fact that SARS-CoV and SARS-CoV-2 S proteins both contain highly conserved amino acid residues essential for ACE2 receptor binding[12], the two viruses share the same entry mechanism[13].

Lipid Rafts and Their Role in Viral Entry

The infectivity of coronaviruses depends on the lipid composition of host cell membranes. For example, infection by the Semliki Forest virus requires both cholesterol and sphingolipids. Retroviruses and filoviruses use cholesterol-rich membrane microdomains as platforms for assembly and/or cell entry[14]. These lipid microdomains are called lipid rafts and serve as entry sites for certain viruses.

Lipid rafts are rich in cholesterol and sphingolipids and concentrate membrane-associated proteins, including receptors and signaling molecules[9]. In polarized cells, lipid rafts are concentrated at the apical surface, whereas in non-polarized cells they are dispersed over the cell surface. There is evidence that lipid rafts are involved in coronavirus entry. In the case of human coronavirus 229E, viral entry was inhibited by depletion of cholesterol, disrupting viral association with the cell receptor, CD13[9]. Thorp and Gallagher showed that lipid rafts were crucial for

entry of mouse hepatitis virus[14]. An early step in the entry process of SARS-CoV into target cells is initiated by engagement of the cell receptor, angiotensin-converting enzyme 2 (ACE2), by the spike glycoprotein. SARS-CoV receptor ACE2 is found in lipid rafts of Vero E6 cells. Productive entry of SARS-CoV into Vero E6 cells requires intact lipid rafts[15]. In the case of SARS-CoV, lipid rafts provide a convenient platform to concentrate receptor-ACE2 in clusters on the host cell membrane, so as to dock it efficiently with the spike protein on the viral envelope[16,17]. This mechanism is identical to that of SARS-CoV-2[18].

Cholesterol Supplementation and Depletion

Cholesterol supplementation increases the cytopathic effects of murine hepatitis virus, an enveloped coronavirus, in tissue culture, and intensifies its pathogenicity *in vivo*. To investigate this phenomenon, the researcher used growth media enriched with methyl cyclodextrin or cholesterol to reduce or elevate cell membrane sterols, respectively[14]. These variable cholesterol levels were directly correlated with spike protein-mediated membrane fusion activity. Thus, cholesterol is considered an essential membrane fusion cofactor[14]. Receptors for several cholesterol-dependent viruses appear to reside largely in rafts[19]. For several other coronaviruses, infectious bronchitis virus, human coronavirus 229E, SARS-CoV and SARS-CoV-2, it has been shown[5,14,20] that cholesterol depletion in the plasma membrane of target cells reduces the efficiency of infection.

Manipulating the Cell Membrane

The outer membrane leaflet of mammalian cells is composed primarily of sphingomyelin and phosphatidylcholine. In contrast, the inner or cytoplasmic leaflet consists mostly of aminophospholipids[21]. Asymmetric arrangement of lipids in the cell membrane affects various biological properties, such as membrane permeability, membrane potential, surface charge, the mechanical stability of membranes and membrane shape. The possibility of manipulating the lipid composition of living cell membranes could therefore be useful in the prevention of cell membrane-mediated pathological diseases[21].

Alpha-Cyclodextrin

Changing lipid composition by lipid exchangers, like cyclodextrins, is an interesting mechanism. Cyclodextrins are natural compounds that may be produced by bacteria[22]. They are sugar rings, hydrophilic on the outside and lipophilic on the inside, and are classified as α-cyclodextrins (six sugars), β-cyclodextrins (seven sugars) and γ-cyclodextrins (eight sugars). Alpha- and β-cyclodextrins scavenge phospholipids from the plasma membrane[23-25]. Alpha-cyclodextrins are considered safe and have been approved as an excipient/food for special medical purposes in the EU[26].

Some researchers have exploited cyclodextrins to carry out efficient outer leaflet lipid exchange in membrane vesicle models, replacing the entire outer leaflet with exogenous lipids *in-situ* by means of hydroxypropyl-α-cyclodextrin, without disturbing the lipid composition of the inner leaflet[27,28]. Another study[29,30] established a procedure to efficiently exchange phospholipids and sphingolipids in the plasma membrane outer leaflet of living mammalian cells with exogenous lipids, by means of lipid-loaded methyl-α-cyclodextrin. After this exchange, the membranes had an asymmetric distribution of lipids[21].

Alpha-cyclodextrins are also able to reduce serum concentrations of phospholipids. Phospholipids serve dual functions, as they activate endocytosis and participate in the regulation of phospholipid metabolism in cell membranes[31]. Serum phospholipids are involved in variations in the endo/exocytosis pathway, as well as in variations in phospholipid influx into the phosphoinositide system, which controls endo/exocytosis. Reducing serum phospholipids reduces the activity of the phosphoinositide system. Since coronaviruses enter host cells *via* endocytosis and α-cyclodextrin scavenges serum phospholipids (substrate of the phosphatidyl-inositol system that regulates endocytosis), viral endocytosis may be impaired by means of α-cyclodextrins[26,31].

Hydroxytyrosol

Hydroxytyrosol is a small (molecular weight 153) natural phenolic compound found in olives and their derivatives. It has strong antioxidant activity[32]. Hydroxytyrosol can also have antiviral activity. Certain research studies[33] suggest that the mechanism of this effect might require the presence of a viral envelope.

Hydroxytyrosol is able to inactivate influenza A viruses, including H1N1, H3N2, H5N1 and H9N2 subtypes. Hydroxytyrosol is considered to be useful against viruses with type I transmembrane envelope glycoproteins and effectively lowers the titer of these viruses in a dose-depen-

dent manner. The viral envelope is presumably involved in the hydroxytyrosol antiviral mechanism. The influenza virus protein has a binding site for hydroxytyrosol in the domain essential for cell entry, which means that hydroxytyrosol affects the influenza virus. The results obtained in this study also suggest that the antiviral effect of hydroxytyrosol on the H9N2 virus can be attributed to the direct effect of hydroxytyrosol on the virus[33]. The morphology of the hydroxytyrosol-treated H9N2 virus has been analyzed under the electron microscope. The results suggested that the structure of the viral envelope could be disrupted by hydroxytyrosol. Suppression of viral mRNA synthesis and lack of viral nucleoprotein observed in cells inoculated with hydroxytyrosol-treated H9N2 virus may be the result of poor viral binding, viral uncoating or problems with other steps of viral infection[33].

Hydroxytyrosol also interacts with the conserved hydrophobic pocket on the surface of the central trimeric coiled-coil of HIV-gp41 fusion complex, the six helical bundle and the catalytic core domain of the HIV-1 integrase active site[34]. It shows dose-dependent inhibition of HIV-1 integrase. Hydroxytyrosol is not cytotoxic in the effective dose ranges[34,35]. It acts against viral entry and integration outside and inside the cell environment. These studies suggest that hydroxytyrosol may be useful against other viruses with type I transmembrane envelope glycoprotein, including SARS-CoV, respiratory syncytial virus, Ebola virus, measles virus and avian flu virus[34,36].

SARS-CoV-2 also recognizes another receptor, the cell-surface heat shock protein A5 (HSPA5). On infection with the virus, HSPA5 is upregulated and translocates to the cell membrane where it binds the spike protein. *In silico* studies show that hydroxytyrosol is able to bind HSPA5 and possibly inhibits entry of SARS-CoV-2 into the cell[37].

Studies[38-40] of unilamellar membranes have revealed that hydroxytyrosol remains at the lipid membrane surface, almost parallel to the bilayer[38]. Its incorporation into the membrane may cause a concavity in the membrane surface[39,40].

Furthermore, the ability of hydroxytyrosol to decrease serum lipids and cholesterol is considered to be significant in the lowering of plasma membrane cholesterol and could lead to changes in plasma membrane structure and composition. This, in turn, is thought to affect entry of enveloped viruses through the plasma membrane[32]. Further studies are required to confirm these observations.

Molecular Docking Studies

Modeling of Alpha-Cyclodextrin and Hydroxytyrosol

To complete our study, we used UCSF Chimera 1.14 to view the interaction between alpha-cyclodextrin and hydroxytyrosol. We found that hydroxytyrosol interacts with only one side of alpha-cyclodextrin (Figure 2). The complex was created by overlapping the two molecules by means of the software.

Molecular Docking and Visualization

The YASARA Z-score of a molecular docking study revealed that alpha-cyclodextrin, hydroxytyrosol and the complex showed significant binding affinity with the spike protein and ACE2 receptor (Table I). The high binding energy and dissociation constant showed that the docked complex is stable[8]. The binding energy suggested that alpha-cyclodextrin binds more strongly with both targets than hydroxytyrosol.

Molecular Docking and Visualization Study of Alpha-Cyclodextrin with Spike Protein

YASARA scoring indicated that molecular docking of alpha-cyclodextrin with the spike protein (PDB ID: 6VXX) had a significant binding affinity of 6.40 kcal/mol. Alpha-cyclodextrin showed different 3D-2D interactions with the spike protein. It formed conventional and carbon-hydrogen

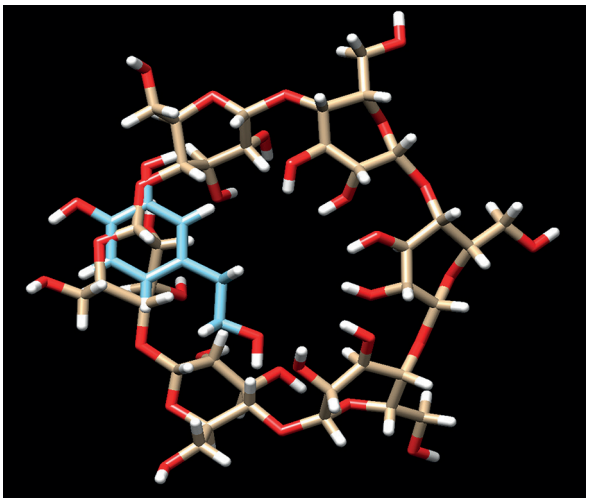

Figure 2. Modelling of alpha-cyclodextrin and hydroxytyrosol (hydroxytyrosol in blue).

Table I. Binding energy of α-cyclodextrin and hydroxytyrosol for Spike protein and ACE2 receptor.

Compounds	Target molecules	
	Spike protein (PDB ID: 6VXX)	ACE2 (PDB ID: 6M0J)
	Binding energy (kcal/mol)	
α-cyclodextrin	6.40	7.91
Hydroxytyrosol	6.41	6.10
Complex X (α-cyclodextrin + hydroxytyrosol)	6.37	7.88

bonding with residues Thr286, Asp287, Lys278, Gly219 and Thr602. The other residues showed Van der Waals interactions (Figure 3).

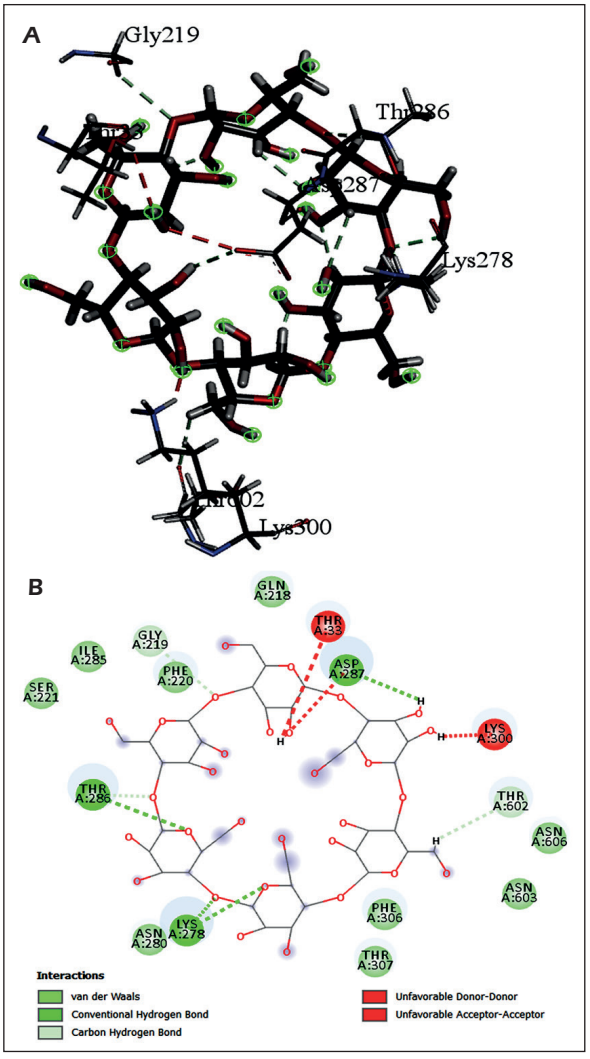

Figure 3. A, 3D interaction of alpha-cyclodextrin with spike protein (PDB ID: 6VXX); **B,** 2D interaction of alpha-cyclodextrin with spike protein (PDB ID: 6VXX).

Molecular Docking and Visualization Study of Hydroxytyrosol with Spike Protein

The molecular docking study of hydroxytyrosol with the spike protein (PDB ID: 6VXX) showed a significant binding affinity of 6.41 kcal/mol. Hydroxytyrosol showed different 3D-2D interactions with the spike protein. It formed conventional hydrogen bonding with Ala 1056 and Thr 778, an π-alkyl interaction with Ile 870 and the remaining residues showed Van der Waals interactions (Figure 4).

Molecular Docking and Visualization Study of Alpha-Cyclodextrin with ACE2

The molecular docking study of alpha-cyclodextrin with ACE2 (PDB ID: 6M0J) showed a significant binding affinity of 7.91 kcal/mol. Alpha-cyclodextrin showed various 3D-2D interactions with the spike protein. It formed conventional and carbon-hydrogen bonding with Asn 394, Asp 206, Leu 73, Asn 77, Lys 74, Asn 103 and Asp 509. The remaining residues formed Van der Waals interactions (Figure 5a, b).

Molecular Docking and Visualization Study of Hydroxytyrosol with ACE2

The molecular docking study of hydroxytyrosol with ACE2 (PDB ID: 6M0J) showed a significant binding affinity of 6.10 kcal/mol. Hydroxytyrosol showed different 3D-2D interactions with the spike protein. It formed conventional and carbon-hydrogen bonding with Ile 291 and Thr 434, an π-alkyl interaction with residue Pro 415, π-π stacking with residue Phe438, and the remaining residues showed Van der Waals interactions (Figure 6).

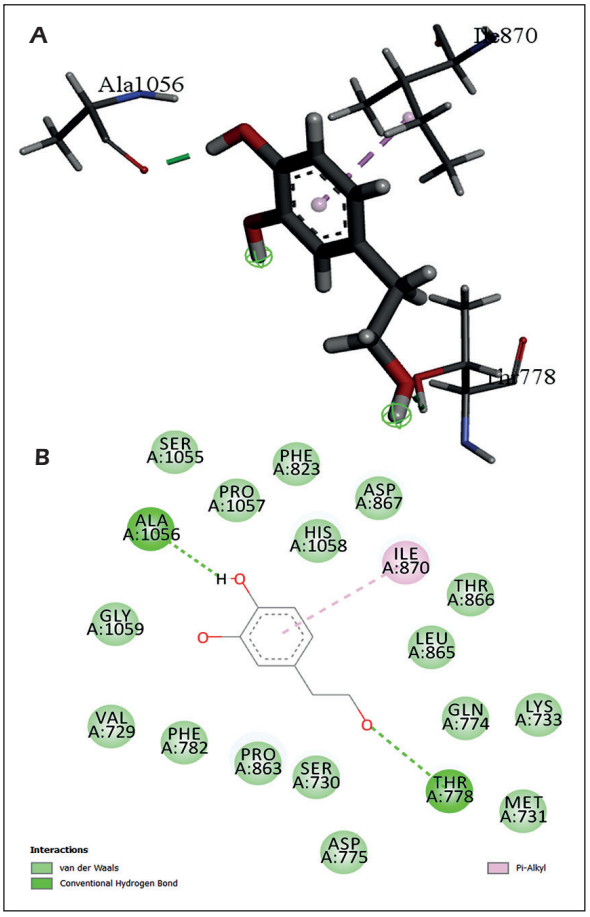

Figure 4. A, 3D interaction of hydroxytyrosol with spike protein (PDB ID: 6VXX); **B,** 2D interaction of hydroxytyrosol with spike protein (PDB ID: 6VXX).

Molecular Docking and Visualization Study of the Complex (Alpha-Cyclodextrin + Hydroxytyrosol) with the Spike Protein

A molecular docking study of the complex (alpha-cyclodextrin + hydroxytyrosol) with the spike protein (PDB ID: 6VXX) showed that its binding energy (6.37 kcal/mol) was very close to those of alpha-cyclodextrin and hydroxytyrosol. It showed a binding energy of 6.37 kcal/mol. It showed different 2D-3D interactions with the receptor protein: conventional and carbon-hydrogen bonding with Lys 535, Leu 533, Asn 532 and Gln 580, and Van der Waals interactions with the other residues (Figure 7).

Molecular Docking and Visualization Study of the Complex (Alpha-Cyclodextrin+ Hydroxytyrosol) with ACE2

Analysis of the docking position of hydroxytyrosol to α-cyclodextrin demonstrated a potential interaction between the two compounds[41-43]. The molecular docking study of the complex (alpha-cyclodextrin + hydroxytyrosol) with ACE2 (PDB ID: 6M0J) showed a significantly higher binding energy of 7.88 kcal/mol than those of the individual compounds. It also showed various 2D-3D interactions with the receptor protein: conventional and carbon-hydrogen bonds with residues Ser 44, Ser 47, Ala 348, Asp 350, Glu 402, His 345 and Asn51 and Van der Waals interactions with the remaining residues (Figure 8).

In conclusion, molecular docking studies of alpha-cyclodextrin to the viral protein (spike protein) and the host-receptor protein (ACE2)

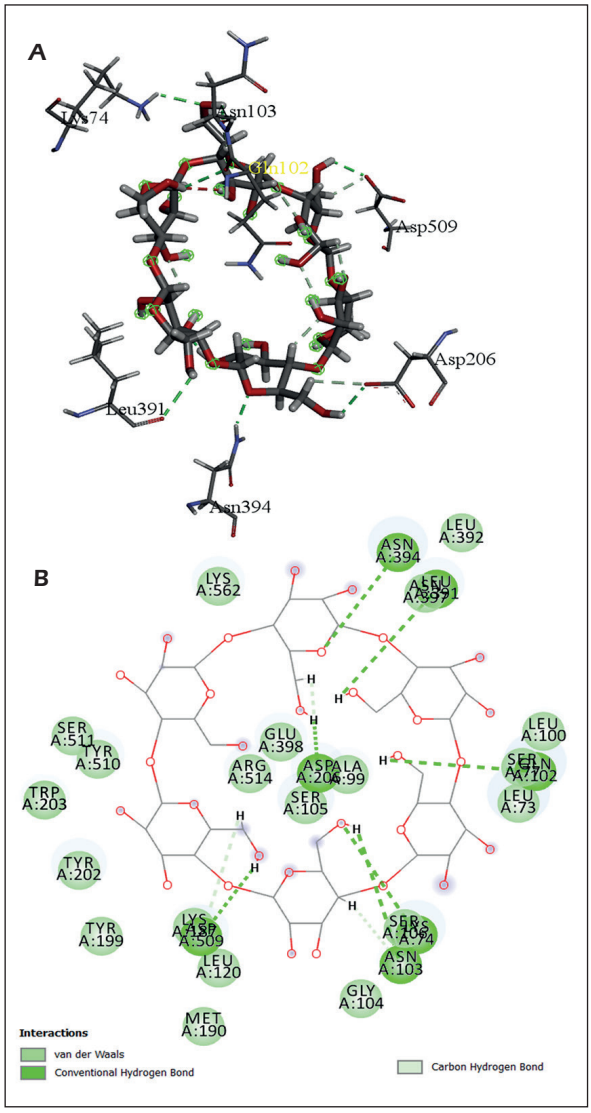

Figure 5. A, 3D interaction of alpha-cyclodextrin with ACE2 (PDB ID: 6M0J); **B,** 2D interaction of alpha-cyclodextrin with ACE2 (PDB ID: 6M0J).

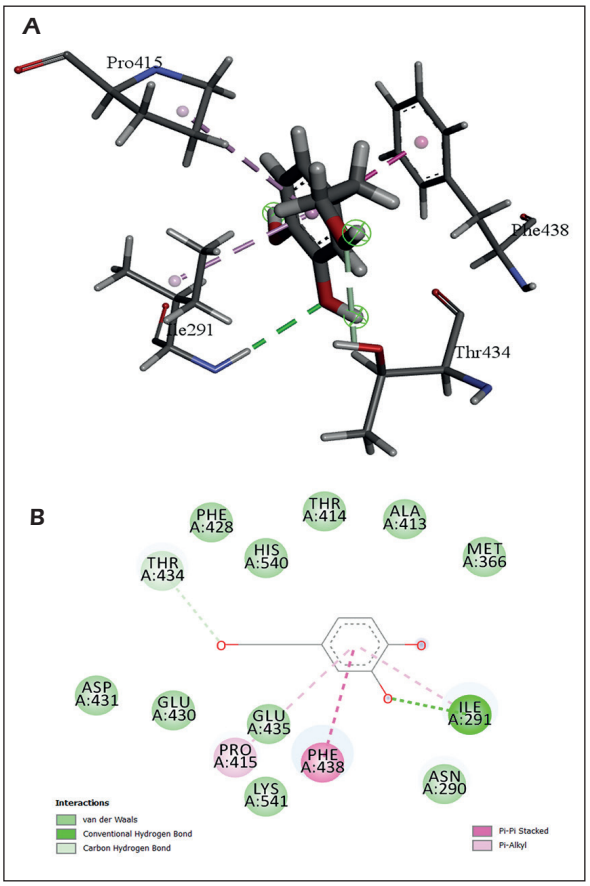

Figure 6. A, 3D interaction of hydroxytyrosol with ACE2 (PDB ID: 6M0J); **B,** 2D interaction of hydroxytyrosol with ACE2 (PDB ID: 6M0J).

showed significantly higher binding affinities than those of hydroxytyrosol to the same proteins. The binding energies of alpha-cyclodextrin and hydroxytyrosol to ACE2 were 7.91 kcal/mol and 6.10 kcal/mol, respectively. The corresponding binding energies to the spike protein were 6.40 kcal/mol and 6.41 kcal/mol, respectively. In conclusion, both compounds could inhibit the two targets by virtue of their significant binding affinities, alpha-cyclodextrin being the better inhibitor for both target proteins.

Other Possible Interactions

Other possible allosteric sites which could have disruptive effects could be considered but would require further characterization to be conclusive. Such sites are the heptad repeat 1 and 2 domains of the spike protein. Hydroxytyrosol and alpha-cyclodextrin could both dock to the S2 region of S protein, using the heptad repeat 1/2 domains as potential binding sites. Hydroxytyrosol and alpha-cyclodextrin both bind to the S2 region with docking scores of -6.2826 kcal/mol and -6.0023 kcal/mol, respectively. They could therefore dock to the S2 region of S protein, inhibiting it from achieving its active post-fusion conformation, which is required for viral reproduction to begin. However, extensive validation will be required to confirm the binding activity of hydroxytyrosol and alpha-cyclodextrin to proteins responsible for entry of SARS-CoV-2 into host cells.

Interestingly, a short docking run showed that sphingosine was incorporated into alpha-cyclodextrin, but its docking score was not high, al-

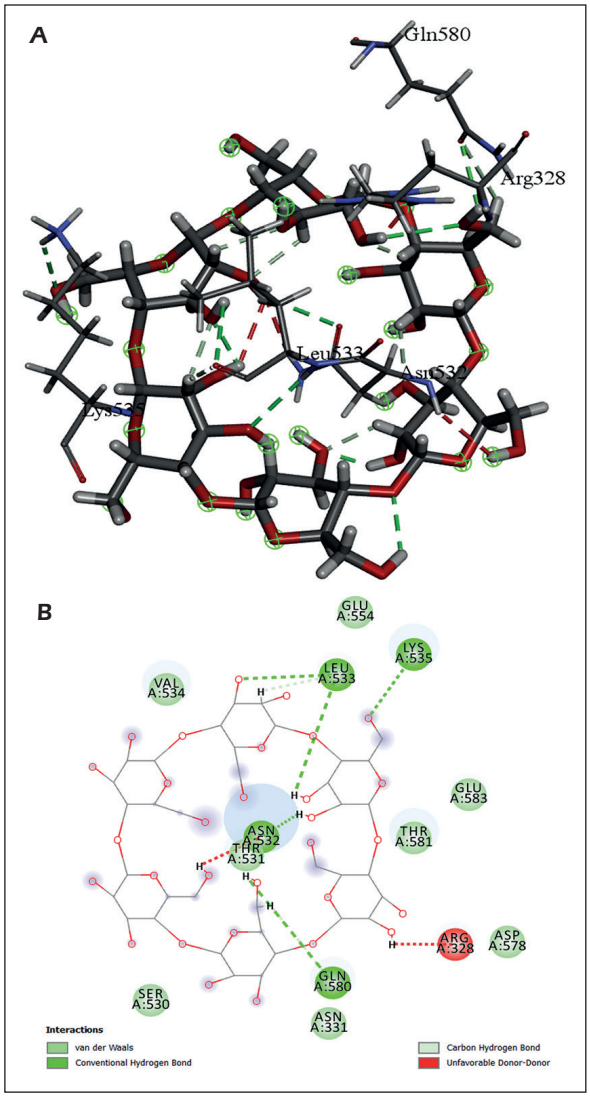

Figure 7. A, 3D interaction of the complex (alpha-cyclodextrin + hydroxytyrosol**)** with the spike protein (PDB ID: 6VXX); **B,** 2D interaction diagram of the complex **(**alpha-cyclodextrin + hydroxytyrosol) with the spike protein (PDB ID: 6VXX).

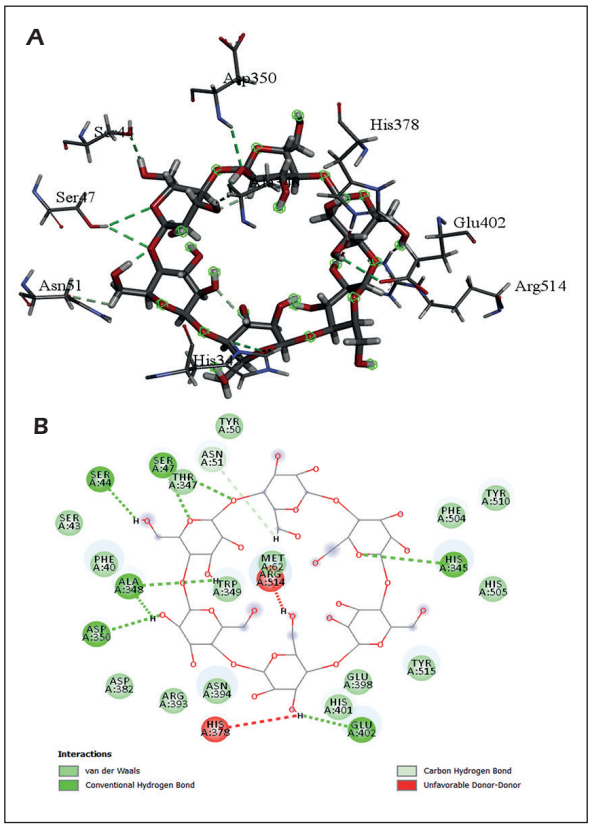

Figure 8. A, 3D interaction of the complex (alpha-cyclodextrin + hydroxytyrosol) with ACE2 (PDB ID: 6M0J); **B,** 2D interaction of the complex (alpha-cyclodextrin + hydroxytyrosol) with ACE2 (PDB ID: 6M0J).

though this could be due to its elongated chain. Alpha-cyclodextrin does indeed interact with sphingolipids, but the thermodynamic stability of the resulting complex is higher with two molecules of α-cyclodextrin[21,44-46].

Discussion

On the basis of this literature review, we developed a complex containing alpha-cyclodextrin and hydroxytyrosol with the purpose of inhibiting entry of SARS-CoV-2 into human cells. Our bioinformatic studies suggest that hydroxytyrosol and alpha-cyclodextrin bind simultaneously to the viral spike protein and the host receptor-protein, ACE2. These findings may support the earlier claims of the authors, based on their clinical results using a spray containing these two natural compounds. The compounds form physical bonds with each other, and the resulting complex is able to bind both proteins.

In previous studies, the mixture containing alpha-cyclodextrin and hydroxytyrosol was first tested for side effects: after using the spray for a week, none of the volunteers showed any side effects. The spray was then tested on a small cohort of six patients whose Real Time PCR swab test was positive for SARS-CoV-2. The two who were given the spray turned negative in five days, while the other four patients turned negative after ten days. In this way, we obtained preliminary confirmation of the safety of the complex and its efficacy against infection by SARS-CoV-2[47,48].

Conclusions

A search of the literature produced two promising naturally occurring compounds that could be useful to inhibit or prevent SARS-CoV-2 infection. The compounds were hydroxytyrosol and alpha-cyclodextrin. Alpha-cyclodextrin may interact with the plasma membrane and modifies its composition by scavenging sphingolipids. Hydroxytyrosol may interact with the viral and the cell membrane and may induce morphological changes in the viral envelope. Hydroxytyrosol is captured in the hydrophobic cavity of alpha-cyclodextrin. The resulting complex has a number of potential activities according to the literature and as confirmed by our *in-silico* studies.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Conflict of Interest

The authors have submitted a patent application to the Italian Patent Office for a product based on hydroxytyrosol and alpha-cyclodextrin.

References

1) Peng X, Xu X, Li Y, Cheng L, Zhou X, Ren B. Transmission routes of 2019-nCoV and controls in dental practice. Int J Oral Sci 2020; 12: 9.
2) Liu L, Wei Q, Nishiura K, Peng J, Wang H, Midkiff C, Alvarez X, Qin C, Lackner A, Chen Z. Spatiotemporal interplay of severe acute respiratory syndrome coronavirus and respiratory mucosal cells drives viral dissemination in rhesus macaques. Mucosal Immunol 2016; 9: 1089-1101.
3) Caruso AA, Del Prete A, Lazzarino AI, Capaldi R, Grumetto L. Might hydrogen peroxide reduce the

hospitalization rate and complications of SARS-CoV-2 infection? Infect Control Hosp Epidemiol 2020; 41: 1360-1361.

4) Marsh M, Helenius A. Virus entry: Open sesame. Cell 2006; 124: 729-740.
5) Glende J, Schwegmann-Wessels C, Al-Falah M, Pfefferle S, Qu X, Deng H, Drosten C, Naim HY, Herrler G. Importance of cholesterol-rich membrane microdomains in the interaction of the S protein of SARS-coronavirus with the cellular receptor angiotensin-converting enzyme 2. Virology 2008; 381: 215-221.
6) Wang H, Li X, Li T, Zhang S, Wang L, Wu X, Liu J. The genetic sequence, origin, and diagnosis of SARS-CoV-2. Eur J ClinMicrobiol Infect Dis 2020; 39: 1629-1635.
7) Dallavilla T, Bertelli M, Morresi A, Bushati V, Stuppia L, Beccari T, Chiurazzi P, Marceddu G. Bioinformatic analysis indicates that SARS-CoV-2 is unrelated to known artificial coronaviruses. Eur Rev Med Pharmacol Sci 2020; 24: 4558-4564.
8) Trott O, Olson AJ. AutoDock VINA: Improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J Comput Chem 2010; 31: 455-461.
9) Lu Y, Liu DX, Tam JP. Lipid rafts are involved in SARS-CoV entry into Vero E6 cells. Biochem Biophys Res Commun 2008; 369: 344-349.
10) Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997; 387: 569-572.
11) Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, Bates P. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci USA 2005; 102: 11876-11881.
12) Li F, Li W, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science 2005; 309: 1864-1868.
13) Chambers JP, Yu J, Valdes JJ, Arulanandam BP. SARS-CoV-2, early entry events. J Pathog 2020; 2020: 9238696.
14) Thorp EB, Gallagher TM. Requirements for CEACAMs and cholesterol during murine coronavirus cell entry. J Virol 2004; 78: 2682-2692.
15) Pralle A, Keller P, Florin EL, Simons K, Hörber JK. Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol 2000; 148: 997-1008.
16) Nomura R, Kiyota A, Suzaki E, Kataoka K, Ohe Y, Miyamoto K, Senda T, Fujimoto T. Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. J Virol 2004; 78: 8701-8708.
17) Jeffers SA, Tusell SM, Gillim-Ross L, Hemmila EM, Achenbach JE, Babcock GJ, Thomas WD Jr, Thackray LB, Young MD, Mason RJ, Ambrosino DM, Wentworth DE, Demartini JC, Holmes KV. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. Proc Natl Acad Sci USA 2004; 101: 15748-15753.
18) Sviridov D, Miller YI, Ballout RA, Remaley AT, Bukrinsky M. Targeting lipid rafts - A potential therapy for COVID-19. Front Immunol 2020; 11: 574508.
19) Krueger DK, Kelly SM, Lewicki DN, Ruffolo R, Gallagher TM. Variations in disparate regions of the murine coronavirus spike protein impact the initiation of membrane fusion. J Virol 2001; 75: 2792-2802.
20) Wang H, Yuan Z, Pavel MA, Hansen SB. The role of high cholesterol in age-related COVID19 lethality. bioRxiv 2020: 2020.05.09.086249. doi:10.1101/2020.05.09.086249.
21) Li G, Kim J, Huang Z, St Clair JR, Brown DA, London E. Efficient replacement of plasma membrane outer leaflet phospholipids and sphingolipids in cells with exogenous lipids. Proc Natl Acad Sci USA 2016; 113: 14025-14030.
22) Fenyvesi E, Szente L. Nanotechnology in the agri-food industry, encapsulations. Academic Press, 2016.
23) Wittkowski KM, Dadurian C, Seybold MP, Kim HS, Hoshino A, Lyden D. Correction: Complex polymorphisms in endocytosis genes suggest alpha-cyclodextrin as a treatment for breast cancer. PLoS One 2019; 14: e0214826.
24) Baglivo M, Baronio M, Natalini G, Beccari T, Chiurazzi P, Fulcheri E, Petralia PP, Michelini S, Fiorentini G, Miggiano GA, Morresi A, Tonini G, Bertelli M. Natural small molecules as inhibitors of coronavirus lipid-dependent attachment to host cells: A possible strategy for reducing SARS-COV-2 infectivity? Acta Biomed 2020; 91: 161-164.
25) Kiani AK, Dhuli K, Anpilogov K, Bressan S, Dautaj A, Dundar M, Beccari T, Ergoren MC, Bertelli M. Natural compounds as inhibitors of SARS-CoV-2 endocytosis: A promising approach against COVID-19. Acta Biomed 2020; 91: e2020008.
26) Wittkowski KM. Use of cyclodextrins to reduce endocytosis in malignant and neurodegenerative disorders. Google Patents, 2020.
27) Vitrac H, MacLean DM, Jayaraman V, Bogdanov M, Dowhan W. Dynamic membrane protein topological switching upon changes in phospholipid environment. Proc Natl Acad Sci USA 2015; 112: 13874-13879.
28) Heberle FA, Marquardt D, Doktorova M, Geier B, Standaert RF, Heftberger P, Kollmitzer B, Nickels JD, Dick RA, Feigenson GW, Katsaras J, London E, Pabst G. Subnanometer structure of an asymmetric model membrane: interleaflet coupling influences domain properties. Langmuir 2016; 32: 5195-5200.
29) Lin Q, London E. Preparation of artificial plasma membrane mimicking vesicles with lipid asymmetry. PLoS One 2014; 9: e87903.
30) Son M, London E. The dependence of lipid asymmetry upon polar headgroup structure. J Lipid Res 2013; 54: 3385-3393.
31) Wittkowski KM. Use of cyclodextrins in diseases and disorders involving phospholipid dysregulation. Google Patents, 2020.

32) Jemai H, Fki I, Bouaziz M, Bouallagui Z, El Feki A, Isoda H, Sayadi S. Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves in cholesterol-fed rats. J Agric Food Chem 2008; 56: 2630-2636.

33) Yamada K, Ogawa H, Hara A, Yoshida Y, Yonezawa Y, Karibe K, Nghia VB, Yoshimura H, Yamamoto Y, Yamada M, Nakamura K, Imai K. Mechanism of the antiviral effect of hydroxytyrosol on influenza virus appears to involve morphological change of the virus. Antiviral Res 2009; 83: 35-44.

34) Lee-Huang S, Huang PL, Zhang D, Lee JW, Bao J, Sun Y, Chang YT, Zhang J, Huang PL. Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: Part I. Fusion [corrected] inhibition. Biochem Biophys Res Commun 2007; 354: 872-878.

35) Lee-Huang S, Huang PL, Zhang D, Lee JW, Bao J, Sun Y, Chang YT, Zhang J, Huang PL. Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: part II. Integrase inhibition. Biochem Biophys Res Commun 2007; 354: 879-884.

36) Wei DQ, Du QS, Sun H, Chou KC. Insights from modeling the 3D structure of H5N1 influenza virus neuraminidase and its binding interactions with ligands. Biochem Biophys Res Commun 2006; 344: 1048-1055.

37) Elfiky AA. Natural products may interfere with SARS-CoV-2 attachment to the host cell. J Biomol Struct Dyn. 2020 May 5:1-10. doi: 10.1080/07391102.2020.1761881. Epub ahead of print

38) Lopez S, Bermudez B, Montserrat-de la Paz S, Jaramillo S, Varela LM, Ortega-Gomez A, Abia R, Muriana FJ. Membrane composition and dynamics: A target of bioactive virgin olive oil constituents. Biochim Biophys Acta 2014; 1838: 1638-1656.

39) Li J, Yang G, Wang S, Jiang L, Liu X, Geng C, Zhong L, Chen M. The protective effects of hydroxytyrosol against ortho-phenylphenol-induced DNA damage in HepG2 cells. Toxicol Mech Methods 2012; 22: 432-437.

40) Zou X, Feng Z, Li Y, Wang Y, Wertz K, Weber P, Fu Y, Liu J. Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: Activation of Nrf2 and JNK-p62/SQSTM1 pathways. J Nutr Biochem 2012; 23: 994-1006.

41) Liu Z, Nalluri SKM, Stoddart JF. Surveying macrocyclic chemistry: from flexible crown ethers to rigid cyclophanes. Chem Soc Rev 2017; 46: 2459-2478.

42) Angelova S, Nikolova V, Pereva S, Spassov T, Dudev T. α-Cyclodextrin: How effectively can its hydrophobic cavity be hydrated? J Phys Chem B 2017; 121:9260-9267.

43) Aree T, Jongrungruangchok S. Structure–antioxidant activity relationship of β-cyclodextrin inclusion complexes with olive tyrosol, hydroxytyrosol and oleuropein: Deep insights from X-ray analysis, DFT calculation and DPPH assay. Carbohydrate Polymers 2018; 199: 661-669.

44) Fauvelle F, Debouzy JC, Crouzy S, Göschl M, Chapron Y. Mechanism of alpha-cyclodextrin-induced hemolysis. 1. The two-step extraction of phosphatidylinositol from the membrane. J Pharm Sci 1997; 86: 935-943.

45) Bernat V, Ringard-Lefebvre C, Le Bas G, Lesieur S. Action of α-cyclodextrin on phospholipid assemblies. J Incl Phenom Macrocycl Chem 2007; 57: 113-119.

46) Cai W, Yu Y, Shao X. Studies on the interaction of α-cyclodextrin with phospholipid by a flexible docking algorithm. Chemometr Intell Lab Syst 2006; 82: 260-268.

47) Ergoren MC, Paolacci S, Manara E, Dautaj A, Dhuli K, Anpilogov K, Camilleri G, Suer HK, Sayan M, Tuncel G, Sultanoglu N, Farronato M, Tartaglia GM, Dundar M, Farronato G, Gunsel IS, Bertelli M, Sanlidag T. A pilot study on the preventative potential of alpha-cyclodextrin and hydroxytyrosol against SARS-CoV-2 transmission. Acta Biomed 2020; 91: e2020022.

48) Paolacci S, Ceccarini MR, Codini M, Manara E, Tezzele S, Percio M, Capodicasa N, Kroni D, Dundar M, Ergoren MC, Sanlidag T, Beccari T, Farronato M, Farronato G, Tartaglia GM, Bertelli M. Pilot study for the evaluation of safety profile of a potential inhibitor of SARS-CoV-2 endocytosis. Acta Biomed 2020; 91: e2020009.