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Molecular Docking Analysis of Phytochemicals from *Polygonum Minus* against SARS-CoV-2

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The COVID-19 pandemic emerged when the novel coronavirus, officially named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), began spreading globally in late 2019. Despite the global scientific community's efforts to develop effective treatments for COVID-19, there remains a critical need for safe, accessible, and productive therapeutics to combat SARS-CoV-2. One approach to investigating alternative treatments involves studying natural compounds that may possess antiviral properties. This study aimed to determine the potential of *Polygonum minus* as in inhibitor of spike, RdRp, M^{pro}, and PL^{pro} activities of SARS-CoV-2 using an in-silico approach. Ligands were obtained from the PubChem database. The Lipinski Rule of Five and pharmacokinetic properties were evaluated using SwissADME prediction tool. Antiviral probability was estimated using the PASS Online website. Molecular docking and screening were performed using PyRx, and Biovia Discovery Studio was used for visualization. The bioactive compounds with the best antiviral potential exhibited the lowest docking scores or highest affinity to the target proteins, including spike, RdRp, M^{pro}, and PL^{pro} of SARS-CoV-2. Paxlovid and Remdesivir were used as positive controls for treating SARS-CoV-2 infection. The results indicate that quercetin, isorhamnetin, and kaempferol from *Polygonum minus*, show strong potential as antiviral agents against SARS-CoV-2 due to their low docking scores. Among these ligands, quercetin, and kaempferol have docking scores lower than control Paxlovid, highlighting their promising antiviral potential. Therefore, these compounds could be further screened via *in vitro* tests against SARS-CoV-2, and the findings could be further validated in an appropriate animal model and, hopefully, developed through subsequent clinical trials to provide additional therapeutic options for patients with COVID-19.

Keywords: SARS-CoV-2; *Polygonum minus;* molecular docking; antiviral

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Coronaviruses are ribonucleic acid (RNA) viruses that cause illness in humans and animals. Among the seven coronaviruses known to infect humans are Human Coronavirus (HCoV-229E), HCoV-NL63, HCoV-HKU1, HCoV-OC43, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), and Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), and the most recent is Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) [1]. SARS-CoV-2 belongs to the Coronaviridae family and is classified within the Nidovirales order. SARS-CoV-2 is characterized by distinctive spike protein molecules protruding from its surface, giving the virus a crown-like appearance, hence the name "coronavirus" [2]. The spike protein is indispensable for viral replication because it mediates the entry of the virus into the cell and is highly correlated with the virus's ability to infect. The spike protein, papain-like protease (PLpro), 3C protease-like protease (3CLpro), and RNA-dependent RNA polymerase (RdRp) play a key role in the entire replication cycle of SARS-CoV-2 [3].

With the widespread impact of COVID-19, individuals experience major symptoms, such as fever, cough, fatigue, dyspnoea [4], abdominal pain, dizziness, nausea, vomiting, and diarrhea [5]. According to the Ministry of Health, individuals with mild to moderate infections who self-isolate and recover within five days may be prescribed the Paxlovid antiviral [6]. Other than Paxlovid, several antiviral drugs have been proposed in emergency as COVID-19 treatments, such as lopinavir/ritonavir, indinavir (anti-HIV), chloroquine (anti-malaria), remdesivir (anti-ebola), oseltamivir (anti-influenza). Nevertheless, no drug or vaccine has shown to be specific and/or effective in treating SARS-CoV-2 [7]. Another promising approach consists of investigating natural resources including medicinal plants. Many phytochemicals have indeed been reported to possess antiviral properties [8]. Some of them have been found to exhibit an inhibitory activity against SARS-CoV-2 main protease [9]. Some of the most potent natural compounds against SARS-CoV-2 are diosmin (flavone glycoside), ellagic acid (polyphenol), quercetin (flavonoid), curcumin (polyphenol) from *Curcuma longa* and sesaminol glucoside (lignin glycoside) [10-12]. Other than that, Sharma et al. [13] reported that catechin (flavonoid) from green tea and piperine (alkaloid) from *Piper*

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nigrum are potent natural compounds that inhibit SARS-CoV-2 by blocking virus entry through interaction with the ACE-2 receptor and enhancing immunity, thus potentially reducing infection severity.

Polygonum minus, known as Kesum, is a perennial herbaceous plant belonging to the Polygonaceae family [14]. It is recognized for its aromatic leaves, which are utilized fresh in various dishes to add flavour and aroma. Beyond its culinary applications, the plant has a rich history in traditional medicine, where its leaves are employed to alleviate various ailments. In folk medicine, *P. minus* has addressed multiple health concerns, including digestive issues, dandruff [15], and inflammatory disorders [16]. Moreover, studies have suggested that extracts of *P. minus* exhibit antimicrobial, anti-inflammatory, antioxidant, antiulcer, antiviral, and antifungal [16, 17]. The antiviral potential of *Polygonum minus* is attributed to several bioactive compounds, particularly polygonumins B, C, and D, which exhibit significant medicinal properties. These compounds, derived from the plant's stem, have demonstrated inhibitory effects on HIV-1 protease and possess antioxidant activities, indicating their potential as antiviral agents [18]. Additionally, the plant contains polyphenols, including flavonoids, which contribute to its broad pharmacological effects, including antiviral activity against Herpes Simplex Virus 1 (HSV-1) [17, 19]. Quercetin is among the major flavonoids in *Polygonum minus* extract. Quercetin and its derivatives can effectively inhibit the binding of the SARS-CoV-2 spike protein to ACE2, with some derivatives showing IC_{50} values as low as 2.22 µM [20]. Quercetin also has demonstrated the ability to inhibit SARS-CoV-2 replication *in vitro*, with IC_{50} values around 145-166.6 µM [21].

Preliminary testing using a molecular docking approach is the optimal method for assessing the potential of plants as drug candidates for finding antivirals against SARS-CoV-2. This approach not only saves time but also reduces costs. By screening other flavonoids, phenolic acid, and terpenes contained in *P. minus* extract, the study aims to explore *P. minus* as alternative sources of potential therapeutic agents that could be more accessible and less toxic than synthetic drugs in inhibiting the key protein and enzyme in the lifecycle of SARS-CoV-2 via molecular docking.

METHODOLOGY

Preparation of Ligands and Target Proteins

Phytochemical compounds from *P. minus* were obtained from previous reports [15, 22]. A total of six compounds (phenolic acid, coumarin, flavonoid, and sesquiterpene) with potential against SARS-CoV-2 were selected for molecular docking studies and downloaded from the PubChem database (**Figure 1**).

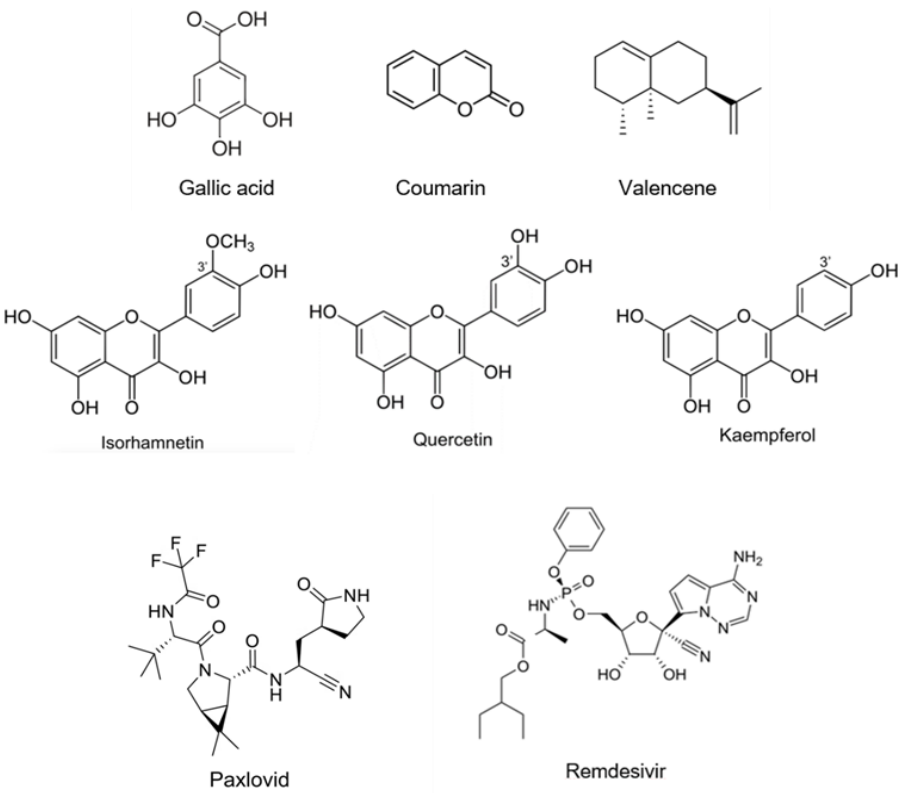

Figure 1. Ligand structures.

Gallic acid exhibits a high binding affinity to ACE-2 receptors which may enhance its efficacy against SARS-CoV-2 by blocking viral entry into host cells [23]. Coumarins exhibit antiviral activity against a range of viruses, including HIV, dengue, hepatitis, and influenza [24]. Furanocoumarins promote the degradation of viral proteins and reduce viral replication by targeting HBx protein in hepatitis B virus [25]. Quercetin is a flavonoid exist in *P. minus*. It exhibits antiviral activity against SARS-CoV-2 through multiple molecular mechanisms, primarily by inhibiting the interaction between the viral spike protein and the ACE2 receptor, as well as targeting viral proteases. This multifaceted approach positions quercetin as a promising candidate in the fight against COVID-19 [20, 26]. Isorhamnetin binds to the ACE2 receptor, inhibiting the entry of SARS-CoV-2 spike pseudotyped viruses into host cells [27]. Kaempferol derivatives have shown promise in inhibiting the 3a channel protein of coronaviruses, suggesting a novel therapeutic avenue [28]. Kaempferol interferes with viral polymerases and prevents viral attachment to host cells [29]. Valencene, a sesquiterpene, has garnered attention for its potential antiviral properties, particularly in the context of viral infections. Recent research indicates that compounds derived from valencene may play a role in inhibiting viral replication and managing viral diseases [30].

Paxlovid and Remdesivir were chosen as the reference molecules because it is a proven synthetic drug recently used in clinical treatment against SARS-CoV-2. Paxlovid is a combination of Nirmatrelvir and Ritonavir, where Nirmatrelvir targets the $3CL^{pro}$ enzyme, and Ritonavir targets the proteolytic enzyme in SARS-CoV-2 [31]. Therefore, Paxlovid was used as reference molecule in this study to target $3CL^{pro}$ and the proteolytic enzyme, PL^{pro}. While Remdesivir is a nucleotide analog that inhibits the RNA-dependent RNA polymerase (RdRp) of SARS-CoV-2 and therefore was used as the reference molecule to target RdRp protein [32]. The selected ligands' Three-Dimensional (3D) conformers were retrieved from PubChem (http://www.pubchem.ncbi.nlm.nih.gov) database in SDF formats. The potential ligands are gallic acid (CID: 370), coumarin (CID: 323), quercetin (CID: 5280343), isorhamnetin (CID: 5281654), kaempferol (CID: 5280863), valencene (CID: 9855795) and Paxlovid (CID:155903259) and Remdesivir (CID: 121304016). The in-built energy minimization parameters of the PyRx 0.8 tool were used to minimize the energy of all the ligands. The energy minimization parameters were kept default (force field = universal force field; optimization algorithm = conjugate; total number of steps = 200; number of steps for update = 1; stop is energy difference is less than = 0.1). The ligands were converted into the PDBQT file format [33].

Similarly, the X-ray 3D structures of proteins were downloaded from the protein data bank (PDB) and were retrieved (www.rcsb.org). Four distinct viral targets were considered for docking studies: Spike protein (PDB ID: 6LZG), RdRp (PDB ID: 6NUS), M^{pro} (PDB ID: 6LU7), and PL^{pro} (PDB ID: 6W9C) (**Figure 2**). The downloaded 3D structures were examined for the presence of water molecules, complex molecules, ions, and protein ligands, which were removed using Biovia Discovery Studio 2021 [34]. Each protein was then prepared by adding polar hydrogen atoms and saved in .pdbqt format using AutoDock Vina to be included as a reference in the virtual screening [35].

Antiviral Probability Prediction

Bioactive prediction of antiviral potential was analyzed using the PASS Online web (https://www.way2drug.com/passonline/predict.php). The category of predictions sought was antiviral. The parameter in this analysis is the probability to be active (Pa) and the probability to be inactive (Pi) value. The potential activity standard is Pa score > 0.3 and Pa>Pi [36].

Protein Binding Pocket Prediction

Sterile protein 3D structure was uploaded to the DoGSiteScorer online tool in PDB format to assess the predicted most likely suitable binding pockets based on durability by considering drug score values [37]. DoGSiteScorer is a grid-based method that applies a difference of Gaussian filter to detect potential binding pockets solely based on protein structure [38]. The pocket structure with the highest drug score pocket was downloaded and visualized in PyMoL to identify the exact residue.

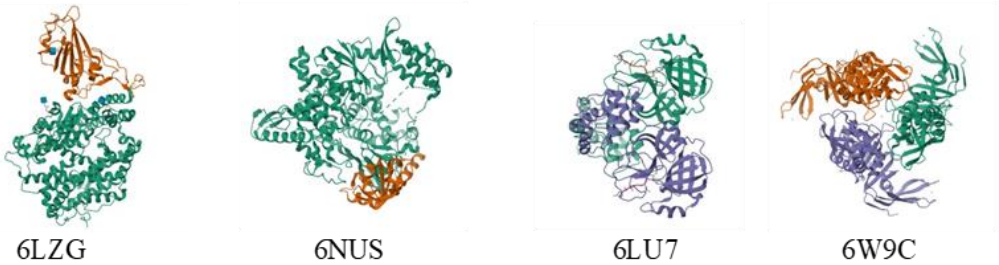

Figure 2. 3D structure of proteins available in protein data bank.

Molecular Docking

The optimized ligand and protein were subjected to molecular docking with the aid of the Auto Dock Vina function based on the predicted binding pocket. The grid box was set with center values of -7.3859, 0.8959, -22.5212 (XYZ coordinates) for (6LUZG) RBD spike protein, 153.9975,150.1565, 140.4328 (XYZ coordinates) for (6NUS) RdRp, 23.9171,26.1785,33.4485 (XYZ coordinates) for (6LU7) M^{pro}, 20.3033,29.7871,22.4854 (XYZ coordinates) for (6W9C) PL^{pro}. Each grid box was set at a dimension of 60 x 50 x 50 to cover the active site [35] that has been predicted in DoGSiteScorer. During docking runs, the 3D structure of the target is fixed while the ligand is moved and rotated to find the best binding modes [39]. The output file of docking was obtained with nine models respective to binding affinity and Root Mean Square Deviation (RMSD). The protein-ligand binding with the highest binding affinity and least RMSD will be selected for further study.

Protein-Ligand Interaction

Biovia Discovery 2021 explored two-dimensional (2D) interaction in that different bonds are formed between amino acid residues of the target protein and ligand. PyMoL used for 3D structure represents the exact location of the binding pocket of the target protein.

Pharmacokinetics and Drug-Likeness Analysis of Ligands

Potential ligands were analyzed to determine the probability of the candidate drug passing through the cell membrane using SwissADME online tools (http://www.swissadme.ch/). Lipinski's rule of five was used as the parameter where the Molecular Weight (MW) of < 500Da, hydrogen donor bond < 5, Hydrogen Bond Acceptor (HBA) < 10, high lipolysis (LogP) < 5, and molar refractory 40-130 [40]. Particular drugs at least achieved two rules out of five [36].

RESULTS AND DISCUSSION

This study aimed to investigate the bioactive compounds from *P. minus* against SARS-CoV-2. SARS-CoV-2, the so-called coronavirus, is an RNA-enveloped virus comprising structural and non-structural proteins. Structural protein includes spike protein, membrane protein, and envelope protein. Non-structural protein (NSP12) includes RdRp, main proteases (M^{pro}), and PL^{pro} [41]. The spike protein is indispensable for viral replication because it mediates the entry of the virus into the cell and is highly correlated with the virus's ability to infect. After SARS-CoV-2 enters the host cell, the released and uncoated large segment (>30 kb) viral RNA genome will produce two open reading frames and polyproteins[42]. These polyproteins are subsequently cleaved by papain-like protease (PLpro) and 3C protease-like protease (3CLpro) to produce nonstructural proteins (NSPs), such as RNA-dependent RNA polymerase (RdRp). Therefore, four viral protein targets, Spike, $3CL^{pro}$, PL^{pro}, and RdRp play a key role in the entire replication cycle of SARS-CoV-2 [43]. As to these four viral targets, there is great hope to develop effective treatment strategies that would interfere with the binding of viral Spike protein to the ACE2 receptor on the surface of host cells to prevent viral infection of cells, inhibit the activity of PL^{pro} and $3CL^{pro}$, interfering with their cleavage of multiple proteins to produce nonstructural proteins (NSPs) such as RdRp, and antagonize RdRp activity and block viral RNA transcription, synthesis and replication. Relying on these four viral protein targets to establish efficient drug screening methods may be convenient for finding natural products with anti-SARS-CoV-2 potential [44]. The positive control used in the study were Paxlovid and Remdesivir. Paxlovid is used as a positive control to assess the binding affinity of ligand with S protein, M^{pro}, and PL^{pro} while Remdesivir is used as a positive control to assess the binding affinity of ligand with RdRp protein and M^{pro} [45].

Pharmacokinetics, Drug-Likeness Analysis of Ligands, and Antiviral Probability Prediction

Polygonum minus compounds were chosen from previous reports [22, 40]. These compounds include gallic acid, coumarin, quercetin, isorhamnetin, kaempferol, and valencene. PASS online analysis results reveal that these six candidates have good antiviral activity with more than three probabilities active (Pa), and Pa values are larger than probability inactive (Pi), as summarised in **Table 1**.

Table 1. Lipinski's rule of five and prediction of antiviral activity of *Polygonum minus* compounds and control.

Molecule	MW	HBA <10	HBD <5	Log P <5	MR	Antiviral probabilities Pa>Pi	
						Pa	Pi
Paxlovid	499.53	8	3	1.09718	125.68	0.531	0.006
Remdesivir	602.58	12	4	2.31218	150.43	0.814	0.004
Gallic acid	170.12	5	4	0.21	39.47	0.654	0.009
Coumarin	146.14	2	0	1.82	42.48	0.358	0.061
Quercetin	302.24	7	5	1.23	78.03	0.403	0.046
Isorhamnetin	316.26	7	4	1.65	82.50	0.365	0.058
Kaempferol	286.24	6	4	1.58	76.01	0.496	0.005
Valencene	204.35	0	0	4.41	68.78	0.434	0.060

*Probability active (Pa), probability inactive (Pi)

Most phytochemical compounds have lower MW, hydrogen bonding capacity, and molar refractivity than Paxlovid and Remdesivir. Additionally, based on the provided data, gallic acid demonstrates a significantly higher antiviral probability (Pa) than Paxlovid but lower than Remdesivir. Paxlovid and Remdesivir has been used as a positive control in clinical trials due to their established effectiveness as a potential prodrug against SARS-CoV-2 infection [46, 47].

Gastrointestinal (GI) absorption and blood-brain barrier (BBB) permeation are two pharmacokinetic behaviors crucial to estimating at various stages of drug discovery. To this end, the Brain or Intestinal Estimated permeation method (BOILED-Egg) is proposed as an accurate predictive model that works by computing the lipophilicity and polarity of small molecules. The white region contains molecules more likely to be absorbed by the GI tract, while the yellow area (yolk) includes molecules more likely to permeate to the brain. Molecules predicted as not absorbed by the GI and BBB non-permeant are located in the grey area or even further outside the range of the plot [48]. Quercetin, kaempferol, isorhamnetin, and gallic acid fall in the white region indicating high gastrointestinal absorption while coumarin falls in the yellow region indicating high BBB permeation (**Figure 3**).

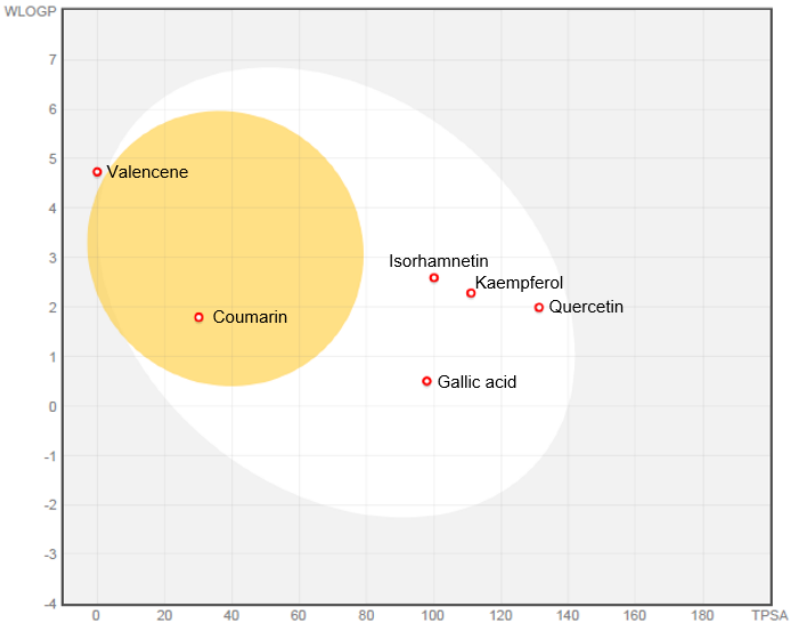

Figure 3. The BOILED-Egg model prediction of the gastrointestinal absorption (white) and blood-brain barrier (BBB) permeation (yellow).

Table 2. The docking score of six phytochemicals from *P. minus*, Paxlovid and Remdesivir with Spike protein, RdRp, M^{pro} and PL^{pro}.

Classification	Ligand	Docking score (kcal/mol)			
		Spike	RdRp	M^{pro}	PL^{pro}
Reference molecule	Paxlovid	-7.8	-6.3	-7.7	-6.8
Reference molecule	Remdesivir	-8.6	-6.2	-8.0	-7.8
Phenolic acid	Gallic acid	-6.3	-5.9	-5.5	-5.0
Coumarin	Coumarin	-7.5	-6.2	-5.1	-5.7
Flavonoid	Quercetin	**-9.7**	-5.9	-7.3	**-7.0**
Flavonoid	Isorhamnetin	-8.4	-5.7	-7.3	**-7.0**
Flavonoid	Kaempferol	-9.1	**-6.7**	**-7.8**	-6.7
Terpene	Valencene	-6.3	-6.0	-5.5	-5.8

Note: The lowest docking score among the phytochemical is highlight in bold

Molecular Docking of Protein-Ligand Interactions

The molecular docking results are discussed based on docking scores, types of interactions, and interactions within the catalytic site. These parameters aim to assess the stability of the protein-ligand interactions. Molecular docking evaluates the strength of interactions between a ligand and its protein target, with stability indicated by the docking score (kcal/mol). Notably, a lower docking score signifies a low binding energy and a high binding affinity with target protein. This exhibits a more stable ligand-protein complex and indicates the ligand's potential inhibitory activity against the SARS-CoV-2 protein [49]. The docking score of protein-ligand interaction (kcal/mol) is shown in **Table 2**.

After analyzing the docking scores, the interactions between the target proteins and ligands were visualized in 2D using Discovery Studio 2021 software. **Figures 4** illustrate the interactions between SARS-CoV-2 proteins and phytochemical compounds with the lowest docking scores, highlighting the various bonds between the viral proteins' amino acid residues and the ligands. For our phytochemical compounds to be selected as potential inhibitors of SARS-CoV-2, they must bind to the active site and interact with the catalytic residues presented in the active site. A compound must interact with essential amino acids of the enzyme via hydrogen bonds and hydrophobic interactions in order to inhibit the enzyme [50].

The spike protein, a homotrimer transmembrane glycoprotein, extends from the viral envelope and is essential for binding to and interacting with the host receptor [51]. In this study, the spike protein chain A was selected for docking simulations, as the receptor-binding domain (RBD) is located within this chain, specifically between ARG319 and PHE541 residues. SARS-CoV-2 variants have exhibited rapid transmission rates, largely due to the high mutation rate of certain viral proteins compared to the original SARS-CoV [52]. Among these, the spike protein is one of the most frequently mutated. The catalytic site of protein is usually less prone to mutation, as the mutation sites are generally located away from this region [49]. Binding at the catalytic site may provide critical insights for therapeutic development, particularly because this region is more conserved. The catalytic domain of the RBD spike protein includes key residues such as GLN493, CYS111, HIS272, and ASN286 [53]. In the molecular docking simulations, quercetin showed the lowest docking score of -9.7 kcal/mol. It was observed that quercetin did not form hydrogen bonds with the catalytic site of the RBD spike. However, it formed hydrogen bonds with ASN290, ILE 291, THR434, ASP431, and carbon-hydrogen bonds with LYS 541 as shown in **Figure 4**. The quercetin phenyl ring also forms pi-pi stacking with PHE438 and pi-alkyl interaction with ALA413 and PRO415. The presence of hydrogen bonds plays a significant role in maintaining interaction stability by resulting in dock score being more negative, whereas weak hydrophobic interactions (pi-pi, pi-alkyl, pi-cation or anion, pi-sigma, pi-amide), facilitate ligand turnover within the target protein during cellular processes [36, 54]. This result is supported by previous studies that report that quercetin exhibits antiviral activity against SARS-CoV-2 through multiple molecular mechanisms, primarily by inhibiting the interaction between the viral spike protein and the ACE2 receptor. This multifaceted approach positions quercetin as a promising candidate in the fight against COVID-19 [20, 26].

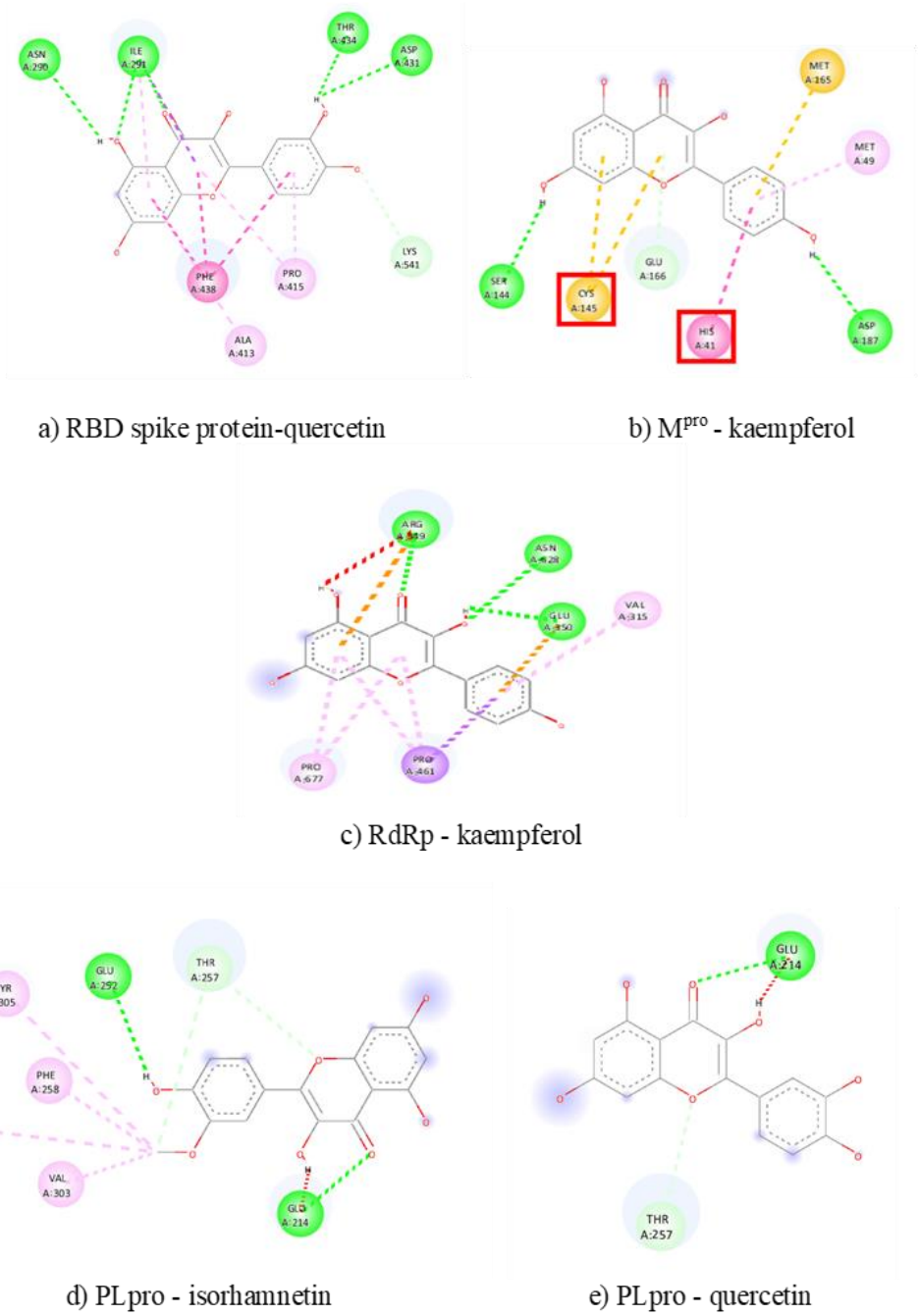

Figure 4. Visualization of molecular interaction between (a) the RBD spike – quercetin, (b) M^{pro} – kaempferol (c) RdRp – kaempferol, (d) PLpro – isorhamnetin and (e) PL^{pro} – quercetin. For the RdRp protein, molecular interactions are observed within the catalytic site shown in the red box. Notes: green = hydrogen bond, light green = carbon-hydrogen bond/ pi-donor hydrogen bond, dark pink = pi-pi stacked, pink = pi-alkyl, purple = pi-sigma, orange = pi-cation, yellow = pi-sulphur, and red = unfavourable bonds.

Inhibition of spike protein disrupts the interaction with angiotensin-converting enzyme 2 (ACE2), inhibiting SARS-CoV-2 cellular entry and preventing viral replication. Binding and entering host cells is the first step in COVID-19 infection. Inhibitors like [2-oxo-2-[2-(3-phenoxyphenyl) ethylamino]ethyl] phosphonic acid and Polydatin bind to the linoleic acid binding pocket in the RBD spike protein, maintaining stable interactions that impede the spike protein's function [55]. Compounds such as those derived from multivalent tryptophan derivatives interfere with the spike protein's binding to ACE2, effectively blocking viral entry [56]. Low molecular weight thiol compounds induce a redox switch in the spike protein, reducing disulfide bonds that are crucial for its interaction with ACE2, thereby inhibiting both entry and replication [57]. FDA-approved drugs against spike protein are Camostat Mesylate, Nafamostat Mesylate, Nelfinavir, Umifenovir (Arbidol),

Fenofibrate, Cefoperazone and Ceftazidime, and Thiostrepton. These drugs primarily function by either inhibiting the proteases involved in viral entry, directly blocking the spike protein's interaction with ACE2 or modulating host cell responses to reduce viral replication.

$3CL^{pro}$ is a non-structural protein that plays a crucial role in viral replication, specifically in the polyprotein cleavage process of SARS-CoV-2 [58]. Also known as M^{pro}, $3CL^{pro}$is a protease enzyme in SARS-CoV-2 [59]. The catalytic site of $3CL^{pro}$ (PDB ID: 6LU7) consists of HIS 41 and CYS 145 [60, 61], which can be targeted for molecular docking in the development of multivariate anti-SARS-CoV-2 therapies. Based on the docking results, kaempferol exhibited the lowest docking score of -7.8 kcal/mol and Paxlovid exhibited a docking score of -7.7 kcal/mol. Kaempferol bound to the catalytic site, forming pi-pi stacked interactions with HIS41 and pi-sulphur interactions with CYS 145, as depicted in **Figure 4**. Kaempferol also forms hydrogen bonds with SER144, ASP187, and pi-donor hydrogen bonds with GLU166. The phenyl ring of kaempferol forms pi-alkyl interaction with MET49 and pi-sulphur interaction with MET165. Kaempferol interacts with two same residues that matches the reference molecule, Paxlovid which are HIS41 and GLU166 as shown in **Table 3**. Paxlovid, an oral antiviral treatment for COVID-19, operates primarily through its two active components: Nirmatrelvir and Ritonavir. Nirmatrelvir binds covalently to the active site of $3CL^{pro}$ (M^{pro}), preventing the cleavage of viral polyproteins necessary for viral replication [62]. Another study also reported that Nirmatrelvir reversibly binds to the catalytic cysteine residue of the SARS-CoV-2 $3CL^{pro}$ protease. Ritonavir, while less potent against SARS-CoV-2, increases the plasma concentration of Nirmatrelvir, ensuring sustained antiviral activity [63]. Early administration of Paxlovid has been shown to significantly lower hospitalization rates and mortality among at-risk patients. Studies indicate that Paxlovid reduces the time required for viral elimination in patients infected with SARS-CoV-2 variants [64].

Table 3. Dock score and overall protein – ligand interaction.

Protein	Ligands	Dock Score (kcal/mol)	H-bond interactions	Other interaction	No. of hydrogen bond
Spike	Remdesivir	-8.6	ILE 291,GLU 406, SER 409,THR 434	MET 366,LEU 370, ALA 423,PRO 415, PHE 438,HIS 540	4
Spike	Paxlovid	-7.8	ASN 290	ASP 367,LYS 441	1
Spike	Quercetin	-9.7	ASN 290,ILE 291, THR434,ASP 431	ALA 413,PRO 415, PHE 438,LYS 541	4
$3CL^{pro}$	Paxlovid	-7.7	THR 26,ASN 142, GLY 143,GLU 166, GLN 189	HIS 41	5
$3CL^{pro}$	Kaempferol	-7.8	SER 144,ASP 187	HIS 41,MET 49, CYS 145,MET 165, GLU 166	2
PL^{pro}	Paxlovid	-6.8	TYR 213,GLU 214, TYR 251	LYS 254	3
PL^{pro}	Quercetin	-7.0	GLU 214, THR 257		2
PL^{pro}	Isorhamnetin	-7.0	GLU 214,GLU 252	LEU 253,PHE 258, VAL 303,TYR 305	2
RdRp	Remdesivir	-6.2	ASN 628,PRO 677	ARG 249,ARG 349, ASN 452,LEU 460, PRO 461	2
RdRp	Kaempferol	-6.7	ARG 349,GLU 350, ASN 628	VAL 315,PRO 461, PRO 677	3

PL^{pro} is a family of proteolytic enzymes associated with polyprotein cleavage, similar to M^{pro} [58]. In this study, isorhamnetin and quercetin were identified as having the lowest docking scores for interactions with PL^{pro} with a docking score of -7.0 kcal/mol, and Paxlovid exhibited a docking score of -6.8 kcal/mol. The catalytic residues of PL^{pro} include CYS 111, HIS 272, and ASP 286 [53]. However, quercetin and isorhamnetin did not exhibit interactions at the catalytic site residues, as shown in **Figures 4**. Additionally, an unfavorable donor interaction was observed at GLU214. Despite this, multiple hydrogen bonds with GLU252, THR257, and GLU214 stabilized the ligand-protein complexes, achieving the desired interaction conformation. Isorhamnetin also form alkyl and pi-alkyl interaction with TYR305, PHE258, LEU253 and VAL303. Isorhamnetin interacts with the same residue as the reference molecule, Paxlovid, which is GLU214 (**Table 3**). Although quercetin has the same docking score as isorhamnetin, quercetin only interacts with two residues of PL^{pro}. Quercetin forms a hydrogen bond with GLU214, a carbon-hydrogen bond with THR257, and unfavorable donor-donor interaction with GLU214. Unfavorable donor-donor interactions can significantly impact the binding stability and energetics of molecular complexes. These interactions often arise when multiple donor sites compete for binding, leading to decreased binding affinity and stability [54, 65]. Previous studies reported that the small molecule inhibitors bind to PL^{pro}, forming hydrogen bonds with residues ASP164, GLN269, and TYR273, while van der Waals interactions dominate the binding free energies, stabilizing the complex and inhibiting PL^{pro}'s function in coronavirus replication and immune evasion. The studies also have identified several compounds with high binding affinities, including dietary compounds like hypericin and rutin [66, 67]. FDA-approved drugs such as imatinib target an allosteric site (BL2 groove), which disrupts access to the catalytic site, thereby inhibiting PL^{pro} function [68]. Paxlovid does not directly inhibit PL^{pro}, but there is potential for combination therapies that target both M^{pro} and PL^{pro} to enhance antiviral efficacy. Studies indicate that using inhibitors for both proteases can lead to a more effective suppression of SARS-CoV-2 replication. This dual-target approach could be particularly beneficial in addressing emerging variants of the virus that may exhibit resistance to single-target therapies [69, 70]. Therefore, based on this study, kaempferol could be combined with isorhamnetin or quercetin to target both M^{pro} and PL^{pro}.

SARS-CoV-2 RdRp is a non-structural protein that plays a significant role in the replication and transcription of SARS-CoV-2 [58]. RdRp 6NUS catalytic site located at SER 709, GLY 774, and SER 784 [53]. Kaempferol has shown the most stable interaction for inhibiting RdRp protein with dock score of -6.7 kcal/mol lower than Remdesivir, -6.2 kcal/mol. Kaempferol has interacted with ARG349, ASN628, and GLU350 through hydrogen bonds. The phenyl group of kaempferol forms pi-alkyl stacking with VAL315 and PRO677, pi-sigma with PRO461, pi-cation, and pi-anion with ARG349 and GLU350. The O-H group on the phenyl ring forms unfavorable donor-donor interaction with ARG349. Thus, the stability and turnover of interactions were achieved and facilitated by weak hydrophobic interactions [36]. Kaempferol interacts with four same residues that match the reference molecule, Remdesivir which are ARG349, ASN628, PRO461, and PRO677 as shown in **Table 3**. Remdesivir acts as a nucleoside analog, specifically inhibiting the RdRp enzyme. When incorporated into the growing RNA strand during replication, it leads to premature termination of RNA synthesis. This occurs after the addition of three nucleotides following Remdesivir incorporation, at which point further elongation is stalled due to a translocation barrier that prevents the addition of subsequent nucleotides [71, 72]. Previous studies indicate that Remdesivir exhibits strong binding affinity not only to RdRp but also to other viral proteins such as the main protease (M^{pro}) and membrane protein (Mprotein). The binding energies suggest that Remdesivir's interaction with RdRp is primarily governed by electrostatic interactions, while van der Waals forces dominate its binding to M^{pro} [73].

However, molecular docking studies face several limitations that can impact their effectiveness. These challenges primarily stem from the inherent complexities of molecular interactions and the computational methods employed. Molecular docking often struggles to accurately predict ligand binding affinities due to oversimplified models and scoring functions [74]. Molecular docking methods also assume rigid protein structures, neglecting the dynamic nature of proteins, which can lead to inaccurate predictions of binding modes [75]. Therefore, further *in-vitro* studies should be conducted to evaluate the properties of these phytochemicals for the development of drug targets against the SARS-CoV-2 protein and enzymes.

Structure-Activity Relationship Between Flavonoid, Phenolic, Coumarin, and Terpene

Quercetin, kaempferol, and isorhamnetin from the flavonoid group exhibit numerous interactions with $3CL^{pro}$ residues. Gallic acid also forms interaction comparable to flavonoids. However, coumarin and valencene only form four and three interactions, respectively with protein residues (**Table 4** and **Figure 5**).

Table 4. Representation of overall interactions between phytochemicals and $3CL^{pro}$.

Phytochem.	HB	Pi-donor HB	Pi-pi	Pi-alkyl	Pi-sigma	Pi-S	Alkyl	Unfav. donor-donor	Total interaction
Gallic acid	5	-	-	-	-	-	-	2	7
Coumarin	-	1	1	2	1	-	-	-	4
Quercetin	5	1	-	1	-	-	-	1	8
Kaempferol	2	1	1	1	-	3	-	-	8
Isorhamnetin	4	-	1	-	-	1	3	-	9
Valencene	-	-	-	3	-	-	-	-	3

Note: HB: hydrogen bond, S: Sulphur

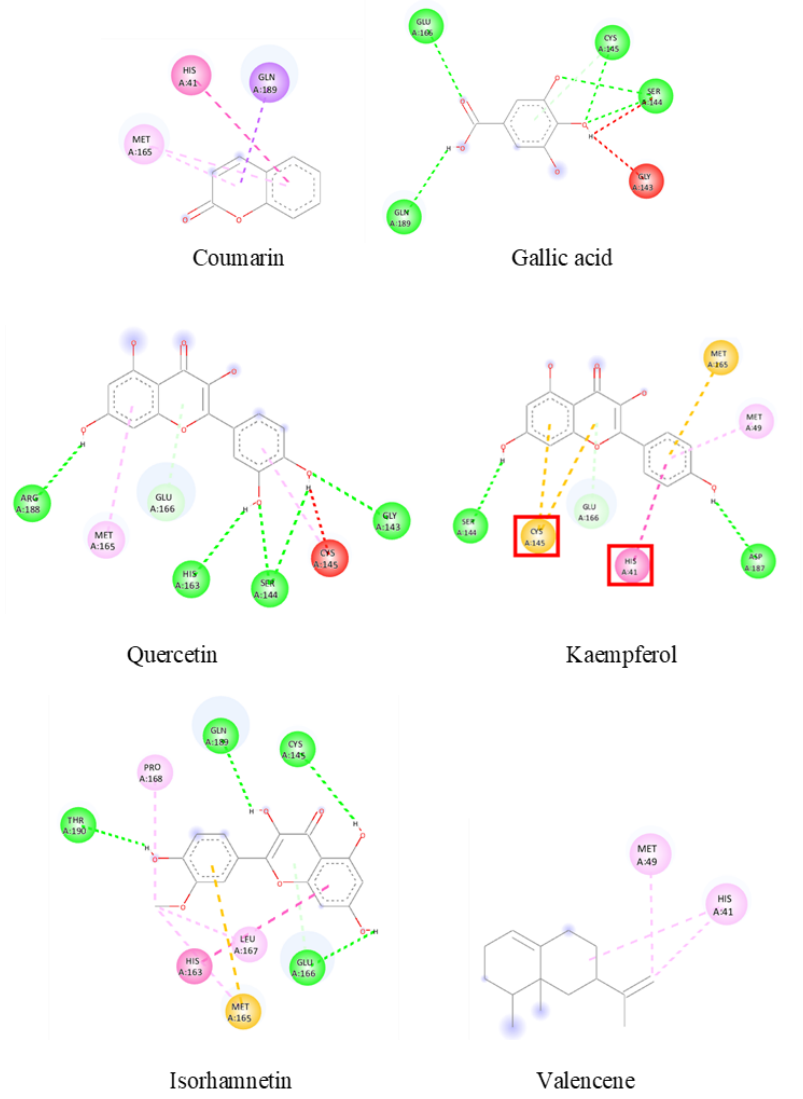

Figure 5. Visualization of phytochemical interaction with $3CL^{pro}$. Note: Green = hydrogen bond; light green = pi-donor hydrogen bond; purple = pi-sigma; dark pink = pi-pi; light pink = pi-alkyl/ alkyl; yellow = pi-sulphur; red = unfavourable donor-donor.

The most populated group of hydrophobic interactions is the one formed by an aliphatic carbon in the receptor and an aromatic carbon in the ligand. This is an indication that aromatic rings are prevalent in small-molecule inhibitors. In fact, 76% of the market drugs contain one or more aromatic rings, with benzene being by far the most frequently encountered ring system. The benzene rings of coumarin, quercetin, kaempferol, and isorhamnetin form pi-alkyl interaction with $3CL^{pro}$ residues [76]. Additionally,

benzene ring of kaempferol exhibit pi-sulfur interaction with MET165 and CYS145. Interaction involving sulfur atom from the side chain of methionine with aromatic carbon from the ligand yields an additional stabilization energy of 1-1.5 kcal/mol compared with a purely hydrophobic interaction. This could be the reason kaempferol has the lowest docking score against $3CL^{pro}$ compared to quercetin and isorhamnetin. The benzene ring of the ligand also forms pi-stacking with protein residues. Interactions involving aromatic rings are major contributors to protein-ligand recognition and drug design. While π-stacking interaction can increase the binding affinity of the inhibitor for its target, it has been pointed out that reducing the number of aromatic rings of a molecule might improve its physicochemical properties, such as solubility. The aromatic ring of quercetin and kaempferol also forms a pi-donor hydrogen bond with glutamic acid. This is a weak hydrogen bond. However, it is increasingly recognized that C–H···O hydrogen bonds play an important role in molecular recognition processes, protein folding stabilization, in the interaction of nucleic acids with proteins, in enzyme catalysis, and in the stabilization of protein–ligand binding complexes. The hydroxyl group and carbonyl oxygen from gallic acid and flavonoids form hydrogen bond with target protein. Hydrogen bonds are the prevailing directional molecular interactions in biological complexes and the predominant contribution to the specificity of molecular recognition. The free energy for hydrogen bonding can vary between -1.5 kcal/mol to -4.7 kcal/mol. Compound without hydroxyl group, carbonyl oxygen group and aromatic ring such as valencene did not exhibit hydrogen bonding and pi-interaction which is important in inhibiting target protein. Therefore, we can conclude that flavonoid has the best structure as an inhibitor against coronaviruses.

Flavonoids may complement existing COVID-19 treatments through various strategies. Clinical studies indicate that quercetin supplementation may alleviate inflammation in early COVID-19 patients [77]. Combining flavonoids with conventional antiviral drugs could enhance therapeutic efficacy. For example, quercetin's synergistic effects with vitamin C have been noted, suggesting potential benefits when used alongside standard treatments. Additionally, incorporating flavonoid-rich foods into the diet may provide a preventive measure against COVID-19. This approach could be particularly beneficial for high-risk populations [78]. However, the effective delivery of flavonoids poses several challenges that need to be addressed. Many flavonoids have low bioavailability due to poor absorption in the gastrointestinal tract. Formulating them in nanoparticles or using liposomal delivery systems could enhance their absorption and therapeutic effectiveness [79]. Flavonoids can be sensitive to light and heat, which may affect their stability in formulations. Developing stable formulations that protect flavonoids from degradation during storage and use is essential for their clinical application [80].

CONCLUSION

In summary, molecular docking of six phytochemicals from *P. minus* or kesum with the spike protein, $3CL^{pro}$, PL^{pro} and RdRP of SARS-CoV-2 demonstrated that flavonoids exhibit better binding affinity to the protein than the reference molecule, Paxlovid, and Remdesivir. The top-ranking interactions, characterized by the highest binding affinity between proteins and ligands from *P. minus* are spike protein-quercetin (-9.7 kcal/mol), RdRp-kaempferol (-6.7 kcal/mol), $3CL^{pro}$-kaempferol (-7.8 kcal/mol), and PL^{pro}-quercetin (-7.0 kcal/mol) and PL^{pro} -isorhamnetin (-7.0 kcal/mol). The analysis of the molecular interactions reveals that kaempferol interacted with both of the catalytic residues (CYS145A and HIS41A) of $3CL^{pro}$ indicating the potential of kaempferol as a potent antiviral agent. Additionally, kaempferol, quercetin, and isorhamnetin interact with essential amino acids of the enzyme via hydrogen bonds and hydrophobic interactions, stabilizing the complex and inhibiting spike protein, PL^{pro} and RdRp. These flavonoids might complement existing treatments through various strategies such as adjunct therapy and dietary supplementation. Although molecular docking studies face several limitations that can impact their effectiveness, the insights gained from molecular docking studies on SARS-CoV-2 can inform broader antiviral strategies applicable to other coronaviruses. Given that many coronaviruses share structural similarities, compounds identified as effective against SARS-CoV-2 may also exhibit activity against related viruses. Continued research using molecular docking can facilitate the rapid identification of potential antiviral agents against emerging coronaviruses by leveraging existing data. This approach not only accelerates drug discovery but also enhances preparedness for future outbreaks by enabling swift adaptation of existing therapies.

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