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Covid-19 and Flavonoids: *In silico* Molecular Dynamics Docking to the Active Catalytic Site of SARS-CoV and SARS-CoV-2 Main Protease

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Abstract

Inhibition of the main protease 3CLpro of SARS-CoV and SARS-CoV-2 is being targeted in the search for antivirals to shorten patient recovery times from Covid-19 disease. We investigated 72 flavonoids for their potential to bind with the active catalytic site of 3CLpro for SARS-CoV and SARS-CoV-2. *In silico* molecular dynamics docking was performed using energy-minimized states of the flavonoids. Three variants of the active catalytic site of the protease 3CLpro were used: one based on x-ray crystallography for SARS-CoV, the second based on x-ray crystallography for SARS-CoV-2, and the third based on a 3D-modeled form of an amino acid sequence alignment of SARS-CoV-2 3CLpro from 8 humans. Docking involved characterization of the best putative pose in the "pocket" of the active site based on altering rotatable bonds in each molecule. The binding energy (kcal/mol) and number of hydrogen bonds were assessed during each pose. Mean binding energy across the 3 variants of 3CLpro was sorted in ascending order to rank each flavonoid, since more negative values indicate stronger binding. The top 10 flavonoids identified were amentoflavone, gallocatechin gallate, diosmin, epigallocatechin gallate, hidrosmin, catechin gallate, elsamitrucin, pectolinaren, quercetin, and isoquercetin. Other flavonoids investigated with significant binding energies were hesperidin, rutin, rhoifolin, and peurarin. In vivo animal research is now needed for evaluating whether these flavonoids can minimize early infection and alleviate symptoms and shorten recovery times for late-stage Covid-19 disease. Human prevention trials for minimizing early infection and combination therapy trials with antivirals for shortening recovery times in late-stage Covid-19 disease could also be pursued.

Keywords: *Docking, SARS-CoV-2, SARS-CoV, Covid-19, Drug discovery, Repurposing, Chemoinformatics, Toxicology, Absorption, Distribution, Metabolism, Excretion, ADME*

1. Introduction

The Covid-19 pandemic that originated in Wuhan China has now spread worldwide and has become an international pandemic (Zhai *et al.*, 2020). Human-to-human transmission has been confirmed to be based on salivary or airway droplets, contaminated hands, and surfaces(Park *et al.*, 2020). Infection can lead to severe respiratory illness following an incubation time of 2-14 days (Ahn *et al.*, 2020). Worldwide prevention measures include cancellation of public

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sporting events and ceremonies, and closure of non-essential business offices, schools, universities, child-care facilities, restaurants and pubs, and sheltering at home. In addition, hospitals and clinics have been instructed to perform only non-elective (emergency) medical procedures (Ahn *et al.*, 2020). Medical treatment for Covid-19 includes early diagnosis, quarantine, and supportive treatments (Huang *et al.*, 2020).

Historically, the 2003 Severe Acute Respiratory Syndrome (SARS) epidemic resulted in 8400 SARS cases and approximately 800 deaths (Ziebuhr *et al.*, 2004). The SARS epidemic was due to a previously unknown coronavirus (SARS-CoV), which was highly infectious and fatal. The original source of SARS-CoV was confirmed to be zoonotic, originating from an animal reservoir which includes horseshoe bats (*Vespertilio ferrum-equinum*) (Zhou *et al.*, 2020) and masked palm civets (*Paguma larvata*) (Guan *et al.*, 2003), although the most recent genomic evidence leads to pangolins (*Manis pentadactyla*) (Zhang *et al.*, 2020). The current Covid-19 pandemic has reached 3.02 million confirmed cases and 207,973 deaths worldwide (WHO, 2019). The zoonotic SARS-CoV continues to be a major threat to humans, and most research groups do not exclude the possibility of continual reemergence of SARS.

Natural plant-derived flavonoids are well known for their beneficial effects on neurodegenerative disease (Solanki *et al.*, 2015), type 2 diabetes (Oh *et al.*, 2017), atherosclerosis(Dalgaard *et al.*, 2019), cardiovascular disease (McCullough *et al.*, 2012; Kim and Je, 2017), and cancer (Zhou *et al.*, 2019; Sankaranarayanan *et al.*, 2019; Navarra *etal.*, 2020; Loung *et al.*, 2019; Feng *et al.*, 2019; Bondonno *et al.*, 2019). Flavonoids have also shown a wide range of antiviral benefits (Goncalves *et al.*, 2005; Li *et al.*, 2008; Friedman, 2007; Cushnie and Lamb, 2005; Bae *etal.*, 2000). Antiviral properties of flavonoids have been established for poliovuris (Shulman *et al.*, 2011; Conti *et al.*, 1990), astrovirus (Superti *et al.*, 1990), HIV(Mahmood *et al.*, 1993; De Clercq, 2000), enterovirus (Genovese *et al.*, 1995), Respiratory Syncytial Virus (RSV), parainfluenza virus type 3 (PIV 3), and influenza virus type A (Flu A) (Wei *et al.*, 2004). Laboratory studies with flavonoids have shown that the proteolytic activity of the original SARS-CoV 3CLpro was inhibited by apigenin, luteolin, quercetin, amentoflavone (Ryu *et al.*, 2010), quercetin, daidzein, puerarin, epigallocatechin, epigallocatechin gallate, gallocatechin gallate (Nguyen *et al.*, 2012), and kaempferol (Schwarz *et al.*, 2014). Jo *et al.* (2020) also published a report on laboratory-based inhibition of the original SARS-CoV main protease 3CLpro (PDB: 4WY3) by 64 flavonoids, and stated that herbacetin, rhoifolin and pectolinarin were found to efficiently block the enzymatic activity of SARS-CoV 3CLpro.

With regard to SARS-CoV-2 (Covid-19), several recent *in silico* Molecular Dynamics (MD) docking studies for drug repurposing have reported that flavonoids were high on their hit list. Chen *et al*. (2020) reported results based on docking 1,500 drugs with SARS-CoV 3CLpro (PDB: 2DUC), and found that diosmin and hesperidin were the two top candidates for one of the chains investigated. In our recently reported *in silico* study (Peterson, 2021), diosmin ranked 22 out of ~4,600 drugs (99th percentile) in terms of Binding Energy (BE) with SARS-CoV-2 3CLpro. When compared with antiviral drugs, diosmin's BE was lower than 97% of the top 30 antivirals and formed more hydrogen bonds with the active site than any of the top 30 antivirals. Adem *et al*. (2020) reported that after evaluating 80 flavonoids for MD docking with the SARS-CoV-2 protease 3CLpro (PDB:GLU7), hesperidin, rutin, and disomin were the top 3 candidates for docking at the active site. In another study, Mishra *et al*. (2020) assessed MD docking properties of 14 flavonoids (herbacetin, rhoifolin, pectolinarin, apigenin, luteolin, amentoflavone, daidzein, puerarin, epigallocatechin, gallocatechin gallate, resveratrol, maslinic acid, piperine and ganomycin B) to target the active site of 3CLpro (PDB:GLU7). Results suggest that amentoflavone, peurarin and maslinic acid were the top binding candidates.

There is clear evidence from empirical laboratory-based inquiry that certain flavonoids reveal an inhibitory effect on 3CLpro of SARS-CoV, while *in silico* MD studies have suggested that 3CLpro of SARS-Cov-2 could potentially be inhibited by flavonoids. Since the solvent-accessible surface (Richmond, 1984; Marsh and Teichmann, 2011) of the active site of 3CLpro of SARS-CoV is different from that of SARS-CoV-2, it is important to understand the differences in predicted binding of flavonoids to both variants. This would help to establish whether they are not specific flavonoids can dock with high affinity to the active site of the newer mutated form as well as the previous form of 3CLpro experienced in 2003. What has eluded systematic investigation, however, is the *in silico* MD docking of flavonoids to both SARS-CoV and SARS-CoV-2 within the same study. This investigation was therefore pursued to perform *in silico* MD docking of flavonoids to the active site of 3CLpro for x-ray crystallography-based forms of 3CLpro from SARS-CoV and SARS-CoV-2, and a 3D modeled form of 3CLpro from SARS-Cov-2, based on alignments with the available human proteomes now available.

2. Materials and Methods

2.1. Ligand Selection

A total of 72 flavonoids were selected for MD docking. Molecular structures of the flavonoids in the form of SMILES strings were obtained from Drugbank (Wishart *et al.*, 2005 and 2018) and PubChem (Kim *et al.*, 2018).

2.2. Ligand Preparation

The canonical SMILES of the flavonoids selected were first desalted, and Open Babel (OB) (O'Boyle *et al.*, 2011) was used to add hydrogens, and transform to 3D. The energy of each ligand was then minimized using the Amber force field (Kini and Evans, 1991; Wang *etal.*, 2004) from within OB, via conjugate gradients (250 iterations), updates at 1 step intervals, and a convergence criterion of 0.0001. Results were saved into 3D SDF format containing partial charges of each atom. PyRx (Dallakyan and Olson., 2015) was then used to input SDF files to correct bonds and hydrogens, and then save in PDBQT format.

2.3. Active Site (3CLpro) 3D Structure

The main protease of SARS-CoV is a chymotrypsin-like protease, 3CLpro, which is important for processing viral proteins and controlling replicase complex activity (Anand *et al.*, 2003). As such, 3CLpro is an ideal target for the design of antiviral therapies because of its vital role in viral replication and infection. The 3D x-ray crystallography models of 3CLpro bound to a novel ligand were obtained for SARS-CoV (PDB: 4WY3, 1.89Å) (Shimamoto *et al.*, 2015) and SARS-Cov-2 (PDB: 6LU7, 2.16Å) (Jin *et al.*, 2020). Swiss Model (Bertoni *et al.*, 2017; Studer *et al.*, 2019; Guex *et al.*, 2009; Waterhouse *etal.*, 2018) was used to obtain a 3D model of SARS-CoV-2 3CLpro based on an alignment of PDB:6LU7 and 8 matching sequences of 3CLpro (6m03.1.A, 6m03.1.B, 5r81.1.A, 5r84.1.B, 6y84.1.B, 6y84.1.A, 5r7y.1.B, 5r82.1.A) from an original pool of 457 templates found to match the target sequence. For the x-ray crystal forms of 3CLpro (SARS-CoV, SARS-CoV-2), PyMol (Rigsby *et al.*, 2016) was used to select amino acid residues of 3CLpro within 5 angstroms (Å) of the ligand (bound to the active catalytic site), and results were saved in PDB format. For the non-crystal modeled form of 3CLpro of SARS-CoV-2, residues within 10 angstroms from the active site were identified and saved as the active site in PDB format. PyRx was used to merge charges and remove non-polar hydrogens, lone pairs, water molecules from the active site, and results were exported to PDBQT format.

2.4. Molecular Ligand-Active Site Docking

Vina (Trott *et al.*, 2010) was used for ligand(flavonoid)-active site docking. A total of 9 ligand poses were assessed at the active site, and the best putative pose was assumed to have the lowest Binding Energy (BE) in kcal/mol. BE values less than -6 kcal/mol were considered to represent significant binding affinity. For each flavonoid, BEs were averaged across the three variants of 3CLpro considered, and ranked in ascending order according to mean BE.

2.5. Toxicology and ADME Predictions

The physical properties of flavonoids and prediction of their toxicology and Absorption, Distribution, Metabolism, Excretion (ADME) was performed using methods described previously (Peterson, 2019).

3. Results

3.1. Findings from this Study

Table 1 lists the top 30 flavonoids and their docking results at the active site of the three forms of 3CLpro. The mean BE for the best putative pose of the top 30 candidates in their energy-minimized state ranged from -8.8 to -7.3. The top 10 candidates were amentoflavone, gallocatechin gallate, diosmin, epigallocatechin gallate, hidrosmin, catechin gallate, elsamitrucin, pectolinaren, quercetin, and isoquercetin. Figures 1 to 4 illustrate the best putative docking poses for the top 32 flavonoids at the active site of 3CLpro for the crystal forms of SARS-CoV (column 1), SARS-CoV-2 (column 2), and the consensus 3D modeled form of SARS-CoV-2 (column 3). Each image also shows the number of strong hydrogen bonds (>3.5 Å) after the name of each flavonoid. Supplement figures S1-S5 also show the remaining 40 flavonoids (33-72) in their best putative pose. Among the 72 flavonoids that were docked, all but one (auraptene) had an average BE which was not < -6 kcal/mol. We describe the top 15 flavonoids and their roles in various diseases and interest in current research in the following section.

Amentoflavin has been investigated for neuroprotection in Alzheimer's disease (Zhao *et al.*, 2019), type 2 diabetes (Su *et al.*, 2019), and oncology (Shen *et al.*, 2019; Lee *et al.*, 2019; Hsu *etal.*, 2019; Chiang *etal.*, 2019). Gallocatechin

gallate is a component of green tea and has been studied for antimicrobial properties (Wan *etal.*, 2005; Sugita-Konishi *et al.*, 1999), cholesterol reduction (Ikeda *et al.*, 2003), and obesity and hypertriglycererides (Cho *et al.*, 2019). Diosmin is used as pharmaceutical (Dafron®) for chronic venous disease to provide soothing relief of leg ulcers, ankle swelling, varicose veins, complications of diabetes, venous insufficiency, hemorrhoids, and other conditions of the lower extremities (Murray, 2007). Epigallocatechin gallate (EGCG) has been investigated for Parkinson's disease (Zhou *et al.*, 2018), fatty liver disease (Chen *et al.*, 2018), diabetes (Feng *et al.*, 2018), cardiovascular and metabolic disorders (Eng *et al.*, 2018), and various cancers (Enkhbat *et al.*, 2018; Negri *et al.*, 2018; Zhu *etal.*, 2019). Hidrosmin has also been investigated for chronic venous insufficiency (Honorato and Arcas, 1990; Dominguez *et al.*, 1992), and chronic lymphedema (Jimenez

Table 1: Molecular Docking Results for Best Putative Pose of Flavonoids at the Active Site of 3CLpro. Values Listed are Binding Energy (BE) in Units of kcal/mol

	SARS-CoV	SARS-CoV-2		
	Crystal	Crystal	Modeled	
Flavonoid	(PDB: 4WY3)	(PDB: GLU7)	Consensus	Average
Amentoflavone	-8.4	-9	-9.1	-8.8
Gallocatechin gallate	-7.9	-8.3	-9	-8.4
Diosmin	-7.5	-9	-8.6	-8.4
Epigallocatechin gallate	-7.4	-8.3	-9	-8.2
Hidrosmin	-6.8	-8.9	-8.9	-8.2
Catechin gallate	-7.1	-8.4	-9	-8.2
Elsamitrucin	-7.3	-8.3	-8.6	-8.1
Pectolinaren	-7.1	-8.3	-8.7	-8.0
Quercetin	-7.5	-7.7	-8.5	-7.9
Isoquercetin	-7	-8	-8.6	-7.9
Orientin	-7.5	-8	-8.1	-7.9
FCLA free acid	-6.9	-7.8	-8.9	-7.9
Silibinin	-7.3	-8.1	-8.1	-7.8
Baicalin	-6.9	-7.9	-8.7	-7.8
Astilbin	-7.1	-7.8	-8.5	-7.8
Hesperidin	-7.2	-8	-8	-7.7
Neohesperidin dihydrochalcone	-8.1	-7.3	-7.7	-7.7
Rutin	-7.3	-7.1	-8.6	-7.7
Rhoifolin	-6.7	-7.9	-8.3	-7.6
Mangiferin	-7.1	-7.9	-7.6	-7.5
Glabridin	-6.5	-7.9	-8.1	-7.5
Monoxerutin	-6.7	-7.5	-8.3	-7.5
Icariin	-6.7	-7.7	-8	-7.5
Peurarin	-7	-7.2	-8.2	-7.5
Genestin	-6.9	-7.3	-8.1	-7.4
Fisetin	-7	-7.3	-7.7	-7.3
Homoplantaginin	-6.8	-7.6	-7.6	-7.3
Hispidulin	-6.5	-7.4	-8	-7.3
Naringin	-6.3	-7.4	-8.2	-7.3
Baicalein	-6.3	-7.6	-7.9	-7.3

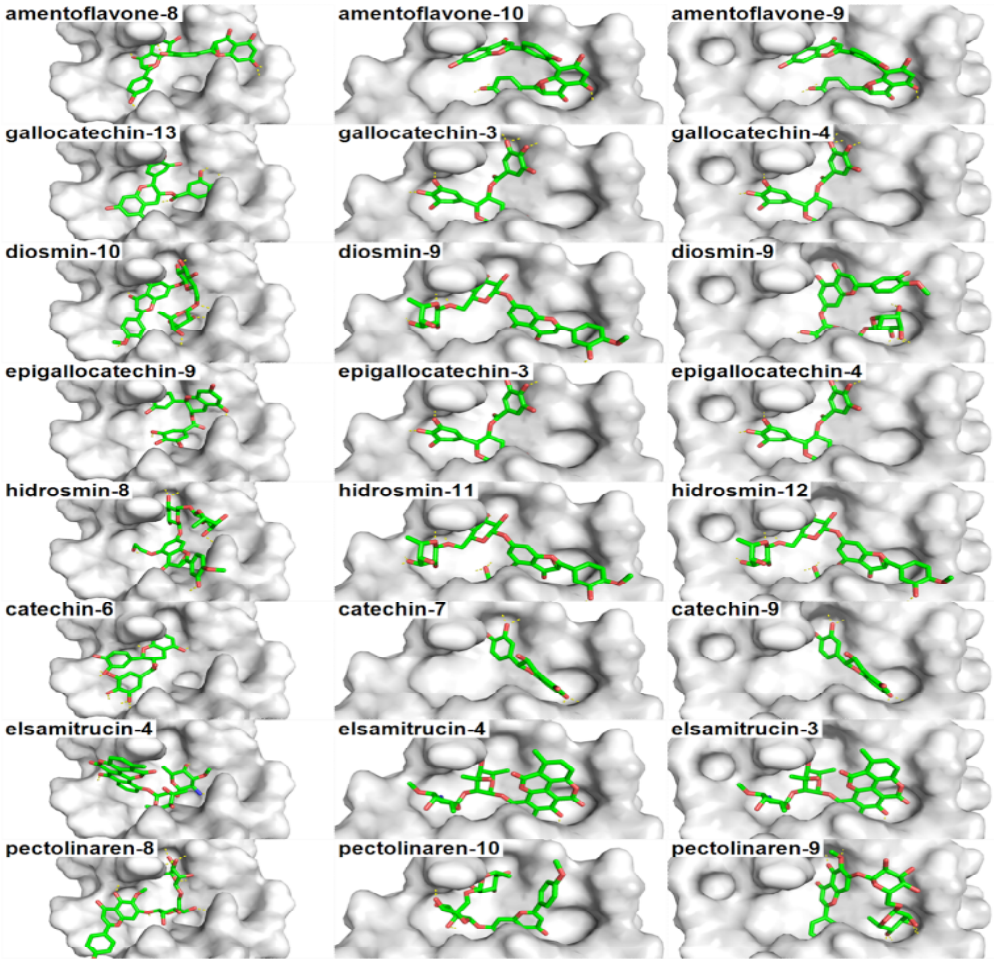

Figure 1: Putative Best Docking Poses at the Active site of 3CLpro for Flavonoids 1-8 in Table 1. First Column: Crystal SARS-CoV (PDB: 4WY3), Second Column: Crystal SARS-CoV-2 (PDB: GLU7), Third Column: Modeled 3D Consensus SARS-CoV-2. Numbers After Each Flavonoid's Name Represent Quantity of Strong Hydrogen Bonds (>3.5 Å)

et al., 1991). Catechin gallate, another green tea flavonoid, has similar properties of EGCG in terms of cholesterol reduction (Ikeda *etal.*, 2003). Elsamatrucin is an antimutor agent that has been previously explored but has not been of interest nearly as much as other flavonoids. As an example, the latest citation dates back to 2011 (Verweij *et al.*, 1994; Raber *et al.*, 1992; Gosse*tal.*, 1994; Allen *et al.*, 1996; Fiocchi *et al.*, 2011). Pectolinarin is mostly known as an anti-inflammatory and has been studied in rheumatoid arthritis (Martinez-Vazquez *et al.*, 1998; Lim *et al.*, 2008; Wang *et al.*, 2019). Quercetin is a major flavonoid found in berries, apples, cauliflower, tea, cabbage, nuts, and onions, for which consumption has traditionally been viewed as being protective against cancer, allergies, metabolic disorders, inflammatory disorders, ocular and cardiovascular diseases, arthritis (Ferenczyova *et al.*, 2020; Batiha *et al.*, 2020), fatty liver disease (Qin *et al.*, 2018), and Alzheimer's disease (Zhang *et al.*, 2020). Quercetin has also been documented to possess antioxidant, antifungal, anti-carcinogenic (Vafadar *et al.*, 2020), hepatoprotective, and cytotoxic activity. Isoquercetin complexes with and is an inhibitor of SIRT6, which is an NAD(+)-dependent protein deacylase regulating metabolism and chromatin homeostasis. SIRT6 activation protects against metabolic and aging-related diseases, and SIRT6 inhibition is considered as being therapeutic for cancer (You *et al.*, 2019). Orientin has been explored for its association with Alzheimer's disease (Zhong *et al.*, 2019), carcinoma (Tian *et al.*, 2018; Thangaraj *et al.*, 2019), diabetes and hyperlipidemia (Malik *et al.*, 2019; Li *et al.*, 2020). FLCA free acid is a chemoluminescing flavonoid, mostly used for singlet oxygen and

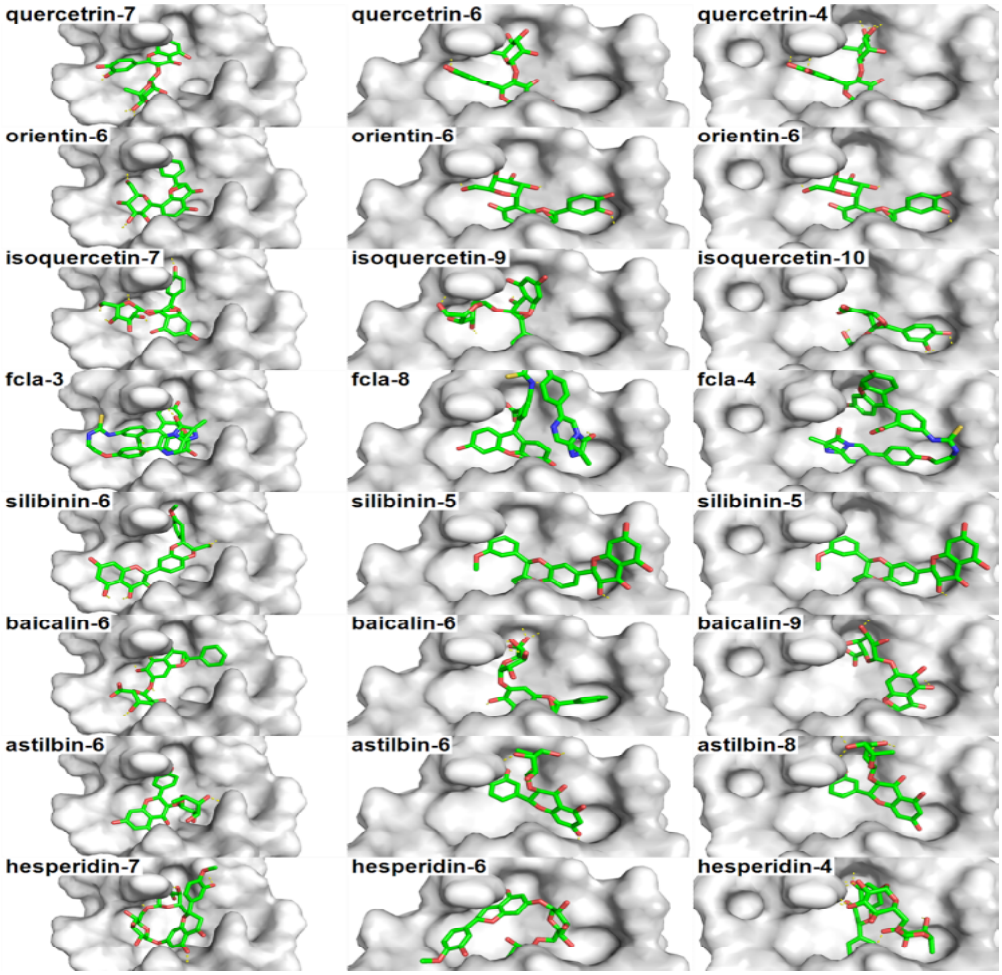

Figure 2: Putative Best Docking Poses at the Active Site of 3CLpro for Flavonoids 9-16 in Table 1. First Column: Crystal SARS-CoV (PDB: 4WY3), Second Column: Crystal SARS-CoV-2 (PDB: GLU7), Third Column: Modeled 3D Consensus SARS-CoV-2. Numbers After Each Flavonoid's Name Represent Quantity of Strong Hydrogen Bonds (>3.5 Å)

superoxide anion detection (He *et al.*, 2002a and 2002b). Silibinin is known for its role in Alzheimer's disease (Shen *etal.*, 2019), cancer (Shi *et al.*, 2019; Mirzaaghaei *et al.*, 2019), and fatty liver disease (Liu *et al.*, 2019). Baicalin has been studied in osteoporosis (Zhao *et al.*, 2020), cancer (Wu *et al.*, 2020), and obesity and hyperlipidemia (Wang *et al.*, 2020). Astilbin (Xu *et al.*, 2020; Xin *et al.*, 2020; Han *et al.*, 2020) has been primarily investigated for its role in cellular oxidative stress (Xu *et al.*, 2020; Xin *et al.*, 2020; Han *et al.*, 2020).

The predicted toxicology and ADME results for the 71 flavonoids whose average BE was less than -6 kcal/mol are shown in Supplement Figure S6. As one can note, the best docked flavonoids (lowest BE) populate the top half of the list and have greater Molecular Weight (MW), Total Partial Surface Area (TPSA), and a greater number of Hydrogen Bond Donors (HBD) and Acceptors (HBA). Most of the drug-like flavonoids occupied the lower half of the list as they possessed a much lower TPSA, which is required for being drug-like. Only one flavonoid was lead-like (Chrysin), since its MW<300 and its other properties met the criteria for being lead-like. The majority of flavonoids also had high predicted probabilities of being toxic to fathead minnows (FHM), honey bees (HBT), and Tetrahymena pyriformis (TPT), which should not be a concern for humans as they are all commonly consumed flavonoids.

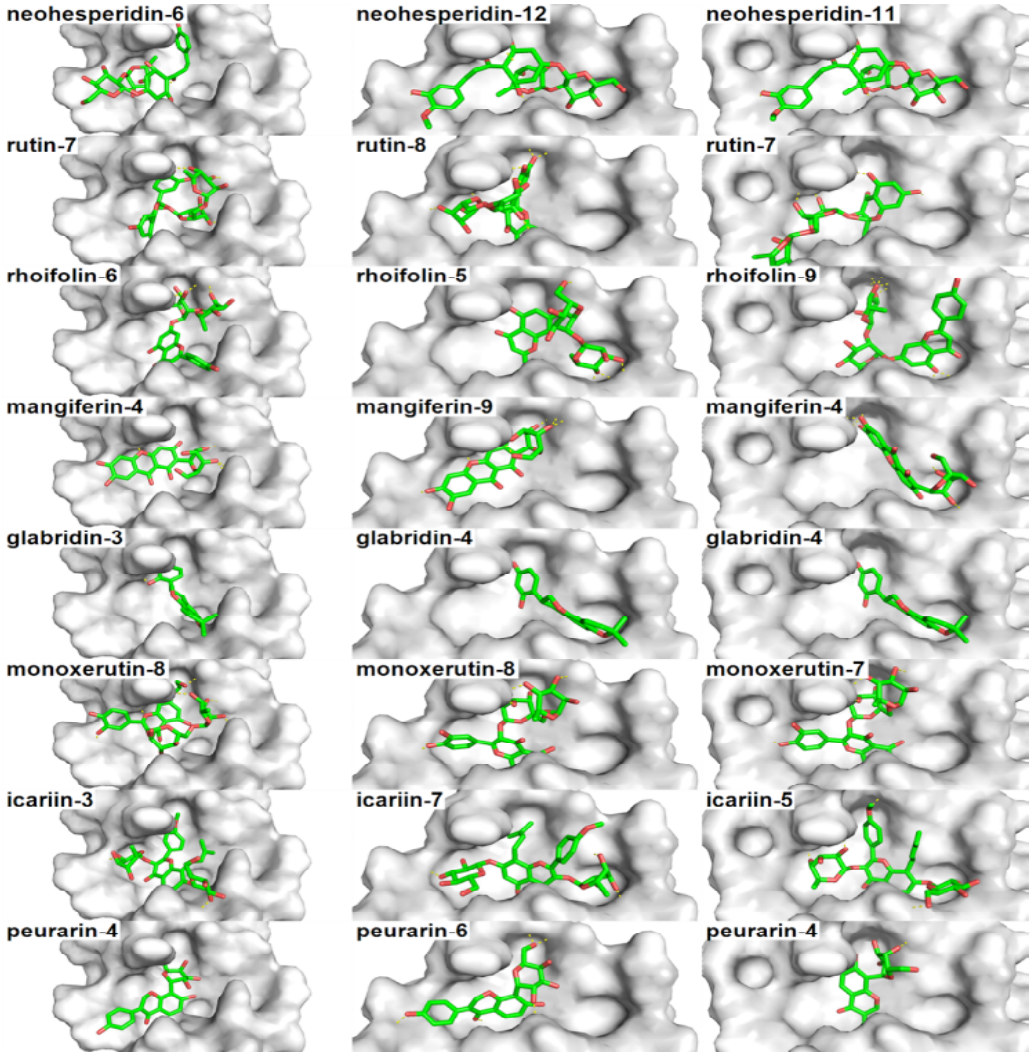

Figure 3: Putative Best Docking Poses at the Active Site of 3CLpro for Flavonoids 17-24 in Table 1. First Column: Crystal SARS-CoV (PDB: 4WY3), Second Column: Crystal SARS-CoV-2 (PDB: GLU7), Third Column: Modeled 3D Consensus SARS-CoV-2. Numbers after Each Flavonoid's Name Represent Quantity of Strong Hydrogen Bonds (>3.5 Å)

3.2. Findings from Multiple Studies

Table 2 provides a compilation of top flavonoid hits reported by previous *in vitro* laboratory-based SARS-CoV 3CLpro inhibition studies published since 2003, and top hits from more recent *in silico* docking studies involving 3CLpro of SARS-CoV-2, including this investigation. Within the constellation of all flavonoids investigated to date for inhibiting (docking) with 3CLpro of either SARS-CoV or SARS-CoV-2, the top hit list includes 14 flavonoids: amentoflavone, daizden, diosmin, epigallocatechin gallate, gallocatechin gallate, herbacetin, hesperidin, luteolin, naringin, peurarin, pectolinarin, quercetin, rhoifolin, and rutin. Table 3 lists a summary of common dietary sources of each of the 14 flavonoids and their hit counts from multiple studies of *in vitro* inhibition and *in silico* docking of the active site of 3CLpro protease of SARS-CoV and SARS-CoV-2 3CLpro.

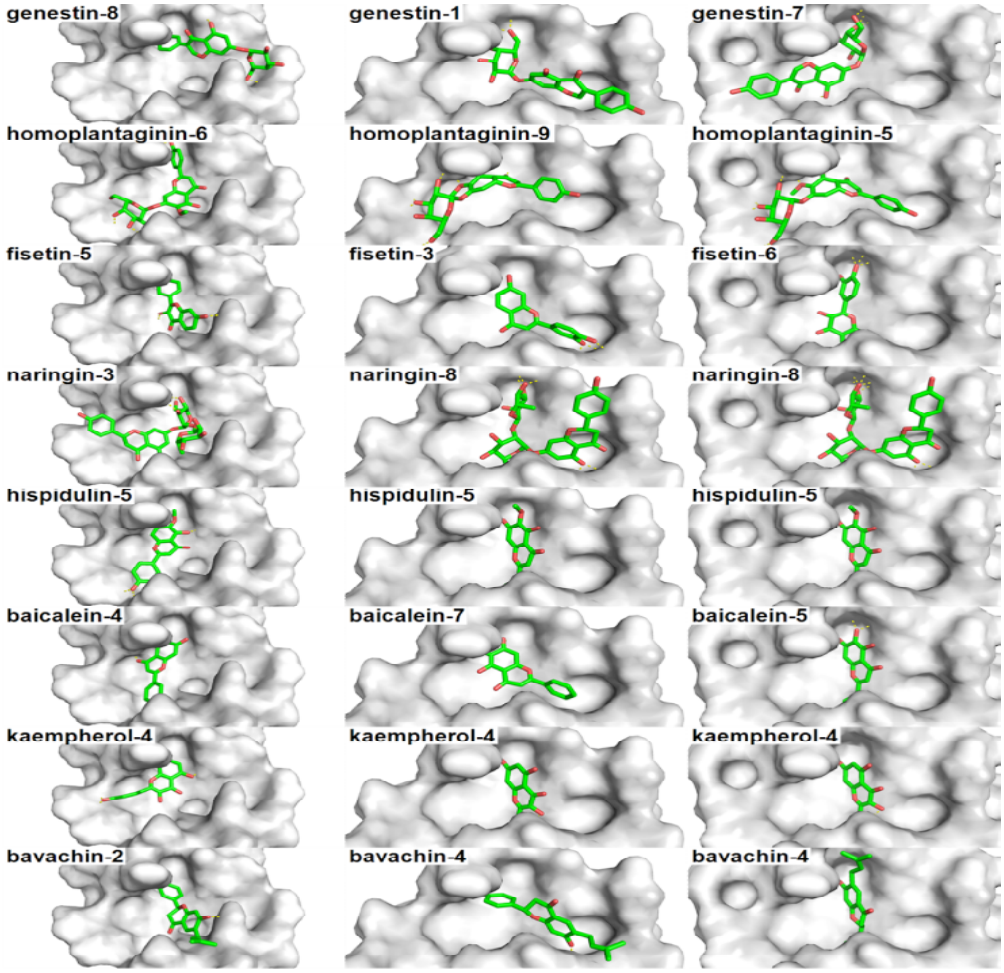

Figure 4: Putative Best Docking Poses at the Active Site of 3CLpro for Flavonoids 25-32 Ranked by Average BE. First Column: Crystal SARS-CoV (PDB: 4WY3), Second Column: Crystal SARS-CoV-2 (PDB: GLU7), Third Column: Modeled 3D Consensus SARS-CoV-2. Numbers After Each Flavonoid's Name Represent Quantity of Strong Hydrogen Bonds (>3.5 Å)

4. Discussion

Covid-19 disease is highly transmissible and has been shown to result in acute respiratory failure in patients who are elderly, immune-compromised, and have pre-existing conditions. Two important hallmarks of Covid-19 are the rapidity in the onset of symptoms and the magnitude of resources required for intensive care for patients. Together, these factors directly and indirectly support the need for prevention of pandemics on a global scale.

Our approach employed two *in silico* levels of computation, one that involved MD docking and another based on toxicology and ADME predictions. MD docking results indicate that many flavonoids yielded high-quality BE's which were less than the threshold of -6 kcal/mol, for which significant binding is assumed. Specifically, MD docking was considered significant for most of the ligands employed. It is important to realize that our approach to MD docking was targeted and hypothesis-driven, in that we focused on flavonoid binding within in the known active catalytic site on 3CLpro. We also did not employ a data-driven approach that is similar to "blind" docking, in which BE's are sought for ligands binding in any pockets found on the solvent-accessible surface of a protein.

One of the main benefits of flavonoids is they are found ubiquitously in plants and are the most common polyphenols found in the human diet. Many of the top flavonoids identified from studies of *in vitro* inhibition and *in silico* docking

Table 2: List of Top Hits from Multiple *In vitro* Inhibition and *In silico* Docking Investigations of the Main Protease 3CLpro of SARS-CoV and SARS-CoV-2

Type Investigation	Protease 3CL Pro variant	Compounds Studied	Top Inhibitory/ Docked Flavonoids	Reference
In vitro	SARS-CoV (cloned)	12 flavonoids	Apigenin, luteolin, quercetin, amentoflavone	Ryu *et al.*(2010)
	SARS-CoV (cloned)	6 flavonoids	Quercetin, daidzein, puerarin, epigallocatechin, epigallocatechin gallate, gallocatechin gallate	Nguyen *et al.* (2012)
	SARS-Cov (cloned)	64 flavonoids	Pectolinarin, rhoifolin, herbacetin	Jo *et al.* (2020)
In silico	SARS-CoV(PDB:2DUC)	1,500 drugs	Diosmin (rank 1), hesperidin (rank 2)	Chen *et al.* (2020)
	SARS-CoV-2 (PDB:6LU7)	4,634 drugs	Diosmin (rank 22), epigallocatechin gallate (rank 134), hydrosmin (rank 163),	Peterson (2021)
	SARS-CoV-2 (PDB: 6LU7)	80 flavonoids	Hesperidin, rutin, diosmin, apiin, diacetyl curcumin, myricetin, flavone23, naringin, neohesperidin, scutellarin	Adem *et al.* (2020)
	SARS-CoV-2 (PDB: 6LU7)	14 flavonoids	Amentoflavone, puerarin, maslinic acid, piperine, gallocatechin gallate, luteolin, apigenin, resveratrol, herbacetin, daidzein, rhoifolin, ganomycin B, epigallo-catechin, pectolinarin	Mishra *et al.* (2020)
	SARS-CoV (PDB: 4WY3), SARS-CoV-2 (PDB: 6LU7), Consensus SARS-CoV-2	72 flavonoids	Amentoflavone, gallocate-chin gallate, diosmin, epigallocatechin gallate, hidrosmin, catechin gallate, elsamitrucin, pectolinaren, quercetin, isoquercetin, hesperidin*, rutin*, rhoifolin*, peurarin*	This study

Note: * From top 30 candidates in Table 1, the preceding candidates are the top 10 in Table 1.

Table 3: Common Dietary Sources of Flavonoids Reported in Multiple *In vitro* Inhibitory and *In silico* Docking Studies of the Active Site of 3CLpro Protease of SARS-CoV and SARS-CoV-2

Flavonoid	Common Dietary Source	Hit Count in Publications
Amentoflavone	*Ginko Biloba* (supplement) (Lobstein-Guth *et al.*, 1988)	3 hits (1 *in vitro*, 2 *in silico*)
Daidzein	Raisins (Liggins *et al.*, 2000)	2 hits (1 *in vitro*, 1 *in silico*)
Diosmin	Diosmin (supplement), citrus peels (Nogata *et al.*, 2006)	4 hits (4 *in silico*)
Epigallocatechin gallate	Green tea extract (supplement) (Sugita-Konishi *etal.*, 1999; Wang *et al.*, 2019)	3 hits (1 *in vitro*, 2 *in silico*)
Gallocatechin gallate	Green tea extract (supplement) (Sugita-Konishi *et al.*, 1999; Wang *et al.*, 2019)	3 hits (1 *in vitro*, 2 *in silico*)
Herbacetin	Flaxseed hulls (Struijs *et al.*, 2007)	2 hits (1 in vitro, 1 in silico)
Hesperidin	Dried peppermint (Dolzhenko *et al.*, 2010), orange juice (Senorans *etal.*, 2001), tangerine peels (Nogata *et al.*, 2006), lemon peels (Liu *et al.*, 2020)	3 hits (3 in silico)
Luteolin	Luteolin (supplement), celery seeds (spice) (USDA, 2021)	2 hits (1 *in vitro*, 1 *in silico*)
Naringin	Bergamot orange (oil, juice), grapefruit juice (Nogata *et al.*, 2006)*, "Earl Grey" tea	2 hits (*in vitro*)
Peurarin	Kudzu (supplement) (Selvakumar *et al.*, 2007; Reppert *et al.*, 2008)	3 hits (1 *in vitro*, 2 *in silico*)
Pectolinarin	Korean thistle (*Cirsium setidens*) (Jeong *et al.*, 2008), dried Gondre	3 hits (1 *in vitro*, 2 *in silico*)
Quercetin	Quercetin (supplement) (Li *et al.*, 2016)	3 hits (2 *in vitro*, 1 *in silico*)
Rhoifolin	Bergamot orange (oil, juice) (Nogata *et al.*, 2006), "Earl Grey" tea	3 hits (1 *in vitro*, 2 *in silico*)
Rutin	Rutin (supplement), buckwheat (Ganeshpurkar and Saluja, 2017)	2 hits (2 *in silico*)

Note: **Grapefruit juice is known to be an inhibitor of cytochrome P-450, and therefore interacts with many drugs (Bailey *et al.*, 1998).

of 3CLpro can be readily obtained in the form of a supplement. Amentoflavone can be found in high concentration in Ginko Biloba, which is available as a supplement (Lobstein-Guth *et al.*, 1988). Daidzein can be found in raisins (Liggins *et al.*, 2000) . Diosmin is found in citrus rinds (Nogata *et al.*, 2006), and is manufactured by extracting hesperidin from citrus rinds of bitter orange, sweet orange, blood orange, tangerine, grapefruit, or lemon, followed by conversion of hesperidin to diosmin (Londoño-Londoño *et al.*, 2010). Gallocatechin gallate, epigallocatechin gallate (EGCG), and catechin gallate are all found in high concentrations in green tea (Sugita-Konishi *etal.*, 1999; Cho *et al.*, 2019; Wang *et al.*, 2019). The herbal supplement known as green tea extract can contain anywhere from 25 mg to 1 g of EGCG -- a 200-250 mg capsule of green tea extract possesses the phytonutrient content of approximately 2-3 cups of green tea. Herbacetin exists primarily in flaxseed hulls (Struijs *et al.*, 2007). On the other hand, hesperidin can be found in dried peppermint (Dolzhenko *et al.*, 2010), orange juice (Senorans *et al.*, 2001), tangerine peels (Nogata *et al.*, 2006), and lemon peels (Liu *et al.*, 2020). Luteolin is available as a supplement and exists in high concentration in celery seeds, which are used as a cooking spice (USDA, 2011). Naringen and rhoifolin can be found in high concentrations in the oil and juice of Bergamot oranges (Nogata *et al.*, 2006). The black tea known as "Earl Grey," acquires its spicy aroma from oil of Bergamot. Naringen is also found in high concentrations in grapefruit juice (Nogata *et al.*, 2006), however, grapefruit juice is known to be an inhibitor of cytochrome P-450 (Bailey *et al.*, 1998), and therefore interacts with many prescribed pharmaceuticals—so it should be consumed with caution. Peurarin is widely available in the supplement known as Kudzu, which is a vine (Selvakumar *et al.*, 2007; Reppert *et al.*, 2008). The strongest concentrations of pectolinarin exist in Korean thistle (Cirsium setidens) (Jeong *et al.*, 2008), which is available as a supplement in the form of dried Gondre. Quercetin is ubiquitously found in many vegetables and can be obtained as a supplement (Li *et al.*,

2016). Rutin is also available as a supplement, and exists in high concentrations in buckwheat (Ganeshpurkar and Saluja, 2017).

We also predicted toxicology and ADME for flavonoids having significant binding to the active site. The toxicological predictions for the flavonoids considered should not be a concern for human consumption, as they are already ubiquitously distributed in vegetables and fruits that are commonly consumed. Such predictions are a requirement for assessing risks and side effects of potential new drugs, as the concerns for safety are becoming increasingly more stringent by regulatory agencies.

The clinical value of our results are established by the potential for testing flavonoids for the treatment of Covid-19, which could prove useful in animal studies, transgenics, and xenograft models, etc., to confirm results of this study and the other MD docking studies which have been recently been reported. Due to the expediency in finding optimal treatments for the global Covid-19 disease pandemic, existing antiviral clinical trials should consider adding an arm for combined therapy involving antiviral+flavonoid(s). The hypothesis for combined therapy is that recovery times for patients would be shorten for combined therapy when compared with monotherapy, due to synergistic efficacy from flavonoids. Group sequential and adaptive human clinical trials should also be rapidly initiated though compassionate use to establish synergistic efficacy for one of more these flavonoids with antivirals. If efficacious synergism is observed, combinations of flavonoids could be consumed by the healthy public for prophylactic protection from SARS-CoV-2 infection providing formularies for dosing and frequency are established. Many of these flavonoids are already available as prescription-free supplements, so they are widely available.

5. Conclusion

Published evidence is now clear that several flavonoids inhibit 3CLpro of SARS-CoV *in vitro*. In addition, recent *in silico* studies predict that many of the same flavonoids bind significantly to the active site of 3CLpro of SARS-CoV-2. The next step was to confirm whether these flavonoids can reduce early infection and late-stage symptoms of Covid-19 disease in vivo using laboratory animals. If promising animal results are obtained in vivo for flavonoids employed singly or in combination, then they could be evaluated in prospective human prevention trials for minimizing early-stage infection, and in combination therapy trials (antiviral+flavonoid(s)) for alleviating symptoms and shortening recovery times among late-stage Covid-19 disease.

Ethical Approval and Consent to Participate

Not applicable.

Consent for Publication

LP authorizes approval for publication.

Availability of Supporting Data

All data used are in the public domain and are available at PubChem (https://pubchem.ncbi.nlm.nih.gov), DrugBank (https://www.drugbank.ca), Protein Data Bank (https://www.rcsb.org), and Swiss Model (https://swissmodel.expasy.org).

Competing Interests

No competing interests declared.

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Author's Contributions

LP designed the study, acquired the data, prepared the active site 3D models, performed the ligand preparation, performed the docking, generated images for the best putative pose, calculated mean binding energy, and preformed the toxicology and ADME predictions.

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