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Inhibitory Activity of Natural Flavonoids against N439K Variant of SARS-CoV-2 Spike Protein: an In Silico Analysis

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ABSTRACT. *Angiotensin-converting enzyme 2 (ACE2) is a functional receptor of the spike protein SARS-CoV-2 that plays an important role in the pathogenesis of COVID-19, especially in providing a passage for the virus to enter human cells. Flavonoids have high bioactivity beneficial for health, such as anti-inflammatory, anticancer, antioxidant, anti-lipogenic, metal-chelating, antimicrobial, and antiviral properties. We predict that flavonoids can be used as inhibitors for spike protein and ACE2 interaction. This study aims to compare the potential of flavonoid compounds in herbal medicine as an inhibitor for the spike protein and ACE2 receptors interaction to prevent COVID-19 transmission. Curcumin, 6-gingerol, luteolin, brazilin and kaempferol were used as ligands to dock with wild-type S protein and N439K variant S protein. Drug likeness properties were also evaluated using SwissADME. Molecular docking analysis of five active compounds of flavonoid to wild-type S protein and ACE2 complex shows kaempferol to have the lowest binding energy, meanwhile for mutant S protein and ACE2 complex, 6-gingerol has the lowest binding energy. Both compounds also form hydrogen bonds at the most active binding site. Kampferol showed the best inhibitory activity for wild-type variant. In contrast, 6-gingerol might have the best potential to interfere S protein-ACE2 interaction for the N439K variant S protein.*

Keywords: *ACE2, flavonoid, N439K spike protein, SARS-CoV-2, wild-type spike protein*

INTRODUCTION

One of the key functional receptor in the pathogenesis of COVID-19 is Angiotensin-converting enzyme 2 (ACE2) which facilitate the entry of SARS-CoV-2 virus to human cells. The viral spike protein (S) of SARS-CoV-2 binds to ACE2 as a cellular receptor, leading to the entry to the viral host cell and S-protein priming by the host cell's TMPRSS2 protease. SARS-CoV-2 cell entry and pathological effects occur on the respiratory tract cell, mainly on the upper respiratory tract. ACE2 is widely expressed in human tissues, both in the primary target organ of SARS-CoV-2 and organs that are likely to play a minor or even unknown role in COVID-19 pathophysiology like heart, kidney, testis, skin, smooth muscle cell of the brain, and various part of intestine [1]. ACE2 expression in each individual varies depending on genetic factors, age, sex, obesity, comorbidities, and immunosuppressive drugs [2].

Currently, there is no specific treatment for COVID-19. Researchers continue to find drugs that can inhibit or prevent transmission of this disease, including using traditional herbal medicines. For centuries, people worldwide have used traditional ingredients abundant in nature to treat various diseases. Traditional herbal medicines are believed to have the ability to treat various kinds of disease, yet are safer than commercially available drugs since it has not gone through a lot of processing [3]. There is even a growing trend in the community to use traditional medicine.

Among the various chemical compounds of traditional herbal medicines, flavonoids are the most abundant. Flavonoids are heterogeneous compounds with various structures found in large quantities in plants, and their presence plays an important role in plant physiological function. Furthermore, flavonoids in various traditional medicinal plants have been extensively studied for their high bioactivity beneficial to health, such as anti-inflammatory [4], anticancer [5], antioxidant [6], anti-lipogenic [7], metal-chelating [8], antimicrobial [9], and antiviral [10] properties. Previous studies showed that flavonoid supplementation decreased upper respiratory infection risk by 33% [10]. Regarding these properties, various herbal medicines are then investigated to prove their ability to inhibit the binding activity of spike protein of COVID-19 and human ACE2. The types of flavonoids used in this research are curcumin in *Curcuma longa*

Linn., 6-gingerol in *Zingiber officinale*, luteolin in *Sonchus arvensis*, a brazilin in *Caesalpinia sappan* L., and kaempferol in *Psidium guajava*. These plants are among the most popular herbal medicines with high natural availability worldwide [11].

We are also interested in comparing the inhibitory activity of these five potential flavonoids in herbal medicine towards the wild-type and mutant SARS-CoV-2 spike proteins. Because of the receptor binding domain (RBD) affinity enhancement, which increases its attachment ability to hACE2, the N439K mutant spike protein was used as a mutant variant because it was thought to be potentially more infectious than the wild-type variant. Moreover, N439K is the second most commonly observed RBD mutation worldwide and the sixth most common S mutation as of January 6th, 2021, this mutation was observed in more than 34 countries [12]. This study aims to compare the inhibiting activity of six flavonoids: Curcumin, 6-gingerol, luteolin, brazilin, and kaempferol against SARS-CoV-2 wild-type and N439K variants spike protein to prevent COVID-19 transmission.

RESEARCH METHODS

Data mining

Data mining included the search for the protein structure of the receptor and wild-type viral S protein subunit 1 (PDB ID: 6LZG), and mutant viral S protein subunit 1 (PDB ID: 7L0N) on the RCSB protein data bank (PDB) website (https://www.rcsb.org/) then downloaded in .pdb format, as well as a search for the 2D structure of the bioactive compound curcumin (PubChem CID: 969516), 6-gingerol (PubChem CID: 442793), luteolin (PubChem CID: 5280445), brazilin (PubChem CID: 73384), kaempferol (PubChem CID: 5280863) on the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and in SDF format.

Physico-chemical analysis

Curcumin, gingerol, luteolin, brazilin, and kaempferol were analyzed for physico-chemical analysis using the SWISS-ADME website (http://www.swissadme.ch/). The investigation was carried out based on the Lipinski Rule of 5, which consists of several parameters including molecular weight (MW), logP, H-Acceptor (HBA), H-donor (HBD), rotatable bonds (RB), and polar surface area (PSA) [11].

Receptor, protein and compound preparation

ACE2 and S protein were prepared by removing water molecules and native ligands using BIOVIA Discovery studio [14]. In contrast, flavonoid compounds of curcumin, gingerol, luteolin, brazilin, and kaempferol were prepared by minimizing the energy using PyRx [15].

Molecular docking

Molecular docking was performed using Hex 8.0.0 [16]. The first docking analysis was performed on the ACE2 receptor, and S protein of SARS-CoV-2 without any chemical compound added to see the original binding pattern. Then, the S protein was docked with flavonoids (curcumin, gingerol, luteolin, brazilin, and kaempferol) to the binding affinity of the flavonoid and the S protein. The docking result of SARS-CoV-2 S protein and flavonoids was then docked to the ACE2 receptor to see how the flavonoids affected the binding activity of S protein and the ACE2 receptor. This molecular docking process was done to both of the S proteins used.

Visualization

The docking results are then visualized using BIOVIA discovery studio [14]. ACE2 structures,S protein, and flavonoids bioactive compounds were given different colors. The receptor binding motives (RBM) regions on the S protein were colored differently even though this part is not in different domains or structures to make it easier to recognize since this area is an active binding site of S protein. Ligand interaction, including chemical bond, binding location, and interacting residues, were collected for further analysis.

RESULTS AND DISCUSSIONS

Drug likeness properties

All of the analyzed compounds have good potential as drug candidates (Table 1), given the suitability to Lipinski's Rule of 5 (LRO 5). There was no incompatibility in molecular weight, LogP value, number of H-bond donor and acceptor, number of rotatable bonds, and Total Polar Surface Area (TPSA) value. These properties demonstrated that seven active compounds from traditional herbal medicine have good permeability and flexibility as drug candidates for carrying out their biological activities.

Potential inhibitory activity against wild type S protein

The molecular docking results between different active compounds of five traditional herbal medicines towards wild-type S protein and ACE2 complex were shown in Figure 1. Among the other compounds, kaempferol appears to have the lowest binding energy (Table 2). This indicates that the amount of energy required for

those compounds to bind was relatively low, increasing the bonding possibility. Lower binding energy tends to increase the effectiveness of bioactive compounds reactions.

Interaction complexes residues of wild-type spike protein and flavonoid class compounds were shown in Table 3. Kaempferol and curcumin tend to have the highest inhibiting potential for wild-type spike protein-ACE2 complex followed by brazilin, luteolin, and 6-gingerol. This analysis was obtained from the hydrogen bond formed between amino acid residues at the most active binding sites. The active binding site of the control complex was known as receptor-binding motif (RBM), placed around the 424-492 and 438-506 amino acid chain.

Table 1. Drug-likeness properties of five analyzed flavonoids compound

Compound	Molecular weight (g/mol)	log P	H-Bonds Acceptor (HBA)	H-Bond Donor (HBD)	Rotatable Bonds (RB):	Polar Surface Area (Å)
Curcumin	368.38	3.27	6	2	8	93.06
6-Gingerol	294.39	3.48	4	2	10	66.76
Luteolin	286.24	1.86	6	4	1	111.13
Brazilin	286.28	1.53	5	4	0	90.15
Kaempferol	286.24	1.70	6	4	1	111.13

Table 2. The binding energy of analyzed ligands interacted with targeted proteins

Ligand	Binding energy (cal/mol)			
	Spike protein wild type	Spike protein mutant	Spike WT-ligand-ACE2	Spike mutant-ligand-ACE2
Control	-	-	-1224.80	-688.12
Curcumin	-288.88	-286.36	-1042.88	-721.19
6-Gingerol	-258.30	-577.07	-1097.85	-827.28
Luteolin	-230.41	-212.27	-1060.21	-669.48
Brazilin	-224.70	-209.40	-1052.88	-698.26
Kaempferol	-228.14	-223.10	-1162.87	-780.72

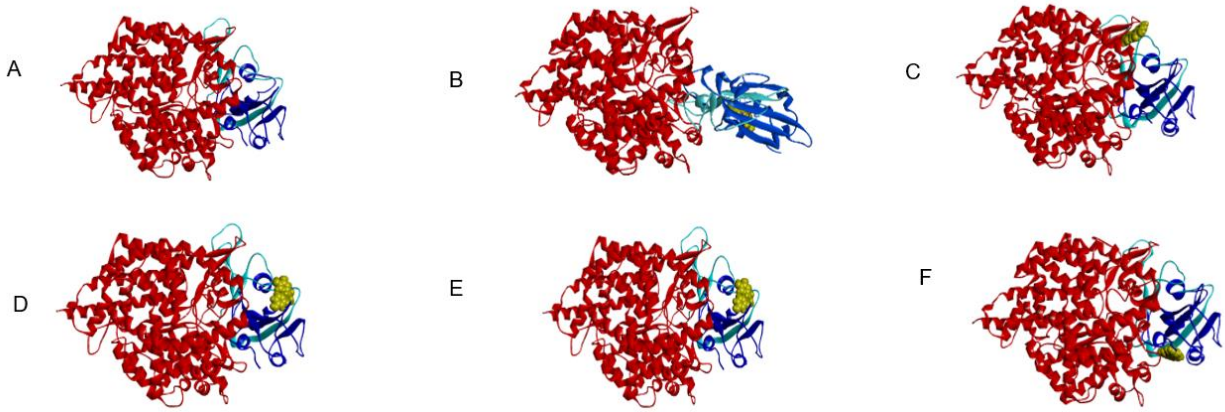

Figure 1. Wild-type S protein-flavonoids complexes with ACE2 SARS-CoV-2 visualization (Red: ACE2, Blue: S protein, Yellow: flavonoids) A. Control; B. Curcumin; C. Luteolin; D. Brazilin; E. 6-Gingerol; F. Kaempferol

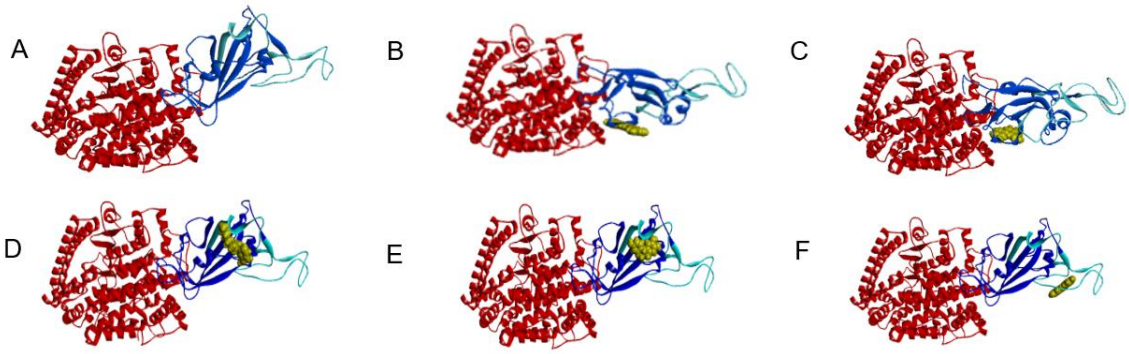

Figure 2. N439K S protein-flavonoids complexes with ACE2 SARS-CoV-2 visualization (Red: ACE2, Blue: S protein, Yellow: flavonoids) A. Control; B. Curcumin; C. Luteolin; D. Brazilin; E. 6-Gingerol; F. Kaempferol.

Potential inhibitory activity against N439K mutant S protein

The molecular docking results between five active compounds of flavonoid class in herbal medicine with N439K mutant S protein and ACE2 receptor were shown in Figure 2. The compound with the lowest binding energy was 6-gingerol, while luteolin had the highest binding energy (Table 2).

Indicating that there were no active compounds that interacted with the active sites of S protein or ACE2 receptor, nor did any compounds bind in the RBM sites. On the other hand, the residue interaction complexes of N439K mutant S protein and flavonoids were shown in Table 3, indicating possible inhibiting activity from those active compounds tested. The compound with the most hydrogen bonds was luteolin, while kaempferol had the fewest. Higher hydrogen compounds represent the stability of the ligand interaction.

Table 3. S protein (wild-type and N439K mutant) and ACE2 residues interaction induced by flavonoid compounds

(Type of Interaction) Interacting Residues Spike-ACE2					
Varian	**Spike-Curcumin-ACE2**	**Spike-6-Gingerol-ACE2**	**Spike-Luteolin-ACE2**	**Spike-Brazilin-ACE2**	**Spike-Kaempferol-ACE2**
Wild type	(HB) Thr345-Asp299 (HB) Arg346-Asp295 (HB) Arg346-Asp292 (HB) Arg346-Asp292 **(HB) Lys444-Asp367** **(ES) Lys444-Asp292** **(HB) Asn487-Asn250** **(HB) Gln493-Ser280** **(HB) Ser494-Ser280** **(HB) Gln498-Asn277** **(HB) Gly502-Glu150** (ES) Arg509-Asp295 (HP) Tyr449-Tyr279	(ES) Thr333-Asp609 (HB) Pro337-Glu483 (HB) Ala344-Glu467 (HB) Thr345-Glu467 (HB) Ala348-Glu224 (HB) Arg346-Glu197 (ES) Arg346-Glu197 (ES) Arg346-Asp201 (HB) Asn354-Glu227 (HB) Asn354-His228 (ES) Lys356-Glu483 (HB) Arg357-Glu231 (ES) Arg357-Glu231 (HP) Arg357-Lys234 (HB) Arg357-Glu238 (ES) Arg357-Glu238 (HB) Ser359-Glu483 **(ES) Arg466-His228** **(ES) Arg466-Glu232** **(HB)Thr470-Gly214**	(HB) Arg346-Asp368 (ES) Arg346-Asp368 (HB) Arg357-Leu156 (HP) Arg357-Leu156 (ES) Arg357-Asp157 **(HB) Lys444-Thr362** **(ES) Arg457-Glu140** **(ES) Lys462-Glu140** **(ES) Arg466-Glu150** **(ES) Arg466-Glu160** **(HS) Ile468-Leu142**	(HB) Leu335-Gln287 (HB) Pro337-Gly286 (HB) Thr345-Asp367 (ES) Arg346-Asp367 (HB) Arg346-Asp368 (ES) Arg346-Asp368 **(ES) Lys444-Asp335** **(HS) Arg457-Glu140** **(HB) Arg466-Glu160** **(ES) Arg466-Glu160** **(HB) Arg466-Glu150** **(ES) Arg466-Glu150** **(HB) Ser469-Cys141**	(HB) Arg346-Asp368 (ES) Arg346-Asp368 **(HB) Arg457-Glu140** **(HB) Lys444-Asp335** **(ES) Lys444-Asp335** **(HB) Lys458-Gln139** **(HB) Arg466-Glu150** **(ES) Arg466-Glu150** **(HB) Arg466-Glu160** **(HB) Ser469-Cys141**
N439K Mutant	(HB) Gly339-Gln287 (HB) Asn388-Asn250 (HB) Ser530-Asn250	(HB) Lys529-Glu150 (ES) Lys386-Asp292 (ES) Lys386-Asp295 (HB) Lys528-Asn277 (HB) Lys528-Asn277 (HP) Pro521-Lys247 (HP) Lys528-Leu281	(HP) Phe329 -Leu156 (HB) Lys386-Pro253 (HB) Asn388-Asn250 (HB) Lys529-Ser155 (HB) Lys529-Asp157 (ES) Lys529-Asp157 (HB) Ser530-Ala251	(HP) Cys391-Val283 (HP) His519-Phe285 (HB) Ala522-Pro284 (HP) Lys528-Leu281 (HB) Lys529-Glu150 (HB) Lys529-Asn154	(ES) Lys386-Asp292 (HB) Cys391-Gly286 (HB) Ala522-Pro284 (HP) Cys391-Ser280 (HP) His519 -Phe285 (HP) Cys391-Val283 (HP) Lys528-Leu281

Discussion

Flavonoids are abundant in traditional herbal medicines. Curcumin, gingerol, luteolin, brazilin, and kaempferol are popular flavonoid compounds in Asia. Before incorporating these ingredients into drugs for specific diseases, their physicochemical properties must be evaluated. According to the LRO 5 law, physico-chemical properties are strongly correlated with the drug similarity to carry out specific biological activity. The analyzed compounds must meet criteria for drug absorption, distribution, efficacy, metabolism, and excretion (ADME).

Flavonoids are an important class of natural products with several subgroups. Flavonoids contain a flavan core with the 15-carbon skeleton. Two benzene rings (A and C rings) are connected by a heterocyclic pyran ring (B ring). The resorcinol moiety of ring A might play a role in ACE2 inhibition. This group might disrupt hydrogen bonds between Glu329/Gln325 of ACE2 and Arg426 of the S protein SARS CoV-2, which form a salt bridge to stabilize their interaction. This hydrophobic interaction occurs in carbon rings with non-polar amino acid residues such as Gly354, Asp355, and Phe 356[15].

Spike protein is a main mediator of SARS-CoV-2 with ACE2. Flavonoids in herbal medicine are known to have the ability to inhibit S protein binding to the receptor. Based on the analysis, kaempferol is known to have the potential as an ACE2-S protein inhibitor since it can bind strongly to the amino acid residues located on RBM spike protein. This analysis is also supported by a report that kaempferol functions as a noncompetitive inhibitor of 3CLpro and PLpro.

Kaempferol also has counteracting virus production activity by blocking the 3a channel and interfere other steps of viral life cycle, so it can be used as an antiviral agents [15]. RBM is a loop-binding in the receptor-binding domain (RBD) of the protein spike subunit 1 and plays an important role in COVID-19 transmission since this area is the most active area to form a binding formation [16]. The hypothesis shows that the spike variation of the N439K protein forms a new salt bridge at the RBD-hACE2 interface (RBD N439K: hACE2E329), causing changes to the active site, which was originally RBM [10]. This evidence suggests that the unusual binding phenomenon visualized in Figure 2 is reasonable since the increasing affinity of RBD and possible salt bridge formation causes different binding positions between S protein and ACE2. As a result, all five tested flavonoids are not bound to the RBM but the RBD of S protein.

Based on the analysis, there are no compounds that interacted with the active sites of S protein mutant, ACE2 receptor, or the RBM sites. However, 6-gingerol has the lowest binding energy among the tested compounds. The lower the energy binding means the higher protein affinity with the stronger ligand [17]. From the residue interaction complexes of mutant S protein and flavonoids, it is known that luteolin has the most hydrogen bond. The more of the hydrogen presence indicated a stronger interaction [18]. Based on that, 6-gingerol and luteolin have the highest potential to interfere with S protein mutant-ACE2 interaction.

Bioactive compounds that bind to crucial amino acid residues inhibit the formation of the S protein-ACE2 protein complex. In comparison, the compounds that do not bind to key amino acid residues can interfere with forming the S protein-ACE2 protein complex [19]. Note that the presence of this kind of interaction does not completely prevent the substrate from binding. It just slows down the rate at which the S protein is bound to the ACE2 receptor. At this point, flavonoids are considered non-competitive ligands that bind to allosteric sites to interfere with the binding activity or even alter the affinity of both proteins.

However, giving curcumin to patients with COVID-19 needs further consideration. Given that several studies show that the highest comorbidity in COVID-19 patients is hypertension (23.7%) [20]. Hypertension sufferers will experience an increase in the production of ACE. A preclinical study indicates that the angiotensin-receptor blocker (ARB) like ACE inhibitor might increase the ACE2 expression. This might cause a highly susceptible individual to get infected with SARS-CoV-2 and eventually become worse. This process occurs because the novel virus has a specific receptor, ACE2, that is also involved in the hypertension mechanism itself [21]. Based on this information, curcumin is not-preferable to be COVID-19 drug candidates. Regardless, all other tested compounds are still preferable and give promising results to be applied as potential COVID-19 medicine and given to the patient as a complementary treatment.

CONCLUSION

In conclusion, all tested flavonoids (curcumin, 6-gingerol, luteolin, brazilin, and kaempferol) have good permeability and flexibility according to drug-likeness prediction using SwissADME. Among five analyzed compounds, kaempferol showed the best potential as an antiviral agent and inhibitor of the S protein-ACE2 interaction for wild-type spike protein. In contrast, the mutant S protein showed the 6-gingerol and luteolin compound might have the best potential to interfere with S protein-ACE2 interaction. The five flavonoids tested still need further research to prove their activity against the SARS-CoV-9 S protein N439k variant.

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