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Original Article

MOLECULAR DOCKING OF BIFLAVONOIDS FROM GENUS ARAUCARIA AS DENV NS5 RNA-DEPENDENT RNA POLYMERASE INHIBITOR USING YASARA AND PLANTS PROGRAMS

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ABSTRACT

Objective: This study aimed to screen 23 biflavonoids (23 BF) from the Araucaria genus to identify the most promising compound for anti-dengue fever antivirus treatment using in silico techniques with yet another scientific artificial reality application (YASARA) Structure and the Protein-Ligand ANT System (PLANTS) programs.

Methods: Predictions of conserved amino acids and potential pockets of the virus dengue NS5 RNA-dependent RNA polymerase (DENV NS5 RdRp) (PDB ID: 5K5M) were examined, while co-crystal ligands were prepared along with 23 biflavonoids. Molecular docking of ligands on the target protein was carried out using the YASARA Structure and PLANTS programs. The interactions were visualized with LigPlot+, Pymol, and Discovery Studio 2021 Client in. pdb format.

Results: The results showed that based on the molecular docking of 23 biflavonoids from the Araucaria genus against the selected DENV NS5 RdRp, the top nine compounds with great potential as antiviral drug candidates were identified. Among these compounds, 7,4',7''-tri-*O*-methylagathisflavone (BF3) was distinct as the best choice based on the analysis conducted using the YASARA Structure and PLANTS programs. Other compounds, including 7,4',4'''-tri-*O*-methylamentoflavone (BF10), 4',4'''-di-*O*-methylamentoflavone (BF11), 7,4',7'',4'''-tetra-*O*-methylamentoflavone (BF12), 7''-*O*-methylamentoflavone (BF13), and 7,7''-di-*O*-methylamentoflavone (BF14), were selected through the YASARA Structure program, while 7,4',7'',4'''-tetra-*O*-methylagathisflavone (BF8) and 7''-*O*-methylrobustaflavone (BF23) were selected from the PLANTS program. All compounds had lower free energy (ΔG), dissociation constant (Kd), and docking scores compared to the reference ligand, balapiravir. Hydrogen and hydrophobic bonds were formed with the protein through conserved amino acid residues, the N-pocket, and the catalytic Gly-Asp-Asp (GDD) site.

Conclusion: The algorithm differences between the YASARA Structure and PLANTS programs led to the selection of the best compound 7,4',7''-tri-*O*-methylagathisflavone (BF3) as a candidate antiviral drug for dengue hemorrhagic fever.

Keywords: Genus araucaria, Biflavonoids, In silico with YASARA structure and PLANTS programs, 7,4',7''-tri-*O*-methylagathisflavone (BF3)

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INTRODUCTION

Dengue virus (DENV) infection is a disease transmitted through an arthropod vector known as an arbovirus. Dengue fever is an Acute Febrile Illness (AFI) caused by one or more dengue viruses that are members of the Flavivirus genus. Two-fifths of the world's population is at risk from dengue sickness, according to the World Health Organization (WHO) [1]. This disease is transmitted by *Aedes aegypti* mosquitoes [2]. DENV accounts for 50 to 100 million human infections annually, with 500,000 cases of dengue fever and 22,000 deaths globally [3]. The prevalence is estimated to exceed 390 million infections per year, with approximately 96 million manifesting a certain level of severity [4]. In Indonesia, reported cases in 2022 amounted to 87,501, with 816 deaths [5].

The dengue virus has different serotypes based on antigenic and biological characteristics, including DENV-1, DENV-2, DENV-3, and DENV-4. The genome consists of a single-stranded positive ribonucleic acid (RNA) molecule containing 11 kilobases (kb) organized into the 5'-untranslated region (5'-UTR), three structural genes (capsid, pre-membrane/membrane, and envelope), seven non-structural protein genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), and 3'-UTR. The NS5 gene is a non-structural protein, which has the largest size of approximately 105 kDa, and the most conserved flaviviral protein, reaching around 900 amino acids [6]. This gene is the largest protein associated with enzymatic activity and virus replication. NS5 consists of a conserved sequence present in all DENV serotypes with a ratio of 67-80% [7]. It has two domains, namely the *RNA-dependent RNA polymerase* (RdRp) located at the C-terminal and the methyltransferase (Mtase) situated at the N-terminal [8]. Furthermore, the NS5 protein is the most extensively studied target for the development of specific antiviral vaccines. The RdRp domain plays a crucial role in the synthesis of positive and negative-sense RNA during the replication process, making it a vital protein target in the virus replication process [9]. Therapies for viral diseases have been extensively developed to reduce mortality, but the results are not entirely satisfactory. The "back to nature" slogan motivates studies to continue exploring active plant components to improve the quality of life and well-being. One of active components with potential antivirus properties is biflavonoid.

Biflavonoid is classified as phenolic compounds composed of two monomer units of flavonoids connected through C-C or C-O-C bonds. Common examples that possess antivirus activity *in vitro* include amentoflavone, cupressuflavone, hinokiflavone, agathisflavone, and robustaflavone. Specifically, robustaflavone reportedly has strong inhibition against Hepatitis B Virus (HBV) in human hepatoblastoma cells chronically infected with HBV, with an EC_{50} of 0.25 µM and a therapeutic index (IC_{50}/EC_{50}) of 153. Cupressuflavone showed antivirus activity against Herpes Simplex Virus (HSV-1) with an SI value of 2.98 [10]. Furthermore, cupressuflavone is known to possess potent activity as a BACE-1 enzyme inhibitor for Alzheimer's disease, with an IC_{50} value of 1.54 µM [11]. Hinokiflavone reportedly showed anti-influenza activity against the H1N1 virus with an IC_{50} value of 41.8 µg/ml [12]. Ryu *et al.* [13] reported that the compound amentoflavone inhibited the SARS-CoV virus by targeting the 3-chymotrypsin-like protease (3CLpro) with an IC_{50} of 8.3 µM. Amentoflavone, cupressuflavone, hinokiflavone, and robustaflavone also inhibit the DENV NS5 RdRp [14] and agathisflavone against the DENV-NS2B-NS3 protease [15].

Previous studies on 26 biflavonoids from the Araucaria genus, exploring the in silico potential as α-glucosidase inhibitors, indicated that agathisflavone, amentoflavone, cupressuflavone, and hinokiflavone were considered toxic based on the assessment of bioavailability and toxicity using admetSAR, while 22 derivatives were found to be non-toxic [16]. Nur [17] added that one derivative, 7''-*O*-methylrobustaflavone, was also classified as non-toxic. These 22 biflavonoids from the Araucaria genus consist of 8, 7, and 7 derivatives of agathisflavone, amentoflavone, and cupressuflavone, respectively. The antidiabetic activity of biflavonoids is determined by the position and number of hydroxyl (-OH) and methoxyl (-OCH_3) groups in the structure. The modification of the-OH group to-OCH_3 and retaining certain-OH groups at specific positions enhanced the activity as α-glucosidase inhibitors. Furthermore, Coulerie *et al.* [14] reported that 7,4',4'''-tri-*O*-methylamentoflavone, 7''-*O*-methylamentoflavone, and 4'''-*O*-methylamentoflavone showed stronger inhibitory activity against DENV-NS5 RdRp virus than the parent compound, amentoflavone. Among these 22 derivatives, seven compounds have been isolated from the leaves of *Araucaria hunsteinii* K. Schum and *Araucaria columnaris* growing in the Bogor Botanical Gardens [18–20]. Therefore, this study aimed to screen the 23 derivatives resulting from the studies of Sugita *et al.* [16] and Nur [17] to identify biflavonoids capable of being developed as potential antivirus candidates using in silico techniques.

In silico techniques, including molecular docking, is a valuable technology for the development of new antivirus agents. Molecular docking is one well-liked computational technique for determining the ligand's preferred orientation of binding to a molecular target [21]. Molecular docking facilitates the assessment of biflavonoids' affinity for the target sites of antiviral agents, hence offering valuable insights into the agents' efficacy. By identifying potential target interactions between the biflavonoid and other proteins, this technique can help gain a better knowledge of the pharmacological properties of the biflavonoid [22]. Despite the potential for predicting the stability and interactions between ligands and target proteins, in silico screening of biflavonoids as antivirals are still limited. In this study, molecular docking between ligands and proteins was performed using two programs, namely Yet Another Scientific Artificial Reality Application (YASARA) Structure and Protein-Ligand ANT System (PLANTS). The protein used was DENV-NS5 RdRp, a crucial metalloenzyme for virus replication. YASARA Structure and PLANTS are programs with different algorithms, hence, the parameters analyzed through YASARA Structure include binding free energy (ΔG), dissociation constant (Kd), and interactions. Meanwhile, PLANTS analyzed docking scores related to poses, as well as the structural relationship and strength of antiviral activity. Both programs are hoped to provide the best ligands based on the two methods' screening results.

MATERIALS AND METHODS

Materials

The protein structure of DENV NS5 RdRp (PDB ID: 5K5M) [23] was obtained from the research collaboratory for structural bioinformatics protein data bank (RCSB PDB) website, along with the 3D structures and canonical smiles of the reference and test ligands from the PubChem website. The 3D structures of the 23 experimental test ligands from the Araucaria genus were obtained from experiments and literature studies (table 1), while the reference ligand was balapiravir [24]. The hardware used had specifications, including an AMD E2-9000 Radeon R2 processor, 8.00 GB RAM, and a Windows 10 Pro 64-bit operating system, or a ThinkPad T420 with an Intel(R) Core (TM) i5-2430M CPU @ 2.40GHz processor, equipped with 8 GB RAM and a Windows 10 Pro 64-bit operating system. The software used included YASARA Structure, Marvinsketch, LigPlot+, and PyMOL, along with virtual platforms such as RCSB PDB, Consurf, PockDrug, PubChem, Plants, Yasara view, ChemDraw 19.1, and Discovery Studio 2021 Client.

Methods

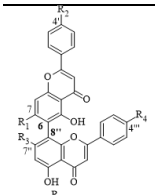
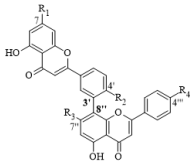
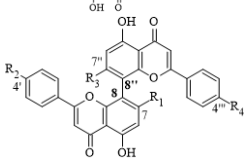
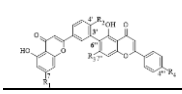
Prediction of conserved amino acids

The protein structure 5K5M was inputted into the Consurf website (https://consurf.tau.ac.il/index_proteins.php). Amino acid conservation was predicted by Consurf, and the results were displayed in the form of a sequence with color-coded labels corresponding to the level of conservation [25].

Potential pocket prediction

The DENV NS5 RdRp protein structure (PDB ID: 5K5M) was downloaded in *.pdb format from the RCSB PDB page (www.rcbs.org/pdb). Potential pocket predictions were carried out on the PockDrug page (http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=Druggability) by selecting druggability prediction using protein. The structures were uploaded to protein (s) information, then "prox" was selected in the pocket estimation method (s) followed by clicking "submit". The results were displayed in druggability probability values and a description of pocket characteristics, including volume, proportion of polar residues and aromatic residues, as well as number of residues [26].

Table 1: Twenty-three biflavonoid derivative ligands from the genus araucaria are safe based on in silico bioavailability tests and admetSAR

Structure	Compound		Functional groups			
	No	Name	R_1	R_2	R_3	R_4
	BF1	7,7''-di-*O*-methylagathisflavone	OCH_3	-OH	OCH_3	-OH
	BF2	4',7''-di-*O*-methylagathisflavone	-OH	OCH_3	OCH_3	-OH
	BF3	7,4',7''-tri-*O*-methylagathisflavone	OCH_3	OCH_3	OCH_3	-OH
	BF4	7,4'''-di-*O*-methylagathisflavone	OCH_3	-OH	-OH	OCH_3
	BF5	7,7'',4'''-tri-*O*-methylagathisflavone	OCH_3	-OH	OCH_3	OCH_3
	BF6	7-*O*-methylagathisflavone	OCH_3	-OH	-OH	-OH
	BF7	7''-*O*-methylagathisflavone	-OH	-OH	OCH_3	-OH
	BF8	7,4',7'',4'''-tetra-*O*-methylagathisflavone	OCH_3	OCH_3	OCH_3	OCH_3
	BF9	7,4',7''-tri-*O*-methylamentoflavone	OCH_3	OCH_3	OCH_3	-OH
	BF10	7,4',4'''-tri-*O*-methylamentoflavone	OCH_3	OCH_3	-OH	OCH_3
	BF11	4',4'''-di-*O*-methylamentoflavone	-OH	OCH_3	-OH	OCH_3
	BF12	7,4',7'',4'''-tetra-*O*-methylamentoflavone	OCH_3	OCH_3	OCH_3	OCH_3
	BF13	7''-*O*-methylamentoflavone	-OH	-OH	OCH_3	-OH
	BF14	7,7''-di-*O*-methylamentoflavone	OCH_3	-OH	OCH_3	-OH
	BF15	7,4'-di-*O*-methylamentoflavone	OCH_3	OCH_3	-OH	-OH
	BF16	7-*O*-methylcupressuflavone	OCH_3	-OH	-OH	-OH
	BF17	7,4',7''-tri-*O*-methylcupressuflavone	OCH_3	OCH_3	OCH_3	-OH
	BF18	7,7''-di-*O*-methylcupressuflavone	OCH_3	-OH	OCH_3	-OH
	BF19	7,4'''-di-*O*-methylcupressuflavone	OCH_3	-OH	-OH	OCH_3
	BF20	7,4',7'',4'''-tetra-*O*-methylcupressuflavone	OCH_3	OCH_3	OCH_3	OCH_3
	BF21	7,7'',4'''-tri-*O*-methylcupressuflavone	OCH_3	-OH	OCH_3	OCH_3
	BF22	4',4'''-di-*O*-methylcupressuflavonne	-OH	OCH_3	-OH	OCH_3
	BF23	7''-*O*-methylrobustaflavone	-OH	-OH	OCH_3	-OH

Note: BF1 to BF22 [16], and BF23 [17]

Protein preparation

The 3D structure of the DENV NS5 RdRp protein (PDB ID: 5K5M) was prepared using YASARA Structure. Water molecules and unnecessary residues were removed from the protein structure. The co-crystal ligand was separated from the target protein, and hydrogen atoms were then added. The results were saved in PDB file format (*.pdb) [27] and protein. mol2 file format [28].

Preparation of co-crystal and test ligands on target proteins with the YASARA structure program

The 3D structure of the target protein was opened in *.pdb format using YASARA. The co-crystalline ligand was separated from the target protein and saved in a PDB file (*.pdb) format. Preparation of reference and test ligands was carried out using 3D structures in *.sdf format of the parent compounds biflavonoids, agathisflavones, amentoflavones, cupresuflavones, and robustaflavones obtained from the PubChem page (https://pubmed.ncbi.nlm.nih.gov/). For the 23 test ligands, the 2D structures were drawn manually using MarvinSketch, while the 3D structure was saved in *.sdf format. Subsequently, the structures of the test and reference ligands were minimized using YASARA and saved in PDB file format (*.pdb) [29].

Preparation of co-crystal and test ligands on target proteins with the PLANTS program

The co-crystal and test ligands were manually drawn in two dimensions using MarvinSketch. Subsequently, the ligands were subjected to protonation optimization using the Major Microspecies method, and the structures were adjusted under pH 7.4 conditions. After the optimization, the conformation most suitable for the DENV NS5 RdRp virus protein (PDB ID: 5K5M) was determined by selecting the conformers method. The number of conformations for each ligand could be adjusted to produce docking scores with the best poses relative to the protein using PLANTS docking. The number of conformations represents the ligand's positions within the pocket [30].

Validation of molecular docking methods

Validation of the anchoring method for both programs (YASARA Structure and PLANTS) was carried out by determining the Root mean Square Deviation (RMSD) parameter, which was carried out in the YASARA program (Analyze>RMSD>Molecule). The results of protein validation were saved in *.pdb format and co-crystal ligands in *.pdb format. The gridbox size ranged from 1.0 Å to 7.0 Å with an interval of 0.5 Å. The ligand co-crystal structure was removed for redocking, and then the protein was saved in YASARA Scene (.*sce) format. Redocking was carried out with the prepared protein in *. sce format and the co-crystal ligand prepared in *.pdb format was inserted. Validation was carried out 100 times and the resulting parameters include binding ΔG, Kd, and amino acids in the *.txt file. The validation results in *.yob format was converted to *.pdb, then the RMSD values were determined using PyMOL. The validation parameters with PLANTS yielded docking score values. Validation was performed, with redocking results consisting of 50 poses of the co-crystal ligand against the protein. Redocking of the co-crystal ligand to the protein in. mol2 format produced the best pose with the lowest docking score. The docking results were considered valid when the RMSD value was ≤ 2.5 Å [31]. A lower RMSD value indicates greater similarity between the docking poses of the test and the crystal ligand [32].

Molecular docking of ligands with proteins using the YASARA structure and PLANTS programs

Molecular docking of ligands with the YASARA Structure program was performed on the protein prepared in .pdb format and the grid box size from the best validation results. The protein was then saved in YASARA Scene format (.sce). The analyzed parameters were the same as during validation [32]. Molecular docking of ligands with the PLANTS program was carried out on the Windows operating system, while the protein and ligands were prepared in. mol2 format. The next process entailed searching for the binding site by entering the command "plants--mode bind ref_ligand. mol2 5 protein. mol2". For the docking, the command "plants--mode screen pc_5k5m. txt" was used. The docking results could be viewed by entering the command "cd results" followed by "more bestranking. csv", in the form of a "results" folder containing the bestranking. csv file. This file contains the docking values or scores from the results [33].

Visualization of ligand-protein interactions

The visualization of interactions between ligands and proteins from molecular docking results was conducted using LigPlot+for 2D and PyMOL for 3D. The molecular docking results in *.yob format was converted to *.pdb using YASARA Structure. Structures in *.pdb format were then visualized using LigPlot+, PyMOL, and Discovery Studio 2021 Client.

RESULTS AND DISCUSSION

Conserved amino acids

The prediction was performed using the ConSurf virtual platform, aiming to identify amino acids playing a role in the crucial sites of the DENV NS5 RdRp protein (PDB ID: 5K5M). The results provided information on the amino acids contained in the protein, categorized by conservation levels ranging from score 1 to 9. Amino acids were considered conserved when the score ranged from 7-9, while scores of 1-3 indicated residues with low conservation levels [25]. Ligand 68T had the highest number of conserved amino acids, with 19 residues, including crucial four at the N-pocket site, namely Arg729, Arg737, Trp795, and His798 (table 2). Visualization of the crystallographic ligand on the DENV NS5 RdRp protein (PDB ID: 5K5M) and the amino acid interactions with the ligand is shown in fig. 1.

Table 2: Conserved amino acids that interact with DENV NS5 RdRp

Ligand	Amino acid
MES	Val321, Asn322[A], Gly323, Val324[A], Arg326, Leu327, Leu748, Leu873
68T	Gly511[A], Leu512[A], His513[A], Leu515[A], Cys709[A], Ser710[A], His711[A], Arg729[A], Arg737[A], Met761[A], Met765[A], Tyr766[A], Thr793[A], Thr794[A], Trp795[A], Ser796[A], His798[A], Ala799, Lys800, His801, Glu802[A], Trp803[A]
Description:	
Blue highlight =	Important N-pocket residues
[A] =	Conservation level 7-9

Lim *et al.* [23] reported that the binding of 68T to the DENV NS5 RdRp protein was located at the N-pocket site, allowing the ligand to alter the active site conformation [34]. According to Kumar *et al.* [35], the test ligands desmopressin, rutin, lypressin, and lanreotide interact similarly to the ligand 68T with crucial residues in the N-pocket (Arg729 and Arg737) and the priming loop residues (Lys800 and Glu802).

Potential pocket

This prediction was conducted using the virtual platform PockDrug, a platform capable of predicting the druggability potential of a pocket on a protein in binding drug molecules. The predicted results from the DENV NS5 RdRp protein (PDB ID: 5K5M) showed two pockets, namely MES and 68T, as observed in table 3. The best pocket was selected based on druggability probability parameters. The 68T pocket was superior to MES, as it had a druggability probability value of 0.71 and comprised 24 conserved amino acid residues. A pocket with a druggability probability greater than 0.5, binding more than 14 conserved amino acid residues, is predicted to have a good probability [26]. Based on these parameters, the 68T binding pocket was selected for further molecular docking steps, both with the YASARA Structure and PLANTS programs.

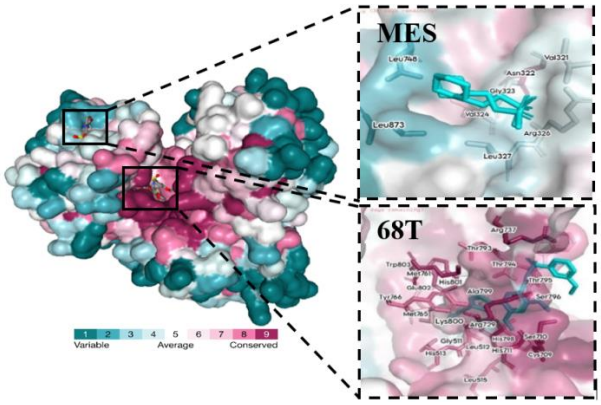


Fig. 1: Conserved amino acids at the critical site of the DENV NS5 RdRp protein (5K5M)

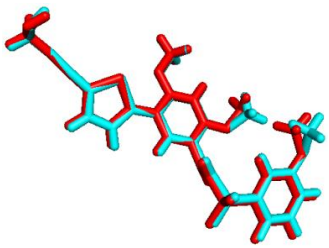
Table 3: Predicted potential pockets using pock drug

Pocket	Pocket volume	Hydrophobicity	Polar residue	Aromatic residue	Otyr	Total residue	Druggability probability
68T	1916.69	-0.73	0.71	0.33	0.02	24.0	**0.71**
MES*	412.08	1.43	0.25	0.0	0.0	8.0	**0.98**

Description: * = small pocket (number of residues less than 14)

Gridbox and gridbol size

Determination of the Gridbox with the YASARA Structure program resulted in asize of 5.5 Å, which was considered optimal grid box as it had the most negative ΔG value, namely-9.5800 kcal/mol, and an RMSD of 0.370 Å. Additionally, the superimposed results of ligand 68T on the structure 5K5M, and the validated ligand 68T showed the most suitable positional similarity with an RMSD value of 0.2596 Å, as presented in fig. 2. Nursamsiar *et al.* [36] stated that a good gridbox size for molecular docking should have an RMSD value of less than 2 Å. Meanwhile, determination of the Gridbol with the PLANTS program occurred in the protein-ligand binding site region with x=-15.8828, y=-43.8665, z=-18.6448, and a binding site radius of 14.991 Å. The best RMSD value was 2.0899 Å on the pose RNA-dependent RNA polymerase_entry_00011_conf_01, which had a docking score of-117.5500. In line with previous reports, the RMSD value was acceptable as it fell within the range of 2.0 to 3.0 Å [37]. According to Rollando [31], the validation is considered successful when the RMSD value obtained is less than or equal to 2.5 Å. Fig. 3 shows the superimposed results of both ref_ligand redocking and the experimental co-crystal ligand with closely matched poses.

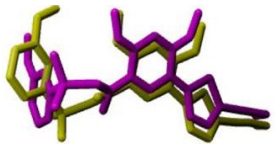


Red structure = Ligand 68T in the 5K5M structure,
Cyan structure = Ligand 68T results from 5.5 Å gridbox validation

Fig. 2: The position of the 68T ligand on the protein in a gridbox size of 5.5 Å with an RMSD value of 0.370 Å based on the YASARA Structure program

Selected biflavonoids by molecular docking

Previous studies have evaluated derivatives of biflavonoids against the α-glucosidase enzyme [16]. To expand the scope, molecular docking of DENV NS5 RdRp (PDB ID: 5K5M) was carried out using two in silico programs, namely YASARA Structure and PLANTS. The molecule was evaluated against 68T and balapiravir (cytidine nucleoside) as a comparative ligand. The docking results showed an interaction with the N-pocket site, which inhibited the initiation stage in the de novo RNA synthesis process, while an IC_{50} value of 0.048-0.172 μM was obtained in *in vitro* evaluation. The use of balapiravir as a reference ligand refers to the study by Bhattarai *et al.* [24] where the drug was docked to the DENV NS5 RdRp protein using Genetic Optimization for Ligand Docking (GOLD). The result showed a ΔG value of-12.1 kcal/mol. Furthermore, Nguyen *et al.* [38] reported that the EC_{50} value of balapiravir in inhibiting the DENV NS5 RdRp protein *in vitro* ranged from 1.3-3.2 μM. Table 4 presents the measurable molecular docking results from the YASARA program in the form of ΔG and dissociation constant (Kd), while PLANTS provides docking scores.



Green color = Experimental 68T ligand, Purple color = Ligand 68T results from redocking of 50 confirmations

Fig. 3: The position of the 68T ligand on the protein in gridbol size with a docking score of-117.5500 and the best RMSD of 2.0899 Å based on the PLANTS program

Molecular docking with the YASARA program indicated that all derivatives of bioflavonoids were selected as inhibitors of the DENV NS5 RdRp protein. Derivative compounds BF2, BF7, BF13, BF14, BF16, BF19, BF22, and BF23 with ΔG in the range of-9.6040 to-10.3540 kcal/mol had lower ΔG values than 68T (ΔG-9.5800 kcal/mol) and balapiravir (ΔG =-8.3530 kcal/mol). Meanwhile, other compounds including BF1, BF3, BF4, BF5, BF6, BF8, BF9, BF10, BF11, BF12, BF15, BF17, BF18, BF20, and BF21 with ΔG in the range of-8.9260 to-9.5130 kcal/mol showed values higher than 68T but lower compared to balapiravir as the reference ligand. This implied that all test ligands formed higher stability complexes with the DENV NS5 RdRp protein, resulting in better advantages than balapiravir. Nelson and Cox [39] stated that compounds with more

negative ΔG values have a stronger and more spontaneous affinity for interacting with proteins. The result was consistent with a previous study that screened biflavonoids against the α-glucosidase enzyme protein, with acarbose as the *in silico* reference ligand. Sugita *et al.* [16] reported that 22 derivatives of biflavonoids inhibited the α-glucosidase enzyme protein with ΔG values ranging from-7.2 to-9.5 kcal/mol, lower than acarbose as the reference ligand (ΔG =-6.3 kcal/mol).

Table 4: Results of molecular docking analysis of 68T, balapiravir, and BF1 to 23 with the DENV NS5 RdRp protein using the yasara structure and plants programs

Compound	Yasara structure		Plants
	ΔG (KCAL/mol)	Kd (PM)	Docking score
68T	-9.5800	9.50×10^4	-117.5500
Balapiravir	-8.3530	7.54×10^5	-93.5236
BF23	-10.3540	2.57×10^4	-95.8737
BF13	-10.1820	3.44×10^4	-95.5629
BF14	-10.0190	4.53×10^4	-98.1874
BF16	-9.7920	6.64×10^4	-95.2348
BF2	-9.7840	6.73×10^4	-92.6135
BF19	-9.7610	7.00×10^4	-95.9253
BF22	-9.6880	7.92×10^4	-96.0786
BF7	-9.6040	9.12×10^4	-90.7047
BF6	-9.5130	1.06×10^5	-89.3655
BF10	-9.4970	1.09×10^5	-100.2460
BF15	-9.4920	1.10×10^5	-104.7450
BF11	-9.4720	1.14×10^5	-98.3149
BF3	-9.4610	1.16×10^5	-94.0155
BF4	-9.4410	1.20×10^5	-90.2434
BF1	-9.2350	1.70×10^5	-89.5818
BF12	-9.0500	2.32×10^5	-95.9319
BF5	-9.0430	2.35×10^5	-92.3850
BF20	-9.0400	2.36×10^5	-94.4259
BF21	-9.0190	2.45×10^5	-91.7511
BF17	-8.9860	2.59×10^5	-91.2757
BF8	-8.9140	2.92×10^5	-94.6170
BF9	-8.9580	2.71×10^5	-99.8523
BF18	-8.9260	2.86×10^5	-93.2079

The results of molecular docking with the PLANTS program indicated that only compounds BF3, BF8, BF9, BF10, BF11, BF12, BF13, BF14, BF15, BF16, BF19, BF20, BF22, and BF23 were selected as inhibitors of the DENV NS5 RdRp protein. These compounds had docking scores ranging from-94.0155 to-104.7450, lower than balapiravir (-93.5236) as the reference ligand but behaved differently from 68T (-117.5500). Other compounds, BF1, BF2, BF4, BF5, BF6, BF7, BF17, BF18, and BF21 were not selected due to the higher docking scores (-89.3655 to-93.2079) compared to both 68T and balapiravir. Syahputra *et al.* [40] reported that ligands with more negative docking scores have strong interactions with proteins.

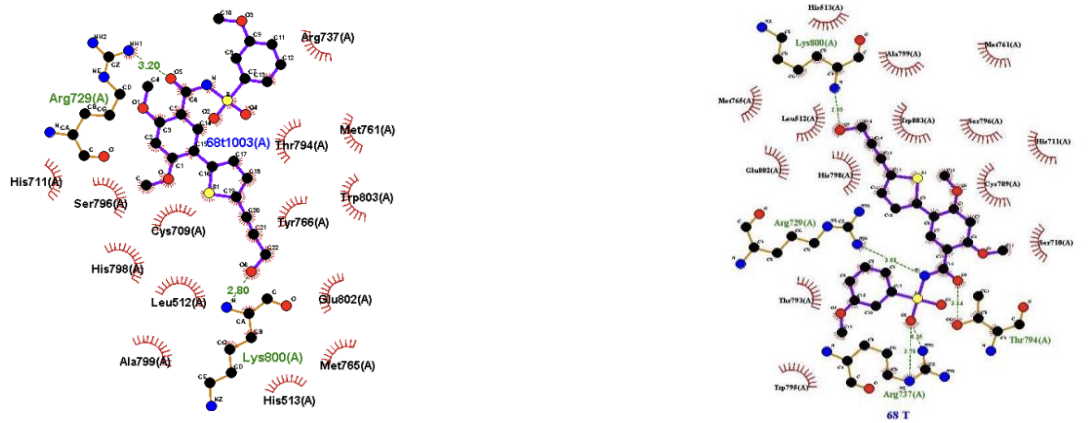
Comparative screening based on ΔG values and docking scores resulted in 14 ligands selected for interacting with the DENV NS5 RdRp protein. These include BF3, BF8, BF9, BF10, BF11, BF12, BF13, BF14, BF15, BF16, BF19, BF20, BF22, and BF23 from both programs. Meanwhile, compounds BF1, BF2, BF4, BF5, BF6, BF7, BF17, BF18, and BF21 passed the selection based on the YASARA Structure program only. The selected ligands from both programs were then analyzed for binding interactions with the DENV NS5 RdRp protein. These compounds interact with the inhibitor through hydrogen bonding, hydrophobic interactions including carbon-hydrogen bonds, alkyl, π-alkyl, π-sigma, and Van der Waals interactions, as well as binding at the active site of the catalytic GDD residues, compared to 68T and balapiravir. YASARA observation resulted in the selection of six ligands, namely BF3, BF10, BF11, BF12, BF13, and BF14, while PLANTS selected three ligands, including BF3, BF8, and BF23. Compounds BF3 and BF8 are agathisflavone, BF10-BF14 are amentoflavone, and BF23 belongs to robustaflavone. The visualization of ligand-receptor interactions is presented in Tables 5 and 6. The selected derivatives of biflavonoids were considered the best ligands capable of being developed as candidate antiviral drugs against the DENV NS5 RdRp protein. The 2D visualization of 68T, balapiravir, one best ligand, BF3 based on YASARA Structure and PLANTS programs, with two ligands from the YASARA Structure program, namely BF10 and BF13, is shown in fig. 4 and 5, respectively.

In vitro studies of selected biflavonoids have been carried out on various biological activities, such as antiviral DENV-NS5 RdRp [14], α-glucosidase, and anticancer enzymes [16, 18, 20]. Furthermore, *in vitro* testing of the amentoflavone derivatives BF10; 4',4'''-di-*O*-methylamentoflavon (BF11); BF13; and 7,4'-di-*O*-methylamentoflavone (BF15) against the DENV-NS5 RdRp target protein showed IC_{50} values of 1.0, 3.12, 0.16, and 1.6 μM, respectively. Another amentoflavone derivative, 4'''-*O*-methylamentoflavon, had an IC_{50} of 0.75 μM [14]. Compounds BF10, BF13, and 4'''-*O*-methylamentoflavone were stronger, while BF11 and BF15 were weaker than the parent amentoflavone (IC_{50} = 1.3 μM). The IC_{50} values of compounds BF10 and BF13 were in line with the ΔG values. Moreover, the docking scores were more negative and the Kd was also lower than the BF11 and BF15 ligands (table 2). Kd is a parameter that shows the tendency for repulsive interactions of ligand-protein complexes; the lower the value, the stronger the ligand-protein interaction [41]. The compound 7''-*O*-methylrobustaflavone (BF23) has never been tested *in vitro* against DENV-NS5 RdRp, but the parent robustaflavone causes inhibition with an IC_{50} of 0.33 μM [14]. The compounds BF3, and 7,4',7'',4'''-tetra-*O*-methylagathisflavone (BF8) have also never been tested *in vitro* against DENV-NS5 RdRp, but the parent agathisflavone inhibits DENV2-and DENV3-NS2B target proteins-NS3 P with IC_{50} of 15.1 and 17.5 μM, respectively [15].

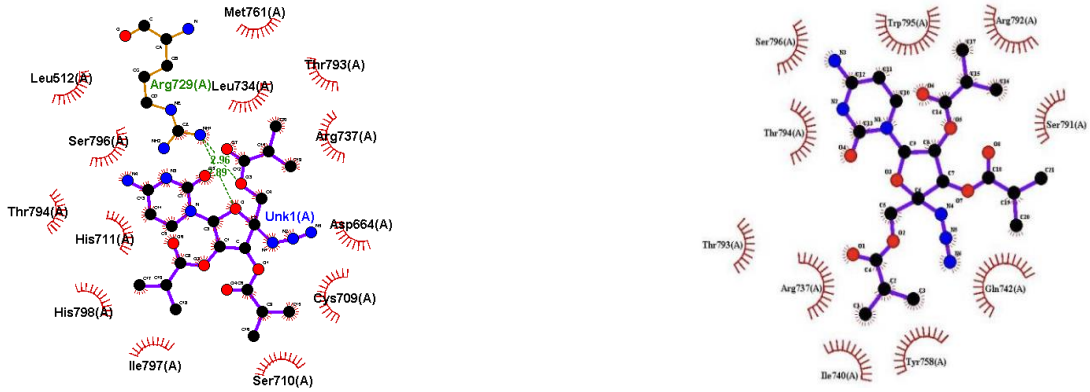
Among the compounds selected as inhibitors of the DENV-NS5 RdRp target protein in silico, seven ligands have been isolated from acetone extracts of *A. hunsteinii* [18, 19] and *A. columnaris* [20] growing in the Bogor Botanical Gardens. Four of the seven selected compounds have potential as inhibitors from the second screening program, namely BF11, 7-*O*-methylcupressuflavone (BF16), 7,4'''-di-*O*-methylcupressuflavone (BF19), and 7,4',7'',4'''-tetra-*O*-methylcupressuflavone (BF20). Meanwhile, the other three compounds 7,7''-di-*O*-methylagathisflavone (BF1), 7,7'',4'''-tri-*O*-methylagathisflavone (BF5), and 7,4',7''-tri-*O*-methylcupressuflavone (BF17) were only selected as having strong potential based on the YASARA Structure program.

Table 5: Visualization of ligand interactions with amino acid residues of the ligand-receptor 68T and balapiravir based on the YASARA Structure program

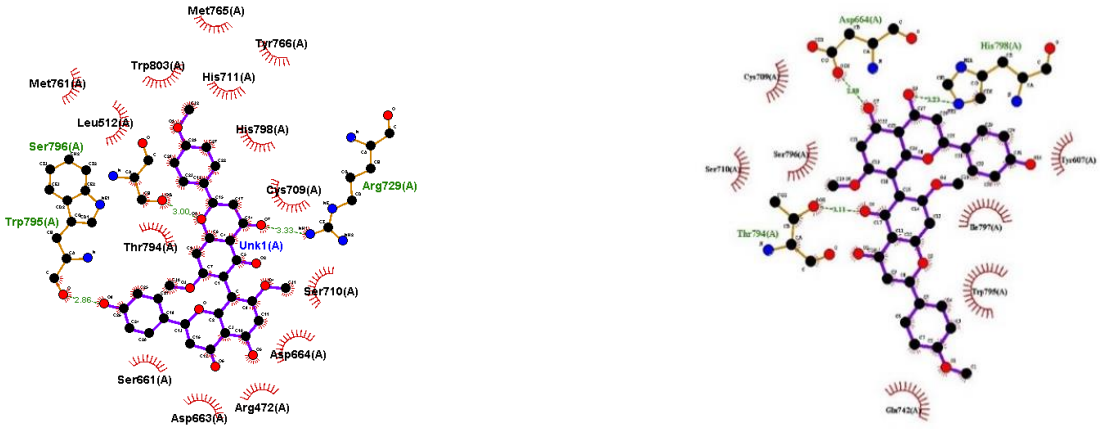
Ligand	Hydrogen bonding	Hydrophobic interactions
68T	Arg729[A](3,20Å), Lys800 (2,80Å)	Leu512[A], His513[A], Cys709[A], His711[A], Arg737[A], Met761[A], Met765[A], Tyr766[A], Thr794[A], Ser796[A], His798[A], Ala799, Glu802[A], Trp803[A]
Balapiravir	$Arg729_1$[A](2,89Å), $Arg729_2$[A] (2,96Å)	Leu512[A], Asp664[A], Cys709[A], Ser710[A], His711[A], Leu734[A], Arg737[A], Met761[A], Thr793[A], Thr794[A], Ser796[A], Ile797, His798[A]
BF3	Arg472, Asp664, Arg729, Trp795, Ser796	Leu512, Asp664, Cys709, His711, Met761, Ala799
BF10	Asp664, Arg729, Tyr766, Thr794, Trp795, Ser796, Ala799	Leu512, His711, Met761, Trp795, Ser796, Ile797, Ala799
BF11	Asp664, Arg729, Met761, Thr794, Ser796, His798	His711, Trp795, Ile797, Ala799
BF12	Asp664, Arg729, Thr794	Arg729, Trp795
BF13	Asp663[A] (3,18Å), Cys709[A] (3,03Å), Arg737[A] (3,10Å), His798[A] (3,08Å)	Leu512[A], Gly662[A], Asp664[A], Ser710[A], His711[A], Arg729[A], Met761[A], Thr794[A], Ser796[A], Ala799, Trp803[A]
BF14	Cys709[A] (2,85Å), Arg737[A] (3,07Å)	Leu512[A], Tyr607[A], Asn610[A], Gly662[A], Asp663[A], Ser710[A], His711[A], Arg729[A], Met761[A], Thr794[A], Ser796[A], His798[A], Ala799, Trp803[A]



a. 68T (co-crystalline ligand)

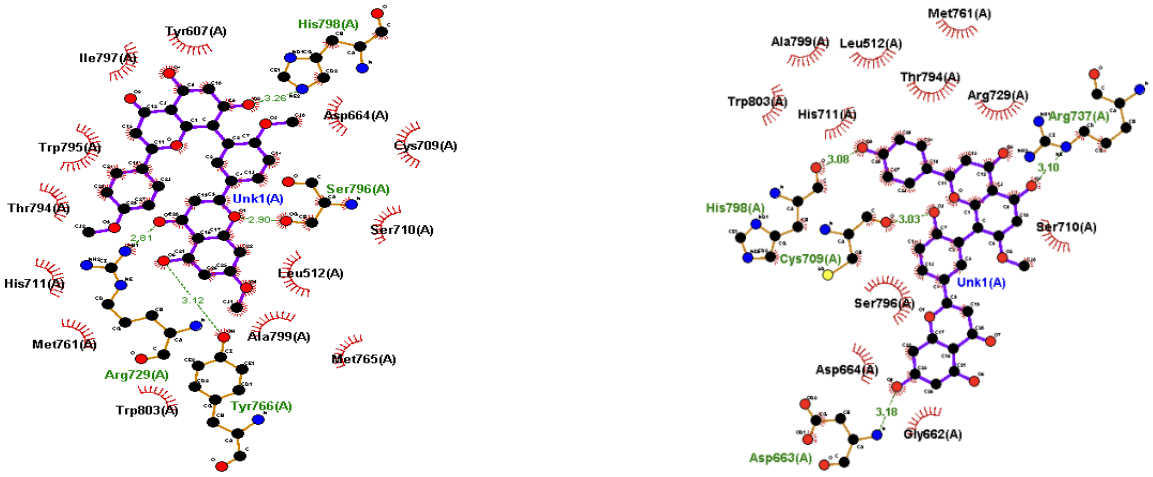


b. Balapiravir (comparison ligand)



7,4',7''-tri-*O*-methylagathisflavone (BF3)

Fig. 4: 2D Visualization of 68T (a), balapiravir (b), and the compound 7,4',7''-tri-*O*-methylagathisflavone (BF3) (c) based on the YASARA structure and PLANTS programs



a. 7,4',4'''-tri-*O*-methyl-amentoflavone (BF10) **b. 7''-*O*-methyl-amentoflavone (BF13)**

Fig. 5: 2D Visualization of the compounds 7,4',4'''-tri-*O*-methyl amentoflavone (BF10) (a) and 7''-*O*-methyl-amentoflavone (BF13) based on the YASARA Structure program

Simultaneous in silico screening of biflavonoid derivatives was also conducted to identify potential anti-rheumatic drug candidates [42]. The screening against the 20S proteasome with target proteins 5LE5 and 5LF7 showed that BF13 and 7,7''-di-*O*-methylamentoflavone (BF14) were the best inhibitor ligands. Other compounds identified as the best inhibitors for the 5LF7 target protein include BF15 and 7,4',7''-tri-*O*-methylamentoflavone (BF9) [42]. The 5LE5 is the crystal structure of the human 20S proteasome without a co-crystallized ligand, while 5LF7 is a structure obtained from the co-crystallization with the covalent inhibitor Ixazomib [43].

In previous studies, selected biflavonoid derivative ligands were tested as inhibitors of the α-glucosidase enzyme. The order of inhibition from strong to weak respectively include BF16, BF1, BF11, BF19, 7,7'',4'''-tri-*O*-methylagathisflavone (BF5) with IC_{50} values of 78.32±0.52, 388.39±0.68, 389.76±1.54, 537.98±2.35, and 12282.04±196.55 µM [16]. Comparative ligand acarbose had IC_{50} values of 607±56 µM(44), and 840±1.73 µM(45). Another *in vitro* test was conducted for inhibition of MCF-7 (ATTC HTB 22), HeLa (ATTC CCL-2), and Calf Pulmonary Arterial Endothelial (CPAE) cancer cells. The inhibitory activity ranged from very active to moderate against the tested cancer cells [46]. Cytotoxicity test data for the three cancer cells are shown in table 7.

The in silico and *in vitro* test results mutually reinforce each other, with the varying activity of biflavonoid derivative compounds being dependent on the parent structure, type, and number of hydroxyl (-OH) or methoxy ($-OCH_3$) substituents [47]. Different substituents indicate specific interactions with the corresponding protein/target molecules. Biflavonoids can bind to respective targets and inhibit the activities, while the attached substituents also play a crucial role in controlling the energy of interactions [48].

Table 7: IC_{50} values of compounds BF1, BF5, BF11, BF16, BF17, BF19, and BF20 against MCF-7 (ATTC HTB 22), HeLa (ATTC CCL-2) and Calf Pulmonary Arterial Endothelial (CPAE) cells

Ligand name	IC50±STD[a] (µM)		
	MCF-7[b]	HELA[b]	(CPAE)[c]
7,7''-di-*O*-methylagathisflavone (BF1)	115.4±36.5	107.6±37,3	ND*
7,7'',4'''-tri-*O*-methylagathisflavone (BF5)	314.4±25.0	337.05±26.7	208.6±54.8
4',4'''-di-*O*-methylamentoflavone (BF11)	2.1±0.6	11.0±2.9	99.2±1.4
7-*O*-methylcupressuflavone (BF16)	3.4±0.5	1.4±1.1	ND*
7,4',7''-tri-*O*-methylcupressuflavone (BF17)	91.7±5.6	ND*	114.0±27.5
7,4'''-di-*O*-methylcupressuflavone (BF19)	11.5±3.4	35.6±1.3	69.8±2.5
7,4',7'',4'''-tetra-*O*-methylcupressuflavone (BF20)	397.9±28.6	528.8±40	403.0±22.7

*ND = Not Detected; [a]STD: standard deviation; [b][18], [c][20].

CONCLUSION

In conclusion, molecular docking of 23 biflavonoids from the Araucaria genus to the DENV NS5 RdRp protein through in silico techniques of YASARA Structure and PLANTS programs resulted in the selection of six and three top ligands, respectively. The six selected ligands based on YASARA Structure were BF3, BF10, BF11, BF12, BF13, and BF14, while the three selected through PLANTS included BF3, BF8, and BF23. The eight overall best ligands were 7,4',7''-tri-*O*-methylagathisflavone (BF3), 7,4',7'',4'''-tetra-*O*-methylagathisflavone (BF8), 7,4',4'''-tri-*O*-methylamentoflavone (BF10), 4',4'''-di-*O*-methylamentoflavone (BF11), 7,4',7'',4'''-tetra-*O*-methylamentoflavone (BF12), 7''-*O*-methylamentoflavone (BF13), 7,7''-di-*O*-methylamentoflavone (BF14), and 7''-*O*-methyl-robustaflavone (BF23) with great potential as antiviral drug candidates. These ligands had lower ΔG, Kd, and docking scores than the reference, balapiravir. All ligands showed non-covalent bindings, such as hydrogen bonding and hydrophobic interactions with the protein through conserved amino acid residues, N-pocket, and the catalytic GDD.

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AUTHORS CONTRIBUTIONS

The experiment was carried out by Najma Aulia Nur, Syifa Silfani Rodotul Zanah, and Kurniawanti, who also prepared, edited, and amended the manuscript. Laksmi Ambarsari and Purwantiningsih

Sugita conceived the design research, supported the analysis, and revised the manuscript. Hanhan Dianhar, Siti Warnasih, and Dyah Utami Cahyaning Rahayu supervised the docking molecular of ligand and receptor interaction and revised the manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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