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Original Article

Flavonoids as potential inhibitors of dengue virus 2 (DENV2) envelope protein

[Flavonoides como posibles inhibidores de la proteína de la cubierta del virus del dengue 2 (DENV2)]

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Abstract

Context: Dengue viruses (DENVs) are the cause of dengue disease, which is one of the most frequent diseases caused by mosquito-borne viral infections. Currently, no specific treatment is available for dengue.

Aims: To identify the most promising inhibitors of dengue virus 2 (DENV2) envelope protein of DENV2 envelope protein from flavonoids compounds through computational methods.

Methods: Structures of 54 flavonoids were collected, then the compounds were screened based on Lipinski's rules, and there were only 34 compounds that passed the screening. Then QSAR analysis was performed, followed by molecular docking analysis, ADMET evaluation, and molecular dynamics simulations to assess the stability of the protein.

Results: Based on the QSAR analysis, only 32 compounds were subjected to molecular docking analysis. Silymarin had the highest docking score, while juglanin had the lowest ACE score compared to positive controls. The ADMET evaluation showed silymarin and juglanin had good absorption and could not penetrate the blood-brain barrier. In contrast to silymarin which had negative results for the Ames test, carcinogenicity, skin sensitization, and eye irritation, juglanin was positive for Ames test and skin sensitization. Even though the molecular dynamic simulation of both ligands with DENV2 envelope protein showed unstable confirmation, it did not necessarily mean that the ligands cannot be used as inhibitors since the molecular docking results provide evidence of the ligands binding to the DENV2 envelope protein.

Conclusions: Based on the favorable results of QSAR analysis, molecular docking, and ADMET evaluation, juglanin and silymarin were chosen as the candidate with the most potential for DENV2 envelope protein inhibitors. However, further analyses such as *in vitro* and *in vivo* analyses are necessary to validate the result of this study.

Keywords: DENV-2; envelope protein; flavonoids; molecular docking; virtual screening.

Resumen

Contexto: Los virus del dengue (DENV) son los causantes de la enfermedad del dengue, que es una de las enfermedades más frecuentes causada por infecciones virales transmitidas por mosquitos. Actualmente, no se dispone de un tratamiento específico para el dengue.

Objetivos: Identificar los inhibidores más prometedores de la proteína de la envoltura del virus del dengue 2 (DENV2) de la proteína de la envoltura del DENV2 a partir de compuestos de flavonoides a través de métodos computacionales.

Métodos: Las estructuras de 54 flavonoides fueron recolectadas. Los compuestos se seleccionaron según las reglas de Lipinski y solo 34 compuestos pasaron la selección. Luego se realizó el análisis QSAR, seguido de análisis de acoplamiento molecular, evaluación ADMET y simulaciones de dinámica molecular para evaluar la estabilidad de la proteína.

Resultados: Según el análisis QSAR, solo 32 compuestos se sometieron a análisis de acoplamiento molecular. La silimarina obtuvo la puntuación de acoplamiento más alta, mientras que juglanina obtuvo la puntuación ACE más baja en comparación con los controles positivos. La evaluación ADMET mostró que la silimarina y la juglanina tenían una buena absorción y no podían penetrar la barrera hematoencefálica. En contraste con la silimarina que tuvo resultados negativos para la prueba de Ames, carcinogenicidad, sensibilización de la piel e irritación de los ojos, la juglanina fue positiva para la prueba de Ames y la sensibilización de la piel. Aunque la simulación de la dinámica molecular de ambos ligandos con la proteína de la cubierta de DENV2 mostró una confirmación inestable, no significa necesariamente que los ligandos no puedan usarse como inhibidores, ya que los resultados del acoplamiento molecular proporcionan evidencia de que los ligandos se unen a la proteína de la cubierta de DENV2.

Conclusiones: En base a los resultados favorables del análisis QSAR, el acoplamiento molecular y la evaluación ADMET, la juglanina y la silimarina fueron elegidas como las candidatas con mayor potencial para los inhibidores de la proteína de la envoltura de DENV2. Sin embargo, se necesitan más análisis, como análisis *in vitro* e *in vivo*, para validar el resultado de este estudio.

Palabras Clave: acoplamiento molecular; DENV-2; flavonoides; proteína de envoltura; proyección virtual.

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INTRODUCTION

Dengue is one of the most frequent diseases caused by viral infection (Tantawichien and Thisayakorn, 2017). The dengue virus (DENVs) transmission relies on the vector mosquitoes, primarily the female mosquitoes of *Aedes aegypti* or with the less common vector of the *Aedes albopictus*. According to World Health Organization (WHO), the number of dengue cases globally has risen dramatically in recent years. Dengue is endemic in more than 140 countries around, mainly affecting 2.5 billion people in the tropical and subtropical regions, including Indonesia (Tantawichien and Thisayakorn, 2017). Based on WHO (2009) guidelines, dengue disease severity can be categorized into dengue with or without warning signs and severe dengue. The criteria for severe dengue are severe plasma leakage, leading to dengue shock syndrome (DSS), severe hemorrhage, and severe organ impairment.

Dengue virus (DENV), the causative agent of dengue disease, belongs to the *Flaviviridae* family and is a single-stranded (ss) RNA enveloped virus. DENV consists of 4 dengue virus serotypes (DENV 1-4), making DENV2 one of the serotypes (Boonyasuppayakorn et al., 2014). DENV2 serotype is associated with severe dengue (Vicente et al., 2016). There are several steps involved in the DENV infection cycle, starting with viral entry followed by viral membrane fusion, viral replication, assembly, and maturation. All of these steps can potentially be targeted (Poh et al., 2009). In this study, the focus is on the entry of the virus into the host cells. The virus entry into the host cells needs the attachment to the receptors of the host cells, followed by the virus fusion with the cellular membrane (Yennamalli et al., 2009). This activity is mediated by the envelope protein, which is involved in receptor binding and fusion. Envelope protein consists of hydrophobic domains I, II, and III (Tian et al., 2018a). From the X-ray structure of E protein, it was found that there is a hydrophobic pocket that can be the target of anti-DENV inhibitors (Tian et al., 2018b).

According to WHO, there is only one approved vaccine for dengue fever for all serotypes (1-4) in the market called Dengvaxia live, a live attenuated-based vaccine. However, there are some controversies that revolve around this vaccine, as Dengvaxia administration might increase people with a more severe form of dengue and the protective effects only apply to people who have had dengue before. Hence, people rely on supportive care such as antipyretics for fever, oral hydration, administration of fluids, blood/platelets/fresh frozen transfusion for patients with thrombocytopenia or hemorrhage (Schaefer et al., 2021; Tantawichien and Thisayakorn, 2017). Therefore, there is still a need to find compounds that have antiviral activity against DENV infection.

Regardless of the general use of modern medicine worldwide, bioactive compounds from plants are still attractive due to their cost-effectiveness in production compared to chemically synthesized drugs (Bekhit and Bekhit, 2014). Many available drugs are derived from plants, such as digoxin for heart disease, vincristine and paclitaxel for cancer, and others (Bekhit and Bekhit, 2014). Bioactive compounds from plants are still an essential source for novel antiviral drugs due to their low adverse effects and are highly available in nature (Zandi et al., 2011).

Several classes of phytochemicals, including phenolics, alkaloids, and terpenoids, are used as antivirals. Phenolic compounds, mostly found in plants, are known to have powerful antioxidant properties due to the phenolic hydroxyls, which are able to neutralize free radicals (Loaiza-Cano et al., 2020). However, in this study, only phenolic compounds, specifically flavonoids, will be investigated. There are 9000 varieties of flavonoids identified in several classes belonging to flavonoids, including flavones, flavonols, flavanones, and others (Wang et al., 2020). Flavonoids have been shown to elicit antiviral activity against many viruses, including DENV-2 (Wang et al., 2020). In addition, epigallocatechin gallate can also inhibit the influenza A, chikungunya virus, and hepatitis B virus (Weber et al., 2015).

Using computational methods, we can screen various compounds with a specific antiviral activity function for the dengue virus (DENV) in a short time. During this pandemic, many computational methods are used since many research institute activities have been limited to reducing disease transmission. There are not many studies that investigated viral dynamics within the host cells. Most studies investigated at the population level. With a computational method, DENV interactions with its natural ligand, in this case, host cell receptors can be analyzed. The data required, such as proteins that are related to DENV, can be obtained easily nowadays, as well as 3D structures of the flavonoid compounds, which will help clarify their potential. Similar *studies in silico* like molecular docking and molecular dynamics for identification of potential DENV inhibitors from phytochemicals have been conducted previously (Qamar et al., 2017; Verma et al., 2015; Vora et al., 2019). Moreover, this pipeline is already standardized for viral inhibitor design in general (Parikesit, 2018; Shiloputra et al., 2021).

This research aims to identify the most promising inhibitors of DENV2 envelope protein from flavonoid compounds through computational methods.

MATERIAL AND METHODS

Protein preparation

The envelope protein (E) of DENV2 (RSCB ID: 1OKE) was retrieved from the RCSB website (https://www.rcsb.org/structure/1oke). The protein was cleaned using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC., to remove its n-octyl-beta-D-glucoside and natural ligands.

Ligand preparation

The sdf files of 54 ligands shown in Table 1 were obtained from PubChem. Molinspiration web server (https://www.molinspiration.com/) was used to predict some molecular properties of each ligand, such as LogP, polar surface area, and numbers of hydrogen bond donors as well as acceptors. Lipinski's rule of five was used to screen the ligands. Ligands that violated more than one rule were eliminated from this study. The ligands were optimized using the Avogadro software (http://avogadro.cc/) for the docking prediction (Hanwell et al., 2012).

Quantitative structure-activity relationship (QSAR) analysis

QSAR analysis was conducted to screen the bioactivity of the ligands using Way2drug/PASS server (http://www.way2drug.com/PASSOnline/) (Badshah et al., 2021). A tabulation related to the bioactivities of the compounds was produced. It is expected that the ligands at least have a general antiviral activity or antiviral against specific positive single-stranded RNA virus families or viral entry inhibitors.

Binding site prediction

The pdb file of enveloped protein (E) of DENV2 (RSCB ID: 1OKE) was downloaded and uploaded to CASTp server (http://sts.bioe.uic.edu/castp/index.html?2cpk) (Tian et al., 2018a). The Computed Atlas of Surface Topography of proteins (CASTp) can identify the surface proteins, interior pockets (active sites) and measure their volumes and areas (Tian et al., 2018b).

Molecular docking

The molecular docking validation of the selected ligands and the envelope protein (E) of DENV2 was performed using PatchDock (https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php) (Schneidman-Duhovny et al., 2005). The clustering root-mean-square deviation (RMSD) parameter was set as 4 Å, while the complex type was set as default. The top one solution from the molecular docking results of each compound was further analyzed. The docking validation was done using natural ligands such as 2-acetamido-2-deoxy-beta-D-glucopyranose (NAG) and octyl beta-D-glucopyranoside (BOG) as the reference compounds.

Absorption, distribution, metabolism, excretion (ADME), and toxicity prediction

The ADME and toxicity of the compounds were also analyzed. The SMILES of the ligands were inputted to the ADMETlab 2.0 webserver (https://admetmesh.scbdd.com/service/evaluation/cal) (Xiong et al., 2021). Several parameters were generated from the server to be analyzed, such as blood-brain barrier, HIA, LC_{50}, AMES toxicity, skin sensitization, carcinogenicity, and eye irritation.

Molecular dynamics simulation

Molecular dynamics (MD) simulation predicts the movement of every atom in the protein over time in order to capture changes in protein conformation, ligand binding, and folding of a protein (Hollingsworth and Dror, 2018). Online molecular dynamics simulation was performed using the CABSFLEX2 server (http://biocomp.chem.uw.edu.pl/CABSflex2/submit) (Kurcinski et al., 2018), and the parameters were set as default. This simulation allows the prediction of protein flexibility by determining the root-mean-square fluctuation (RMSF) (Kurcinski et al., 2018).

Data analysis

The results of the QSAR analysis were inputed to Google spreadsheet, six main bioactivities were focused on such as general antiviral activity, antiviral against negative single-stranded RNA virus, positive single-stranded RNA virus and also viral entry inhibition. Compounds with either a general antiviral activity or specific antiviral activity or viral entry inhibition with threshold Pa >0.3 were chosen for molecular docking.

In the molecular docking, two parameters were going to be focused on the binding score and ACE score. To validate the docking method, the compounds should have a higher binding score and lower ACE score than the reference compounds.

There were multiple parameters for ADME and toxicity generated from ADMElab 2.0; however, only 6 parameters were focused on, such as skin sensitization, eye irritation, Ames test, carcinogenicity, human intestinal absorption, and blood-brain barrier penetra-

tion. The selected compounds were expected to not exhibit any significant toxicity.

In the molecular dynamics simulation results of the selected compounds, the RMSF threshold were expected to be between 1-3 Å to be considered stable conformation (Parikesit and Nurdiansyah, 2021).

Table 1. The flavonoids with antiviral activity reported from the scientific literature.

No.	Name	PubChem	Reference	No.	Name	PubChem ID	Reference
1	Apigenin	5280443	(Ninfali et al., 2020)	28	Delphinidin	68245	(Loaiza-Cano et al., 2020)
2	Luteolin	5280445	(Ninfali et al., 2020)	29	Taxifolin/ dihydroquercetin	439533	(Badshah et al., 2021)
3	Vitexin	5280441	(Ninfali et al., 2020)	30	Sodium rutin sulfate	11851154	(Badshah et al., 2021)
4	Apigenin-7-O-glucoside	44257792	(Ninfali et al., 2020)	31	Pachypodol	5281677	(Badshah et al., 2021)
5	Isoquercetin	347828869	(Ninfali et al., 2020)	32	Quercetin-3- ß-O-D-glucoside	5280804	(Badshah et al., 2021)
6	Quercetin	5280343	(Ninfali et al., 2020)	33	(-) Gallocatechin gallate (GCG)	199472	(Badshah et al., 2021)
7	Quercetin-3-rhamnoside	5353915	(Ninfali et al., 2020)	34	Quercetin 7-rhamnoside	5748601	(Badshah et al., 2021)
8	EGCG	65064	(Ninfali et al., 2020)	35	Pectolinarin	168849	(Badshah et al., 2021)
9	Myricetin-3-rhamnoside	5352000	(Ninfali et al., 2020)	36	Leachianone G	5275227	(Badshah et al., 2021)
10	Catechin	73160	(Ninfali et al., 2020)	37	Kaempferol	5280863	(Badshah et al., 2021)
11	Naringenin	439246	(Ninfali et al., 2020)	38	Juglanin	5318717	(Badshah et al., 2021)
12	Delphinidin-3-rutinoside	192918	(Ninfali et al., 2020)	39	Herbacetin	5280544	(Badshah et al., 2021)
13	Sanggenon O	15479637	(Ninfali et al., 2020)	40	Gossypetin	5280647	(Badshah et al., 2021)
14	Chamaejasmin	390362	(Ninfali et al., 2020)	41	Genistein	5280961	(Badshah et al., 2021)
15	Baicalin	64982	(Ninfali et al., 2020)	42	Galangin	5281616	(Badshah et al., 2021)
16	Baicalein	5281605	(Ninfali et al., 2020)	43	(-) Epicatechin gallate	107905	(Badshah et al., 2021)
17	Tangeretin	68077	(Ninfali et al., 2020)	44	(±) Dihydromyricetin	161557	(Badshah et al., 2021)
18	Nobiletin	72344	(Ninfali et al., 2020)	45	Cyanidin-3-(p-coumaroyl)-rutinoside-5-glucoside	23724699	(Badshah et al., 2021)
19	Kaempferol-7-O-glucoside	10095180	(Ninfali et al., 2020)	46	Amentoflavone	5281600	(Badshah et al., 2021)
20	Quercetagetin	5281680	(Ninfali et al., 2020)	47	7-O-(6-feruoylglucosyl) isoorientin	72193672	(Badshah et al., 2021)
21	Pinocembrin	68071	(Ninfali et al., 2020)	48	3-methyl Quercetin	44259658	(Badshah et al., 2021)
22	Flavone	10680	(Ismail and Jusoh, 2016)	49	(-)Epigallocatechin (EGC)	72277	(Badshah et al., 2021)
23	Fisetin	5281614	(Ismail and Jusoh, 2016)	50	(-)Epicatechin	72276	(Badshah et al., 2021)
24	Glabranine	3144815	(Ismail and Jusoh, 2016)	51	Myricetin	5281672	(Badshah et al., 2021)
25	Hyperoside	5281643	(Ismail and Jusoh, 2016)	52	Silymarin	5213	(Badshah et al., 2021)
26	Ladanein	3084066	(Ismail and Jusoh, 2016)	53	Sorbifolin	3084390	(Badshah et al., 2021)
27	Naringin	442428	(Loaiza-Cano et al., 2020)	54	Pedalitin	31161	(Badshah et al., 2021)

RESULTS

Initially, 54 flavonoid compounds were screened; however, based on Lipinski's rule of five in which only at most one violation was allowed, only 34 compounds passed the screening. These compounds can be potential inhibitors of the envelope (E) protein of DENV2. Table 2 shows the list of flavonoids that passed Lipinski's rules based on the result of the Molinspiration web server. It comprises the compound's 2D chemical structure from PubChem, PubChem ID, the compound names and their SMILES, and the number of violations.

The QSAR analysis using the Way2drug/PASS server is necessary to evaluate the bioactivity of the compound. Delphinidin QSAR analysis showed no bioactivity, and thus this compound was removed for further analysis. From Figs. 1 and 2, it can be observed that most of the selected compounds and the reference compounds have general antiviral activity; only Tangeretin and Flavone did not show general antiviral activity from QSAR analysis. While compounds like vitexin, apigenin-7-O-glucoside, catechin, baicalein, quercetagetin, were shown to have general antiviral activity with threshold Pa of 0.3-0.7 indicating the compounds' activity in the experiment or moderate activity (Filimonov et al., 2014; Lagunin et al., 2000; Parikesit and Nurdiansyah, 2021). Some of the flavonoids like apigenin, luteolin, naringenin, pinocembrin, glabranine, ladanein, taxifolin, pachypodol, leachianone G, genistein, and others showed threshold Pa less than 0.3 that indicated weak activity or unlikely to elicit the activity in the experiment (Filimonov et al., 2014; Lagunin et al., 2000; Parikesit and Nurdiansyah, 2021). In addition to general antiviral activity, the compounds may possess specific antiviral activity such as picornavirus, rhinovirus, retrovirus which are also positive single-stranded RNA viruses (Modrow et al., 2013), and thus compounds with this activity are likely to be a candidate inhibitor of DENV2. While some had antiviral activity against the influenza virus, which is a negative single-stranded RNA virus (Krug and Aramini, 2009). In addition, there were also several compounds that have viral entry inhibitor activity. In this study, the reference compounds were shown to have general antiviral properties and specific antiviral properties against single-stranded RNA viruses. Flavonoids that at least had a general antiviral activity or specific antiviral activity like rhinovirus, retrovirus, picornavirus, or influenza virus or inhibition of viral entry with threshold Pa>0.3 were subjected to further analysis.

Fig. 3 shows the binding pocket of the DENV2 envelope protein predicted by the CASTp web server. The binding pocket area was found to be 322.502 $Å^2$, while the volume was 435.625 $Å^3$. Molecular docking results of the ligands and the envelope protein of DENV2 are shown in Table 3. The NAG and BOG are used as positive controls since both are natural ligands of DENV2 envelope protein. The docking score is described as the geometric shape complementarity score, and it is inversely proportional to the free energy Gibbs (ΔG). In contrast, the ACE score refers to atomic contact energy, which is directly proportional to the ΔG (Filimonov et al., 2014; Krug and Aramini, 2009). The PATCHDOCK output shown in Table 3, juglanin had the lowest ACE score of -376.01 while silymarin had the highest docking score of 6190.

The ADME and toxicity analysis shown in Table 4 show that all the compounds cannot pass through the blood-brain barrier. Juglanin and silymarin were found to be readily absorbed by the human intestine since ≥30% can be absorbed. In contrast, NAG and BOG, less than 30% were absorbed, which might affect their bioavailability (Yan et al., 2008). Furthermore, juglanin is positive for the AMES test and skin sensitization but negative for carcinogenicity and eye irritation. In comparison, silymarin had negative results for all the toxicity tests. Similarly, both reference compounds, NAG and BOG exhibited negative results for all the toxicity tests.

The molecular dynamic simulation of juglanin with DENV2 envelope protein showed unstable conformation since most of the residues did not fall between the RMSF threshold of 1-3 Å (Parikesit and Nurdiansyah, 2021), shown in Fig. 4. Similarly, silymarin showed unstable conformation shown in Fig. 5.

Table 2. The list of flavonoids that passed Lipinski's rules.

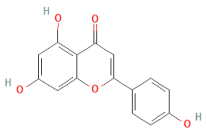
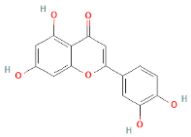
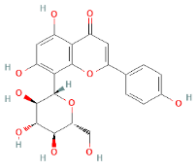
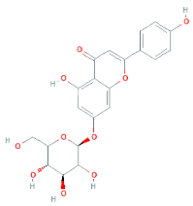
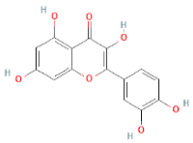
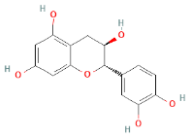
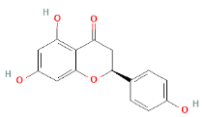
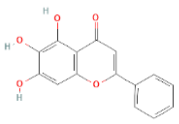
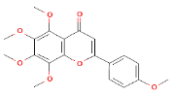
No.	Chemical structure	PubChem ID	Compound	SMILES	No. violation
1		5280443	Apigenin	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O	0
2		5280445	Luteolin	C1=CC(=C(C=C1C2=CC(=O)C3=C(C=C(C=C3O2)O)O)O)O	0
3		5280441	Vitexin	C1=CC(=CC=C1C2=CC(=O)C3=C(O2)C(=C(C=C3O)O)C4C(C(C(C(O4)CO)O)O)O)O	1 **HD > 5**
4		44257792	Apigenin-7-O-glucoside	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O	1 **HD > 5**
5		5280343	Quercetin	C1=CC(=C(C=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O	0
6		73160	Catechin	C1C(C(OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)O	0
7		439246	Naringenin	C1[C@H](OC2=CC(=CC(=C2C1=O)O)O)C3=CC=C(C=C3)O	0
8		5281605	Baicalein	C1=CC=C(C=C1)C2=CC(=O)C3=C(O2)C=C(C(=C3O)O)O	0
9		68077	Tangeretin	COC1=CC=C(C=C1)C2=CC(=O)C3=C(O2)C(=C(C(=C3OC)OC)OC)OC	0

Table 2. The list of flavonoids that passed Lipinski's rules (continued...)

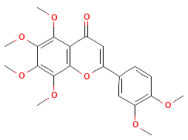
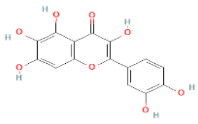
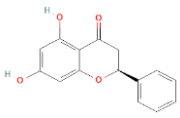
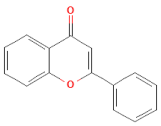
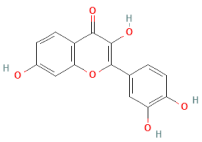
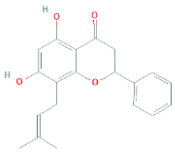
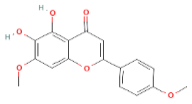
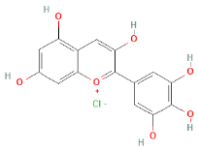
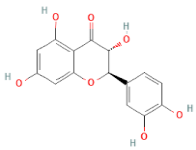
No.	Chemical structure	PubChem ID	Compound	SMILES	No. violation
10		72344	Nobiletin	COC1=C(C=C(C=C1)C2=CC(=O)C3=C(O2)C(=C(C(=C3OC)OC)OC)OC)OC	0
11		5281680	Quercetagetin	C1=CC(=CC=C1C2=CC(=O)C3=C(C=C(C=C3O2)OC4C(C(C(C(O4)CO)O)O)O)O)O	1 **HD > 5**
12		68071	Pinocembrin	C1[C@H](OC2=CC(=CC(=C2C1=O)O)O)C3=CC=CC=C3	0
13		10680	Flavone	C1=CC=C(C=C1)C2=CC(=O)C3=CC=CC=C3O2	0
14		5281614	Fisetin	C1=CC(=C(C=C1C2=C(C(=O)C3=C(O2)C=C(C=C3)O)O)O)O	0
15		3144815	Glabranine	CC(=CCC1=C2C(=C(C=C1O)O)C(=O)CC(O2)C3=CC=CC=C3)C	0
16		3084066	Ladanein	COC1=CC=C(C=C1)C2=CC(=O)C3=C(C(=C(C=C3O2)OC)O)O	0
17		68245	Delphinidin	C1=C(C=C(C(=C1O)O)O)C2=[O+]C3=CC(=CC(=C3C=C2O)O)O.[Cl-]	1 **HD > 5**
18		439533	Taxifolin/ dihydroquercetin	C1=CC(=C(C=C1[C@@H]2[C@H](C(=O)C3=C(C=C(C=C3O2)O)O)O)O)O	0

Table 2. The list of flavonoids that passed Lipinski's rules (continued...)

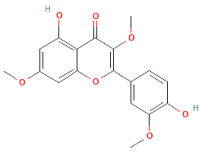
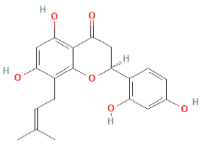
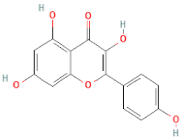
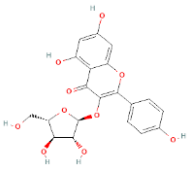
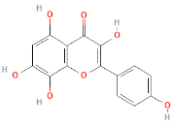
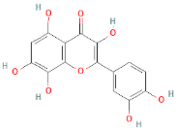
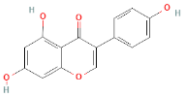
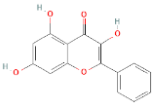
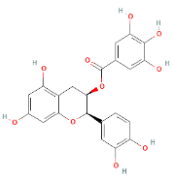
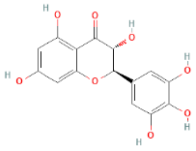
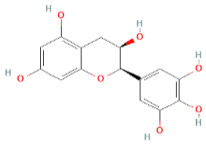
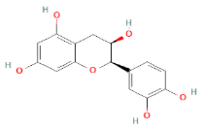
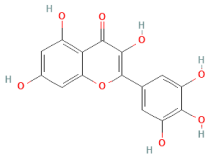
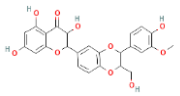
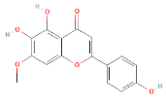
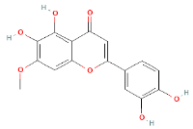
No.	Chemical structure	PubChem ID	Compound	SMILES	No. violation
19		5281677	Ro-090179	COC1=CC(=C2C(=C1)OC(=C(C2=O)OC)C3=CC(=C(C=C3)O)OC)O	0
20		5275227	Leachianone G	CC(=CCC1=C2C(=C(C=C1O)O)C(=O)C[C@H](O2)C3=C(C=C(C=C3)O)O)C	0
21		5280863	Kaempferol	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O)O	0
22		5318717	Juglanin	C1=CC(=CC=C1C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O[C@H]4[C@@H]([C@H]([C@@H](O4)CO)O)O)O	1 **HD > 5**
23		5280544	Herbacetin	C1=CC(=CC=C1C2=C(C(=O)C3=C(O2)C(=C(C=C3O)O)O)O)O	0
24		5280647	Gossypetin	C1=CC(=C(C=C1C2=C(C(=O)C3=C(O2)C(=C(C=C3O)O)O)O)O)O)O	1 **HD > 5**
25		5280961	Genistein	C1=CC(=CC=C1C2=COC3=CC(=CC(=C3C2=O)O)O)O	0
26		5281616	Galangin	C1=CC=C(C=C1)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O	0
27		107905	(-) Epicatechin gallate	C1[C@H]([C@H](OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)OC(=O)C4=CC(=C(C(=C4)O)O)O	1 **HD > 5**

Table 2. The list of flavonoids that passed Lipinski's rules (continued...)

No.	Chemical structure	PubChem ID	Compound	SMILES	No. violation
28		161557	(±) Dihydromyricetin	C1=C(C=C(C(=C1O)O)O)[C@@H]2[C@H](C(=O)C3=C(C=C(C=C3O2)O)O)O	1 **HD > 5**
29		72277	(-) Epigallocatechin (EGC)	C1C(C(OC2=CC(=CC(=C21)O)O)C3=CC(=C(C(=C3)O)O)O)O	1 **HD > 5**
30		72276	(-) Epicatechin	C1C(C(OC2=CC(=CC(=C21)O)O)C3=CC(=C(C=C3)O)O)O	0
31		5281672	Myricetin	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(C=C(C=C3O2)O)O)O	1 **HD > 5**
32		5213	Silymarin	COC1=C(C=CC(=C1)C2C(OC3=C(O2)C=C(C=C3)C4C(C(=O)C5=C(C=C(C=C5O4)O)O)O)CO)O	0
33		3084390	Sorbifolin	COC1=C(C(=C2C(=C1)OC(=CC2=O)C3=CC=C(C=C3)O)O)O	0
34		31161	Pedalitin	COC1=C(C(=C2C(=C1)OC(=CC2=O)C3=CC(=C(C=C3)O)O)O)O	0

The compounds at most only violated 1 Lipinski's rule. HD: Hydrogen donor.

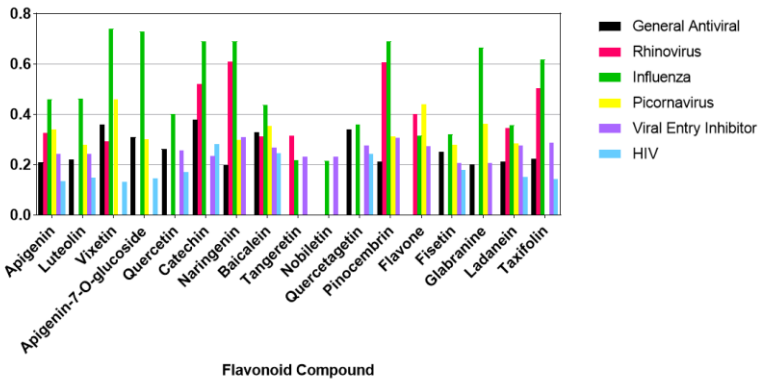


Figure 1. Predicted antiviral activities of the selected compounds using the PASS server.

The X-axis indicates the compound's name, and the Y-axis indicates the Pa (probability) value.

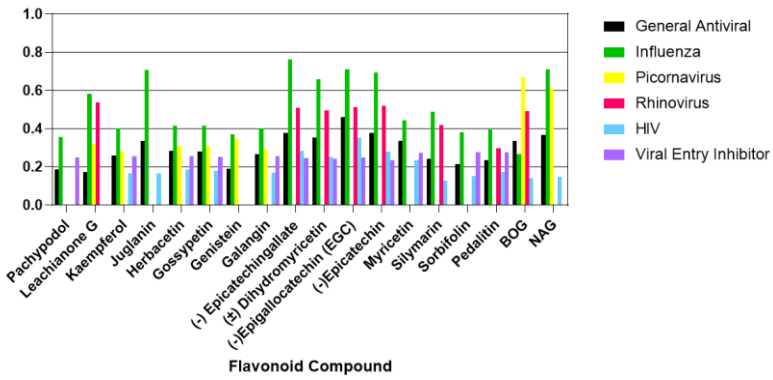


Figure 2. The predicted antiviral activities of the selected compounds using the PASS server.

The X-axis indicates the compound's name, and the Y-axis indicates the Pa (probability) value.BOG: octyl beta-D-glucopyranoside; NAG: 2-acetamido-2-deoxy-beta-D-glucopyranose.

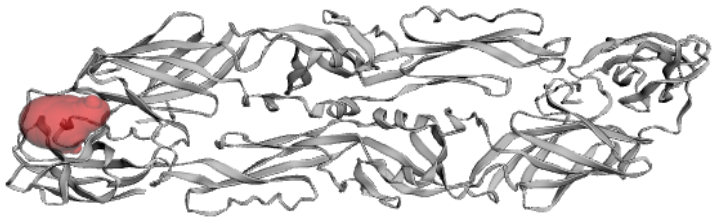


Figure 3. The binding pocket prediction of DENV2 envelope protein using CASTp server.

The red-colored structure indicates the binding pocket.

Table 3. The molecular docking result of selected ligands and DENV2 envelope protein using PATCHDOCK webserver.

No.	Compound	Docking score	ACE value	No.	Compound	Docking score	ACE value
1	Apigenin	4206	-260.04	18	Kaempferol	4402	-280.69
2	Luteolin	4350	-256.79	19	Juglanin	4982	-376.01
3	Vitexin	4634	-96	20	Herbacetin	4426	-261.42
4	Apigenin-7-O-glucoside	5670	-349.24	21	Gossypetin	4790	-286.93
5	Quercetin	4564	-274.3	22	Genistein	4434	-266.6
6	Catechin	4534	-266.02	23	Galangin	4620	-258.74
7	Naringenin	4408	-281.52	24	(-) Epicatechin gallate	4958	-178.21
8	Baicalein	4352	-253.62	25	(±) Dihydromyricetin	4642	-284.82
9	Quercetagetin	4582	-288.59	26	(-) Epigallocatechin (EGC)	5262	-129.53
10	Pinocembrin	4522	-257.38	27	(-) Epicatechin	4642	-259.84
11	Flavone	4312	-233.32	28	Myricetin	4706	-283.48
12	Fisetin	4412	-256.63	29	Silymarin	6190	-379.73
13	Glabranine	4334	-340.8	30	Sorbifolin	4668	-295.96
14	Ladanein	4712	-304.67	31	Pedalitin	4954	-279.44
15	Taxifolin/ dihydroquercetin	4580	-277	32	Tangeretin	5182	-348.5
16	Pachypodol	5152	-326.25	33	NAG	3814	-173.06
17	Leachianone G	4374	-17.43	34	BOG	4918	-191.32

ACE: atomic contact energy; BOG: octyl beta-D-glucopyranoside; NAG: 2-acetamido-2-deoxy-beta-D-glucopyranose.

Table 4. The ADME and toxicity evaluation of selected ligands using ADMETlab 2.0 web server.

No	Compound	Ames toxicity	Skin sensitization	Carcinogenecity	Eye irritation	$LC_{50}FM$	$LC_{50}DM$	HIA	BBB penetration
1	Apigenin	-	+++	--	+++	5.208	5.209	---	---
2	Luteolin	+	+++	---	+++	5.222	5.301	---	---
3	Vitexin	++	--	---	---	4.965	5.177	++	---
4	Apigenin-7-O-glucoside	+	--	++	---	4.804	5.367	++	---
5	Quercetin	+	+++	---	+++	5.222	5.331	---	---
6	Catechin	+	+++	---	+++	4.568	5.228	---	---
7	Naringenin	--	+++	+	+++	6.692	6.41	---	---
8	Baicalein	-	+++	-	+++	4.767	5.607	---	---
9	Tangeretin	-	--	---	+++	5.488	6.734	---	---
10	Quercetagetin	+	+++	---	+++	5.009	5.521	---	---
11	Pinocembrin	--	++	+	+++	6.714	6.36	---	---
12	Flavone	++	--	++	+++	5.186	5.232	---	---
13	Fisetin	++	+++	--	+++	5.305	5.333	---	---
14	Glabranine	--	++	--	++	6.76	6.588	---	---
15	Ladanein	+	++	--	++	5.079	6.445	---	---
16	Taxifolin/dihydro quercetin	+	+++	---	+++	5.581	5.908	---	---
17	Pachypodol	+	--	---	++	5.673	6.137	---	---
18	Lechianone G	--	+++	-	+++	7.025	6.723	---	---
19	Kaempferol	+	++	---	+++	5.223	5.205	---	---
20	Juglanin	++	++	---	-	4.954	5.368	--	---
21	Herbacetin	++	+++	--	+++	5.066	5.146	---	---
22	Gossypetin	++	+++	---	+++	5.095	5.26	---	---
23	Genistein	--	+++	-	+++	5.275	5.632	---	---

Table 4. The ADME and toxicity evaluation of selected ligands using ADMETlab 2.0 web server (continued...)

No	Compound	Ames toxicity	Skin sensitization	Carcinogenecity	Eye irritation	$LC_{50}FM$	$LC_{50}DM$	HIA	BBB penetration
24	Galangin	+	++	--	+++	5.098	5.075	---	---
25	(-) Epicatechin gallate	-	+++	---	+++	5.366	5.744	+	---
26	(±) Dihydromyricetin	-	+++	---	+++	5.388	5.589	---	---
27	(-) Epigallocatechin (EGC)	-	+++	---	+++	4.181	5.111	--	---
28	(-) Epicatechin	+	+++	---	+++	4.568	5.228	---	---
29	Myricetin	-	+++	--	+++	4.982	5.272	---	---
30	Silymarin	-	--	-	--	6.831	6.707	-	---
31	Sorbifolin	-	+++	--	+++	4.839	6.083	---	---
32	Pedalitin	+	+++	---	+++	4.594	0.063	--	---
33	NAG	---	---	---	---	1.182	1.313	+++	-
34	BOG	-	--	---	---	3.079	3.760	++	--

The '-' symbol indicates a negative test while '+' indicates a positive test. The number of '-' and '+' refers to predicted values based on probability (Guan et al., 2019). $LC_{50}FM$ refers to the lethal concentration that causes 50% death of fathead minnow in 96 hours (-log10 [(mg/L)/(1000*Mw)]. $LC_{50}DM$ indicates lethal concentration that causes 50% death of *Daphnia magna* in 48 hours (-log10 [(mg/L)/(1000*Mw)]. BBB refers to the blood-brain barrier. HIA refers to human intestinal absorption where a negative value indicates ≥ 30% absorption and a positive value indicates <30% absorption. BOG: octyl beta-D-glucopyranoside; NAG: 2-acetamido-2-deoxy-beta-D-glucopyranose.

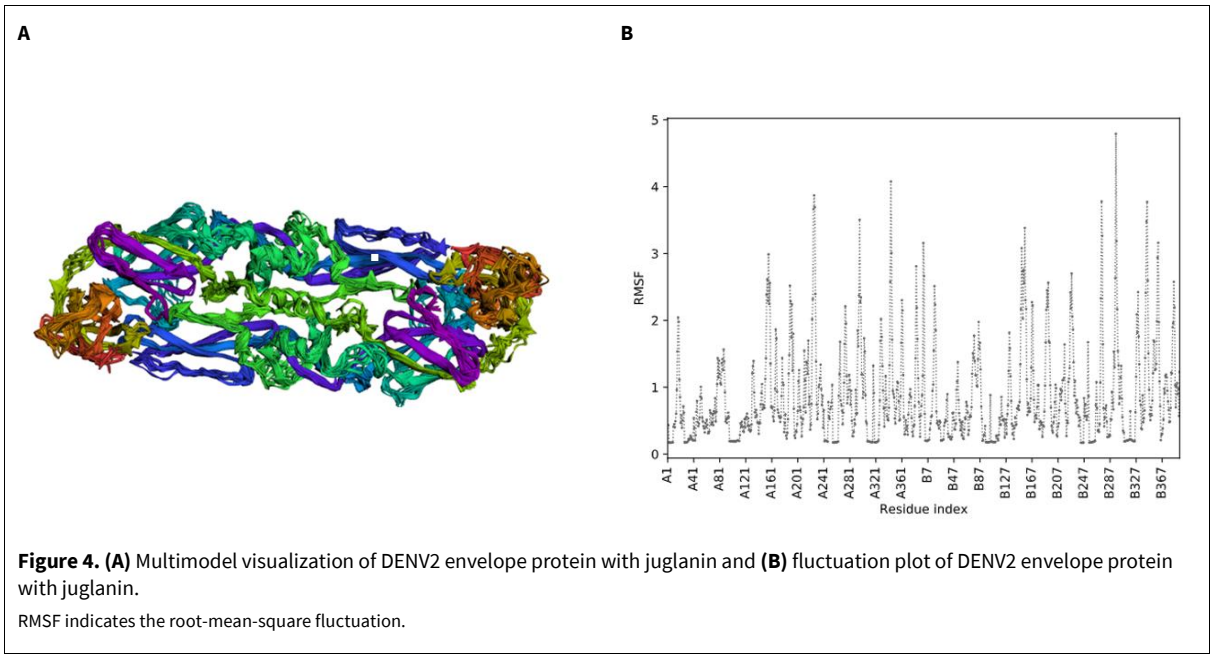


Figure 4. (A) Multimodel visualization of DENV2 envelope protein with juglanin and **(B)** fluctuation plot of DENV2 envelope protein with juglanin.

RMSF indicates the root-mean-square fluctuation.

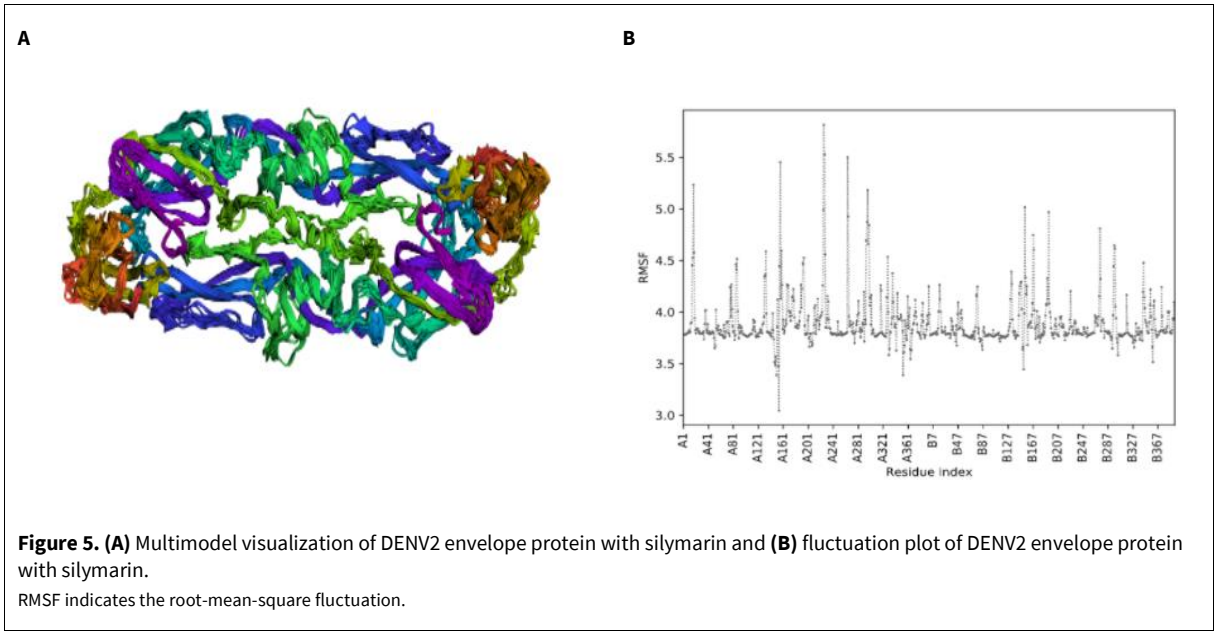


Figure 5. (A) Multimodel visualization of DENV2 envelope protein with silymarin and **(B)** fluctuation plot of DENV2 envelope protein with silymarin.
RMSF indicates the root-mean-square fluctuation.

DISCUSSION

Dengue disease, a viral disease spread by mosquitoes, occurs in around 390 million cases worldwide every year, where 96 million of those cases lead to severe symptoms (WHO, 2022). Currently, no specific treatment is available for dengue (Schwarz et al., 2014). In recent years, there have been several candidates for targets in the discovery of anti-DENV drugs, including essential proteins such as NS3/NS2B protease, NS3 helicase, E protein, methyltransferase (MTase), and RNA-dependent RNA polymerase (RdRp) of NS5 (Rajapakse et al., 2012). This study focuses particularly on the E protein, a class II fusion protein responsible for virion assembly and fusion of the virus with the target cell membrane (Tian et al., 2018a).

In order to identify the possible lead compounds, virtual screening was performed. Virtual screening or *in silico* analysis may accelerate the process of drug discovery and development as it reduces the number of compounds needed to be tested *in vitro* and *in vivo,* leads to a more cost-effective process which may also increase the chance of identifying novel lead compounds (Ho et al., 2007). Screening of phytochemicals remains attractive to many researchers as they may possess low adverse effects and can be readily available in nature (Loaiza-Cano et al., 2020).

Flavonoids, which can be classified into several classes, are shown to have antiviral activity against many types of viruses, including influenza A and B virus, chikungunya virus, hepatitis B and C virus, enterovirus A71, HIV, poliovirus as well as DENV2 (Filimonov et al., 2014). This study selected juglanin and silymarin as the most potential DENV2 envelope protein inhibitors, as shown by their predominantly favorable results in QSAR analysis, molecular docking, and ADMET evaluation.

Juglanin can be isolated from the husks of *Juglans mandshurica* or more commonly known as the green walnut (Dong and Yuan, 2018). Juglanin is also known as the glycoside derivative of kaempferol, which has been proven to inhibit the viral production of SARS-CoV with a significantly low IC_{50} (2.3 μM) (Karim et al., 2021). In concordance with the previous study, the QSAR analysis of juglanin showed that it is likely to exhibit general antiviral activity. Silymarin can also be a potential candidate for a DENV2 inhibitor. Silymarin can be readily extracted from the milk thistle plant (Surai, 2015). Instead of general antiviral activity, it was found to likely have antiviral activity against rhinovirus, a positive ss-RNA virus (Modrow et al., 2013), which may further suggest the possibility of becoming the DENV2 envelope protein inhibitor. A previous *in vitro* study regarding the effects of baicalein and silymarin has shown that silymarin prevented viral entry into the cells and exhibited favorable effects against all four serotypes of dengue (Low et al., 2021). In addition, silymarin can also bind to the viral E protein with a significant binding affinity and form hydrogen bonds with several amino acids on the protein (Low et al., 2021).

One of the essential tools for drug discovery is molecular docking, which predicts a ligand's major binding mode with a three-dimensional protein structure (Morris and Lim-Wilby, 2008). When used effectively, high-dimensional spaces can be identified (Morris and Lim-Wilby, 2008). Molecular docking is also used in lead optimization, where a virtual screening is performed on a library of compounds, ordering the results based on their performance and coming up with

predictions of how the ligands could inhibit the target (Morris and Lim-Wilby, 2008). The docking score and ACE score from molecular docking analysis of juglanin with DENV2 envelope protein were better than the positive controls or the reference compounds used, as it had a higher docking score and lower ACE score. The molecular docking result of silymarin with DENV2 envelope protein also showed similar results. A higher docking score may indicate less steric hindrance (Duhovny et al., 2002), whereas a negative ACE score may suggest a spontaneous reaction or not requiring energy (Zarei et al., 2019).

ADMET consists of chemical absorption, distribution, metabolism, excretion, and toxicity. These factors play crucial roles in the discovery and development of new drugs. A drug can be determined as high quality by having the proper ADMET values when given at a therapeutic dose and having a high efficacy towards the target. One of the most essential aspects of ADME is human intestinal absorption or HIA, which is used to study the precise use of pharmaceuticals in the human body using statistical models. HIA is also an important stage in the delivery of drugs to their intended destination (Yan et al., 2008). Furthermore, because there are several components at play in the oral bioavailability process, it is challenging to determine the oral bioavailability of a myriad of drugs (Wessel and Mente, 2001). To create useful predictive models for human oral bioavailability due to the varied drug absorption pathways need strong descriptors that relate to carrier-mediated transport and first-pass metabolism (Yan et al., 2008). Because HIA is one of the most critical factors regulating oral bioavailability, various efforts have been made to accurately predict it (Yan et al., 2008). This study shows that both silymarin and juglanin had good absorption since more than equal to 30% were absorbed (Wang et al., 2017). Good absorption might lead to higher bioavailability and thus the result also may suggest that the compounds are suitable for oral administration.

Moreover, all the selected compounds had negative results for the BBB barrier, indicating that the compounds will not cause toxicity to the brain as they cannot pass through the BBB barrier (Mangas-Sanjuan et al., 2010). Juglanin and silymarin were shown to have negative carcinogenicity, meaning they are unlikely to cause cancer (Hou et al., 2018). In contrast to silymarin, juglanin had a positive result in the Ames test, suggesting that juglanin can be mutagenic and thus may serve as a potential genetic carcinogen. However, a positive result in the Ames test may be hard to interpret as a mutagen in the Ames test may not certainly cause harmful effects in humans (Hengstler and Oesch, 2001). Besides, the side effects might be minimized by optimizing the drug formulation in the wet lab. In addition, juglanin and silymarin LC_{50}FM showed values of 4.954 and 6.831, respectively, which is a relatively low number that indicates the effective killing of 50% of the fathead minnow. On the other hand, the LC_{50}DM showed values of 5.368 for juglanin and 6.707 for silymarin. LC_{50}DM itself is the value effective to kill 50% of *Daphnia magna*.

Although the result of molecular dynamic simulation showed unstable conformation between the selected ligands with the DENV2 envelope protein, this did not necessarily mean that the ligands cannot be inhibitors because the molecular docking results provide evidence that the juglanin and silymarin actually can bind to the DENV2 envelope protein, however some improvement on the structure of the ligands may be necessary for a stable conformation.

The limitation of this study includes the lack of flavonoid compounds that were being screened, as only 54 compounds were screened. This might affect the prediction of the lead compounds as both ligands were found to be unstable in the molecular dynamic simulation. To overcome this problem is to increase the number of flavonoids being screened. Furthermore, the results from this study are solely based on prediction or virtual screening, which depends on the preparation of the inputs and the output interpretation. Thus, further validation through wet-lab experiments is required.

CONCLUSION

This study showed juglanin and silymarin as the most potential candidate for DENV2 envelope protein inhibitors. Silymarin and juglanin were readily absorbed by the intestines and cannot penetrate the blood-brain barrier. Silymarin passed all the toxicology tests, while juglanin did not fulfill all toxicity criteria. Even though the molecular dynamic results showed unstable conformation for both compounds, the QSAR analysis showed that they were likely to elicit antiviral activity and bind to the target based on the molecular docking. However, further investigations through wet-lab experiments such as *in vitro* or *in vivo* testing are necessary.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Renantha RR	Liga AR	Tanugroho CB	Denovian LX	Budiyanto SLAZ	Parikesit AA
Concepts or ideas	x	x	x	x	x	x
Design	x	x	x	x	x	
Definition of intellectual content	x	x	x	x	x	
Literature search	x	x	x	x	x	
Experimental studies	x	x	x	x	x	
Data acquisition	x	x	x	x	x	
Data analysis	x	x	x	x	x	
Statistical analysis	x	x	x	x	x	
Manuscript preparation	x	x	x	x	x	
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

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