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COMBINED *In-silico* AND *In-vitro* APPROACHES TO EVALUATE THE INHIBITORY THE POTENTIAL OF BIFLAVONOIDS FROM ARAUCARIA PLANTS AGAINST α-GLUCOSIDASE AS TARGET PROTEIN

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ABSTRACT

Biflavonoids are dimers of flavonoids created by a covalent connection of C-C or C-O-C between two flavonoids and have been found to have a variety of pharmacological effects, including the ability to treat diabetes mellitus (DM). In the current investigation, the inhibitory effect of biflavonoids extracted from *Araucaria hunsteinii* K. Schum towards the target protein α-glucosidase was assessed using a combination of *in-vitro* experiments and *in-silico* molecular docking approach. The biflavonoids' inhibition properties were contrasted with those of acarbose, a widely used pharmaceutical for treating type 2 DM. AutoDock Vina was employed to analyze the conformational sites and docking parameters, such as binding affinity and inhibition constant. *In-silico* studies showed that biflavonoids effectively interacted with the active site of the α-glucosidase enzyme, which is in charge of cleaving not only bonding of the α-1,4 but also the α-1,6 glycosidic on the exterior of amylose or amylopectin residues to obtain simple sugars. The docking experiments revealed that biflavonoids had tighter binding forces than acarbose against α-glucosidase. The selected biflavonoids, 7-*O*-methylcupressuflavone; 7,7"-di-*O*-methylagathisflavone, 4′,4‴-di-*O*-methyl-amentoflavone and 4‴,7-di-*O*-methylcupresuflavone showed an IC_{50} of 78.32±0.52; 388.39±0.68; 389.76±1.55 and 537.98±2,35 µM, respectively. These biflavonoids had a low binding affinity and more hydrogen bond interactions with the target enzyme, which had several important amino acid residues. The effectiveness of these compounds in inhibiting the enzyme may be explained by some of their hydrophobic interactions. Therefore, the study comes to the conclusion that biflavonoids are prospective antidiabetic agents and should be taken into consideration when developing candidates for new antidiabetic medicines.

Keywords: Biflavonoids, α-Glucosidase, *In-silico*, *In-vitro.*

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INTRODUCTION

A computer-based or digital simulation-based experiment is known as an *in-silico* experiment. New drug design will save costs and time because the selection stage is done computationally. *In-silico* can predict drug structure through mathematical equations, visualizing three-dimensional shapes, and evaluating compound interactions with the target before synthesizing it into a drug. To create novel compounds with high activity and little steric interference, the lead moiety of the compound is chemically or molecularly changed.[1] Drug design is a vital technique in medicinal chemistry. Critical elements of structure-based drug design include docking small molecules into receptor binding sites is the complex's binding affinity.[2-3]

A group of metabolic diseases collectively referred to as diabetes mellitus (DM) is characterized by chronic hyperglycemia or persistently increased blood sugar levels.[4] By 2030, predicted that there will be 21.3 million individuals globally with DM, and Indonesia is one of a country having a high prevalence rate of

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about 12 million in 2019.[5] The two different types of DMs are type 1 and type 2. However, as many as 90% of diabetics are type 2 DM because an unhealthy lifestyle influences it. Postprandial glucose levels in patients with type 2 DM should be monitored. Its levels can be regulated by delaying glucose absorption by blocking digestive organs' α-glucosidase action, which plays for hydrolyzing of carbohydrates.[6] The American Association of Clinical Endocrinologists has recommended acarbose for therapy of type 2 DM as α-glucosidase inhibitors (AGI). Acarbose can have harmful side effects over time, including flatulence, stomach pain, and diarrhea. α-Glucosidase is an exoenzyme enzyme that acts on the exterior of amylose or amylopectin cleavage residues by cleaving not only α-1,4 glycosidic bonds but also on α-1,6 glycosidic bonding's to produce simple sugars. Human lysosomal α-glucosidase has been studied on the Asp518 residue and other residues near the enzyme's active site. On the other hand, the presence of Trp516 and Asp518 residues also plays an important role in their performance as catalysts.[7] This study used a receptor in the form of maltase-glucoamylase as the α-glucosidase enzyme. The docking procedure must be carried out utilizing their structural homology because the crystal structure of homo sapiens α-glucosidase is not yet available. The structural homology used a pattern of the maltase-glucoamylase crystal structure of homo sapiens afforded from Protein Data Bank (PDB) with 2QMJ PDB code and 1.90 resolution. Many communities are starting to approach back-to-nature treatment by utilizing plant phytochemicals. One of them that has the potential as antidiabetic is biflavonoid which is a phenolic group of compounds. The literature study showed that several biflavonoid compounds have the potential as antidiabetic, including garcinia biflavonoid 1 (GB1) and 2 (GB2), colaviron,[8-9] macrophyloflavones,[10] agathisflavone,[11] and amentoflavones.[12] Biflavonoids are very interesting to study by isolation or laboratory synthesis. In developing countries like Indonesia, besides experimentation in the laboratory, other strategies are needed to discover new drugs. An *in-silico* strategy must be established in addition to an *in-vitro* and *in-vivo* analysis of a compound. *In-silico* screening is an affordable method for identifying possible interactions between compounds and selected targets. *In-silico* screening can enrich a subset of chosen compounds with molecules that are likely to hit a target molecule before any effort is made. Biflavonoids have the potential to be an effective treatment for DM by inhibiting α-glucosidase and reducing blood glucose levels. The current research aims to determine if *in-vitro* and *in-silico* investigations can reveal the inhibitory effect of biflavonoids and derivatives against the α-glucosidase enzyme. The overall findings are crucial in determining the appropriate and optimum chemical structure of biflavonoids towards α-glucosidase enzyme and treating type 2 DM.

EXPERIMENTAL

General Procedures

Twenty-six biflavonoid ligands and their derivatives from the Araucaria plant in 3D structure as test ligand (Table-1) in *.sdf format, crystal structure of homo sapiens maltase-glucoamylase (PDB 2QMJ, Fig.-1A), the 3D structure of the α-glucosidase enzyme as receptor and 3D form of acarbose as DM commercially ligand in *.pdb format (Fig.-1B), and five biflavonoids were isolated from Indonesian *Araucaria hunsteinii* leaves.[13-14] Chemicals used α-glucosidase assay included 0.1 M phosphate buffer (pH 7.0), α-glucosidase, 0.5 mM 4-nitrophenyl α-D-glucopyranoside, and 0.2 M sodium carbonate solutions. Hard and software used: AutoDock Tools 1.5.6 (The Scripps Research Institute, USA), Laptop with specifications Acer One 14-Z1401 Intel® Celeron® Dual Core N2840, 2 GB RAM, Windows 8 operating system, Autodock Vina Tools, Discovery Studio Visualizer, PyMOL, Ligplot+, ChemDraw Ultra 12.0.2, as well as Swissadme and admetSAR websites.

Preparation of Target Receptor, Biflavonoids Ligand, and Gridbox

The α-glucosidase was downloaded in *.pdb format (PDB ID: 2QMJ) from the Protein Data Bank (PDB). The downloaded biflavonoid ligand and its derivatives were derived from PubChem in *.pdb design. Then, the ligand file format was changed to *.pdbqt and saved on the drive (C:) from windows. Before molecular docking, it is necessary to prepare the Gridbox size in Autodock Tools 1.5.6. The grid box is computed from the active site coordinates of -glucosidase using the Autodock Tools software.

Docking Method Validation

Validation was carried out using the Autodock Vina program on the α-glucosidase enzyme receptor with acarbose using a spacing of 1 Å. The prepared receptor and ligand structures are stored in the "Vina

Docking" folder, which is already stored on the drive (C:), then a configuration file (conf) is created by entering the receptor name, ligand, box size, and center box, and setting the energy and amount the mode used. Validation was carried out using the command prompt program with 20 repetitions.

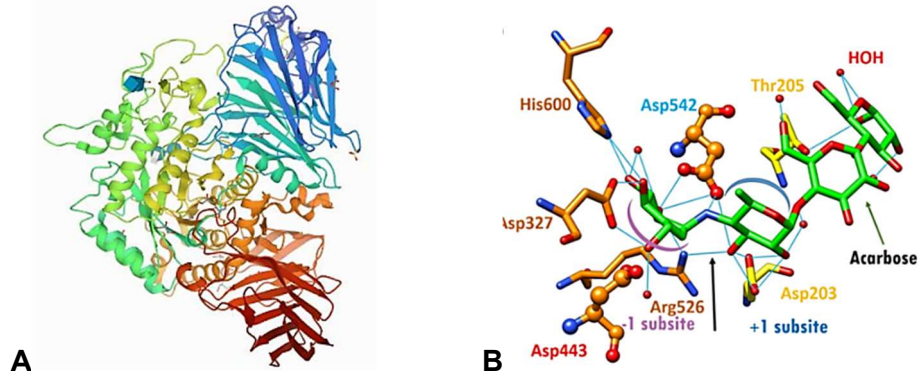


Fig.-1: Crystal Structure of Homo Sapiens Maltase-Glucoamylase (PDB 2QMJ, 1.90 a resolution) (A), 3D Structure of the α-Glucosidase Enzyme and Acarbose in *.pdb format (B)[15]

In-silico α-Glucosidase Inhibition

Molecular docking was performed using the AutoDock Vina program using a method of targeted docking. The grid box was run on sizes of x= -21.741, y= -6.434, and z= -5.063, and the dimensions are x= 14, y= 14, and z= 20 with a spacing of 1 Å. AutoDock Vina was run by the command prompt in a form file with *.pdbqt and a log-in *.txt. The parameters of binding affinity (kcal/mol) and inhibition constant (μM) were examined for all biflavonoids. We ran AutoDock Vina many times to get different docked conformational positions. The docking parameters found using AutoDock Vina were used to select the biflavonoids for subsequent *in-vitro* study. The Discovery Studio Visualizer software visualized the interaction between enzymes and ligands for 3D and Ligplot+ for 2D. The parameters determined were hydrogen bond interactions and hydrophobic interactions.

Prediction of Pharmacokinetic and Toxicity

Pharmacokinetic and toxicity analyzes were performed on acarbose and all biflavonoids. This process is a process of eliminating ligands with hazardous physicochemical properties. The structure of these ligands was downloaded to the PubChem database in SMILES format or converted manually through the Open Babel program. Pharmacokinetic predictions were carried out using Lipinski's rules through the website http://www.swissadme.ch/index.php. Meanwhile, the toxicity prediction was carried out using several parameters through the website http://lmmd.ecust.edu.cn/admetsar1/predict/. The ligand structure in SMILES format is uploaded on the page, and the compound's pharmacokinetic and toxicity prediction data appear on the page.

In-vitro Inhibition of the Enzyme Glucosidase

The *in-vitro* enzyme inhibition level was determined using the experiment refers to by Elya *et al.*[16] The reaction was carried out by mixing 250 μL of 4-nitrophenyl α-D-glucopyranoside 5 mM, 250 μL of α-glucosidase (0.15 unit/mL) and 10 μL of a sample at a varying concentration (62.5-1000 ppm). The mixture was then incubated for 15 minutes at 37 °C. The process was then stopped by adding 2000 μL of sodium carbonate 200 mM solution. The amount of p-nitrophenol emitted in the mixture can be read by using a microplate reader to monitor the enzymatic hydrolysis of the substrate at 400 nm wavelength. All experiments were carried out in triplicates. Acarbose was utilized as a positive control for the inhibitor of α-glucosidase. The IC50 was determined as the extract concentration necessary to block 50% of α-glucosidase activity under the test conditions.

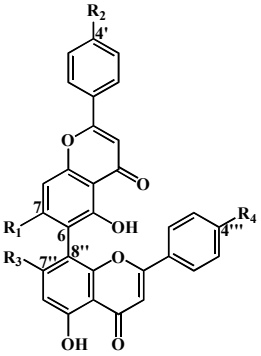
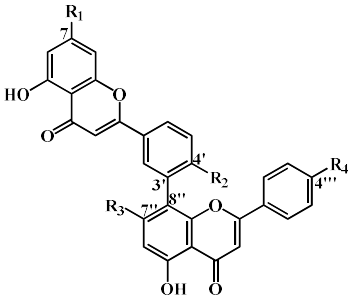
RESULTS AND DISCUSSION

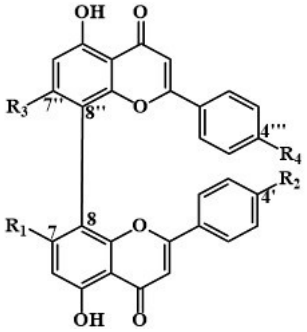
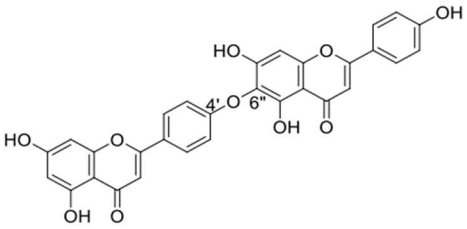
Molecular Docking Validation Results

The validation stage begins with preparing the receptor and ligand. The receptor and ligand must be free of water and other molecules complexed on the receptor and ligand that will inhibit the docking process. The

next step is to determine the size of the grid box as a docking site between the receptor and the ligand, which must cover all the active areas of the target protein used. From 20 replications, the value of the root means square deviation (RMSD) and affinity energy were 1.668 and -6.3 kcal/mol, respectively. Under these conditions, the grid box contains the best pose for the ligand because it minimizes ligand rotation and produces a ligand alignment that does not differ at the acarvocine position from the acarbose that binds the most residues to the active site of the receptor. An indirect correlation exists between binding affinity and RMSD (root mean square deviation). The docking strategy was accepted because the grid box's RMSD value was less than 2.0.[17] According to Sim *et al.*[15] binding acarbose ligand to the 2QMJ receptor, seven important amino acid residues play a role, namely Asp443, Asp203, Thr205, Asp327, Arg526, Asp542, and His600. One of the amino acid residues, Asp443, acts as a nucleophile of receptors in catalytic reactions that play a major role in hydrolyzing oligosaccharides into monosaccharides.

Table-1: Biflavonoid and Their Derivatives Ligands from the Genus Araucaria

Structure	R_1 (*7*)	R_2 (*4′*)	R_3 (*7″*)	R_4 (*4‴*)	Compound	
					Name	No
	-OH	-OH	-OH	-OH	Agathisflavone	**1**
	-OH	-OH	OCH_3	-OH	7"-*O*-methylagathisflavone	**2**
	OCH_3	-OH	OCH_3	-OH	7,7”-di-*O*-methylagathisflavone	**3**
	OCH_3	-OH	-OH	OCH_3	7,4’’’-di-*O*-methylagathisflavone	**4**
	OCH_3	-OH	-OH	-OH	7-*O*-methylagathisflavone	**5**
	OCH_3	-OH	OCH_3	OCH_3	7,4’’’,7”-tri-*O*-methylagathisflavone	**6**
	OCH_3	OCH_3	OCH_3	-OH	7,4’,7”-tri-*O*-methylagathisflavone	**7**
	OCH_3	OCH_3	OCH_3	OCH_3	7,4’,7”,4’’’-tetra-*O*-methylagathisflavone	**8**
	-OH	OCH_3	OCH_3	-OH	4′.7”-di-*O*-methylagathisflavone	**9**
	-OH	-OH	-OH	-OH	Amentoflavone	**10**
	OCH_3	OCH_3	OCH_3	-OH	7,4′,7″-tri-*O*-methylamentoflavone	**11**
	OCH_3	OCH_3	-OH	OCH_3	7,4′,4‴-tri-*O*-methylamentoflavone	**12**
	-OH	OCH_3	-OH	OCH_3	4′,4‴-di-*O*-methylamentoflavone	**13**
	-OH	-OH	OCH_3	-OH	7″-*O*-methylamentoflavone	**14**
	OCH_3	-OH	OCH_3	-OH	7,7″-di-*O*-methylamentoflavone	**15**
	OCH_3	OCH_3	OCH_3	OCH_3	7,4′,7″,4‴-tetra-*O*-methylamentoflavone	**16**
	OCH_3	OCH_3	-OH	-OH	7,4′-di-*O*-methylamentoflavone	**17**
	-OH	-OH	-OH	-OH	Cupressuflavone	**18**
	OCH_3	-OH	-OH	-OH	7-*O*-methyl-cupressuflavone	**19**
	OCH_3	-OH	OCH_3	-OH	7,7″-di-*O*-methylcupressuflavone	**20**
	OCH_3	OCH_3	OCH_3	-OH	7,4′,7″-tri-*O*-methylcupressuflavone	**21**

	-OH	OCH_3	-OH	OCH_3	4′,4‴-di-*O*-methylcupressuflavone	**22**
	OCH_3	OCH_3	OCH_3	OCH_3	7,4′,7″,4‴-tetra-*O*-methylcupressuflavone	**23**
	OCH_3	-OH	OCH_3	OCH_3	7,7″,4‴-tri-*O*-methyl-cupressuflavone	**24**
	OCH_3	-OH	-OH	OCH_3	7,4‴-di-*O*-methylcupressuflavone	**25**
					Hinokiflavone	**26**

***In silico* α-Glucosidase Inhibitory Profiling**

The binding affinity, inhibition constant, and final docked conformation against the enzyme were used to evaluate *in-silico* α-glucosidase inhibitory profiling. All the biflavonoid compounds showed excellent docking parameters compared to the acarbose standard against α-glucosidase. Table-2 shows that the biflavonoid compounds have lower binding affinity than the acarbose (-6.3 kcal/mol). The binding affinities of these molecules range from -9.5 kcal/mol to -7.2 kcal/mol. Consequently, these biflavonoids have more potential binding sites for inhibiting α-Glucosidase than acarbose. Binding affinity describes the Gibbs free energy (ΔG). ΔG is one of the thermodynamic parameters that can determine the occurrence of chemical reactions based on changes in entropy (ΔS) and enthalpy (ΔH) at certain pressures and temperatures.[18] The lower the ΔG value, the more stable the conformation of a molecule and the more spontaneous chemical reactions, and the protein-ligand complex gets better affinity and activity.[19-20] The negative value of binding energy indicated the spontaneous and exothermic reaction in bond forming.[21] The inhibition constants of all the biflavonoids were lower than those of the acarbose standard (23.82 μM). Proenca *et al*.[22] reported that standard acarbose had inhibition constant (Ki) values of 457 ± 11 μM, and the type of inhibition was competitive to the yeast of α-glucosidase. The inhibition constant and binding affinity are closely related.[23] This research shows a decrease in the Ki of all the biflavonoid compounds along with a decrease in their binding affinity. Biflavonoids inhibit at concentrations ranging from 0.107 μM to 5.206 μM. The low Ki value indicated that the inhibitory activity is effective because the attention required to impede it is getting smaller. However, all biflavonoids compounds' binding site similarities (BSS) were lower than that of acarbose (100%). BSS is useful in drug repurposing, protein-ligand, protein-protein complexes analysis, and function prediction in chemical biology and biochemistry.[24] BSS value of biflavonoids at ranging from 37.5% to 81.25%. Table-3 provides an overview of all biflavonoid binding affinities, hydrogen bonding interactions, and amino acid interactions with α-glucosidase.

The biflavonoids demonstrated good binding sites like the acarbose standard against α-glucosidase. The biflavonoids also have potential α-glucosidase inhibitory binding sites like the acarbose standard. The amino acid residues play a key function in the target enzyme's binding site. Biflavonoid 24 had the highest crucial site similarity of 81.25%, with 17 amino acid hydrophobic bonded residues but no hydrogen bonded amino acid residues. The other biflavonoid with binding site similarities greater than 50% obtained 13 biflavonoids and less than 50% seven biflavonoids (Table-3). Acarbose interacts with all critical amino acid residues, namely Asp443, Asp203, Thr205, Asp327, Arg526, Asp542, and His600, and has 16 interactions divided into two hydrogen bonding and 16 hydrophobic bonding. One of the essential components of molecular recognition is the specificity and directionality of contact that hydrogen bonds between a protein and its ligands give. The energy of hydrogen bonds gives the target structure stability and the specificity

required for preferred macromolecular interactions. On the other hand, hydrophobic interactions are crucial to the power in the strength of α-glucosidase because those interactions are responsible for maintaining proteins in a spherical shape.[25]

Table-2: Summary of the Molecular Docking Studies of the Biflavonoids Against α-Glucosidase

Compound No./Ligand	Binding Affinity (ΔG) (kcal/mol)	Inhibition Constant (kI) (μM)
26	-9.5	0.107
10	-8.8	0.349
5	-8.6	0.489
1	-8.5	0.579
2	-8.5	0.579
3	-8.4	0.685
4	-8.3	0.812
23	-8.2	0.961
24	-8.2	0.961
18	-8.2	0.961
6	-8.1	1.138
22	-8.1	1.138
25	-8.1	1.138
19	-8.0	1.347
20	-7.9	1.595
21	-7.7	2.237
9	-7.7	2.237
14	-7.6	2.648
13	-7.4	3.713
16	-7.4	3.713
11	-7.3	4.397
12	-7.3	4.397
15	-7.3	4.397
17	-7.3	4.397
7	-7.2	5.206
8	-7.2	5.206
Acarbose	-6.3	23.82

The biflavonoids with BSS greater than 50% with several hydrogen bonding like acarbose and greater than 2 were biflavonoids of **2**, **3**, **5**, **9**, **19**, and **22**, whereas biflavonoids with BSS less than 50% were biflavonoids **11**, **13**, **14**, **15**, **16**, and **17**. On the other hand, biflavonoids with BSS greater than 50% with several hydrophobic bonding like acarbose and greater than 14 were biflavonoids number **4**, **6**, **21**, **22**, and **23**, but not found for biflavonoids with BSS less than 50%. This study showed that 17 biflavonoids (structure numbers **1** to **9** and **19** to **26**) had practical binding orientations > 60% based on the visualization data, with biflavonoids **2**, **3**, **4**, **5**, and **6** having the best visualization (Fig.-2).

In silico Pharmacokinetics and Toxicity Analysis

Medicine development and production take an extended period, and significant human and material resources are needed. The pharmacokinetic properties of medications can now be assessed utilizing computer-assisted *in-silico* screening methods.

Table-3: The % Binding Site Similarities (BSS), Interacting Group (Hydrogen and Hydrophobic Bondings) Formed with the Group Interaction Atoms of the Corresponding Amino Acids

Compound No./Ligand	(%) Binding Site Similarities (BSS)	Group interaction/ hydrogen bond		Bond length (Å)	Amino acid interactions Hydrophobic
		Amino acid	Oxygen No.		
17	37.5	Thr544	3	2.96	Leu473, Thr204, Asp203, Asn207, Tyr605, Tyr299, Asp443, Trp406, Phe575, dan Thr205.
		Arg202	5	3.04	
14	37.5	Asn209; Asn207	10	3.27; 2.80	Tyr605, Asp203, Phe450, Tyr299, Arg202, Phe575, Trp406, Leu473.
		Asp542	6	2.77	
		Thr205	8	3,14	
13	43.75	Asn209; Asn207	10	3.20; 2.81	Tyr605, Ala576, Thr204, Tyr299, Asp542, Phe575, Trp406, Arg202, dan Leu473
		Asp203	5	3.06	
		Thr205	8	3.02	
16	43.75	Asn474; Arg202	9	2.73; 3.00	Thr205, Gln603, Phe450, Ala576, Asp203, Asn207, Lys480, Leu473, Tyr299, Asp542, Phe575, Trp406, dan Thr544.
11	43.75	Asp474; Arg202	9	2.79; 3.05	Thr205, Gln603, Ala576, Asp203, Asn207, Lys480, Leu473, Tyr299, Asp542, Trp406, Phe575, dan Phe450
		Thr544	3	3.13	
12	43.75	Arg202	5	3.04	Thr205, Leu473, Thr204, Ala576, Asp203, Asn207, Tyr299, Tyr605, Asp542, Trp406, Phe575, Leu577, dan Thr544.
15	43.75	Thr544	3	2.91	Leu473, Thr204, Ala576, Asp203, Tyr605, Tyr299, Asp542, Trp406, Phe575, Thr205, dan Asn207.
		Arg202	5	3.13	
18	50	Tyr605, Asp203, Thr205, Asp443,	6; 10; 8; 5	3.03; 3.09; 2.63; 2.90	Phe450, Asp542, Met444, Tyr299, Phe575, Trp406
10	56.25	Thr205, Gln603, Asp443, Arg202, Arg526,	6; 2; 9; 5; 9	2.98; 3.22; 2.91; 2.91; 2.96	Phe450, Lys480, Thr204, Asp203, Met444, Phe575, Tyr299, Trp406, Asp542, Tyr605
1	62.5	Thr544, Asn543, Asp203, Asp542, Asp327,	9; 9; 7; 9; 6	2.85; 3.04; 3.21; 3.04; 2.93	Ala576, Thr205, Trp406, Trp441, Tyr299, Asp443, Ile364, Phe575
3	62.5	Ser448	5	2.83	Thr205, Phe450, Asp203, Trp406, Phe575, Asp542, Asp443, Trp441, Tyr299, Ile364, Lys480.
		Asp327	10	3.20	
4	62.5	Asp327	10	3.12	Thr205, Phe450, Arg202, Asp203, Trp406, Phe575, Asp542, Asp443, Trp441, Tyr299, Ile364, Asn449, Ser448, Lys480

6	62.5	Asp327	10	3.12	Thr205, Lys480, Phe450, Asn449, Ser448, Ala576, Asp203, Arg202, Trp406, Phe575, Asp542, Asp443, Tyr605, Tyr299, Ile364
8	62.5	-	-	-	Thr205, Ala576, Phe450, Asp203, Arg202, Lys480, Ile364, Asn449, Tyr299, Trp441, Asp542, Asp443, Phe575, Trp406, Ser448.
20	62.5	-	-	-	Tyr605, Thr205, Gln603, Ala576, Asp203, Met444, Phe450, Tyr299, Asp542, Asp443, Arg526, Trp406, Phe575, Thr204.
21	62.5	Tyr605	2	3.30	Thr205, Arg334, Gln603, Ala576, Asp203, Phe450, Met444, Thr204, Tyr299, Asp542, Asp443, Arg526, Trp406, Phe575.
25	62.5	Tyr605 Thr205	2 8	3,29 2,82	Phe450, Met444, Asp443, Asp542, Phe575, Ala576, Arg526, Tyr 299, Arg334, Asp203 and Trp406
26	62.5	His600, Asp327, Asp203, Asn207, Thr205	10; 10; 9; 7; 7	3.30; 3.06; 2.92; 2.80; 3.19	Trp441, Asp443, Phe575, Asp542, Tyr299, Phe450, Trp406, Leu473, Thr204
5	68.75	Asp542; Asn543; Thr544	7	2.92; 2.97; 2.80	Thr205, Ala576, Asp203, Arg202, Trp406, Phe575, Asp327, Asp443, Trp441, Tyr299, dan Thr204
2	68.75	Asp203, Asp542, Asn534, Thr544	7; 9; 9; 9	3.21; 3.03; 2.92; 3.02	Thr205, Tyr299, Asp327, Asp443, Ile364, Trp441, Phe575, Trp406, Ala576
7	68.75	-	-	-	Thr205, Arg202, Ala576, Lys480, Asp203, Ile364, Tyr299, Trp441, Asp542, Asp443, Phe575, Trp406, Asp327.
19	68.75	Asp327	5	3.22	Ala576, Asp203 Met444, Phe450, Tyr299, Asp542, Asp443, Arg526, Trp406, dan Phe575
		Thr205	10	2.88	
		Tyr605		3.17	
9	68.75	Asp542	7	3.28	Thr205, Thr204, Ala576, Lys480, Met444, Tyr299, Asp443, Phe575, Trp406, Ile328, Asp327, Tyr605.
		Asp203, Arg202	9	2.93; 2.85	
22	75	Thr205	6	2.70	Gln603, Arg334, Ala576, Asp203, Met444, Phe450, Tyr299, Trp441, Asp443, Arg526, Asp542, Phe575, Trp406, Asp327
		Tyr605	4	3.24	
23	75	Tyr605	2	3.33	Thr205, Arg334, Gln603, Ala576, Asp203, Met444, Phe450, Tyr299, Asp443, Arg526, Asp542, His600, Phe575, Trp406, Asp327, Thr204

24	81.25	-	-	-	Thr205, Gln603, Ala576, Asp203, Met444, Phe450, Tyr299, Trp441, Asp443, Arg526, Asp542, His600, Phe575, Trp406, Asp327, Tyr605, Thr204.
Acarbose	100	His600	4	3.22	Thr205, Trp406, Asp327, Phe575, Ile328, Asp542, Trp539, Asp443, Trp441, Tyr299, Ile364, Met444, Asp203, dan Ala576.
		His600	3B	2.97	
		Arg526	2B	2.97	
		Arg526	3	2.8	

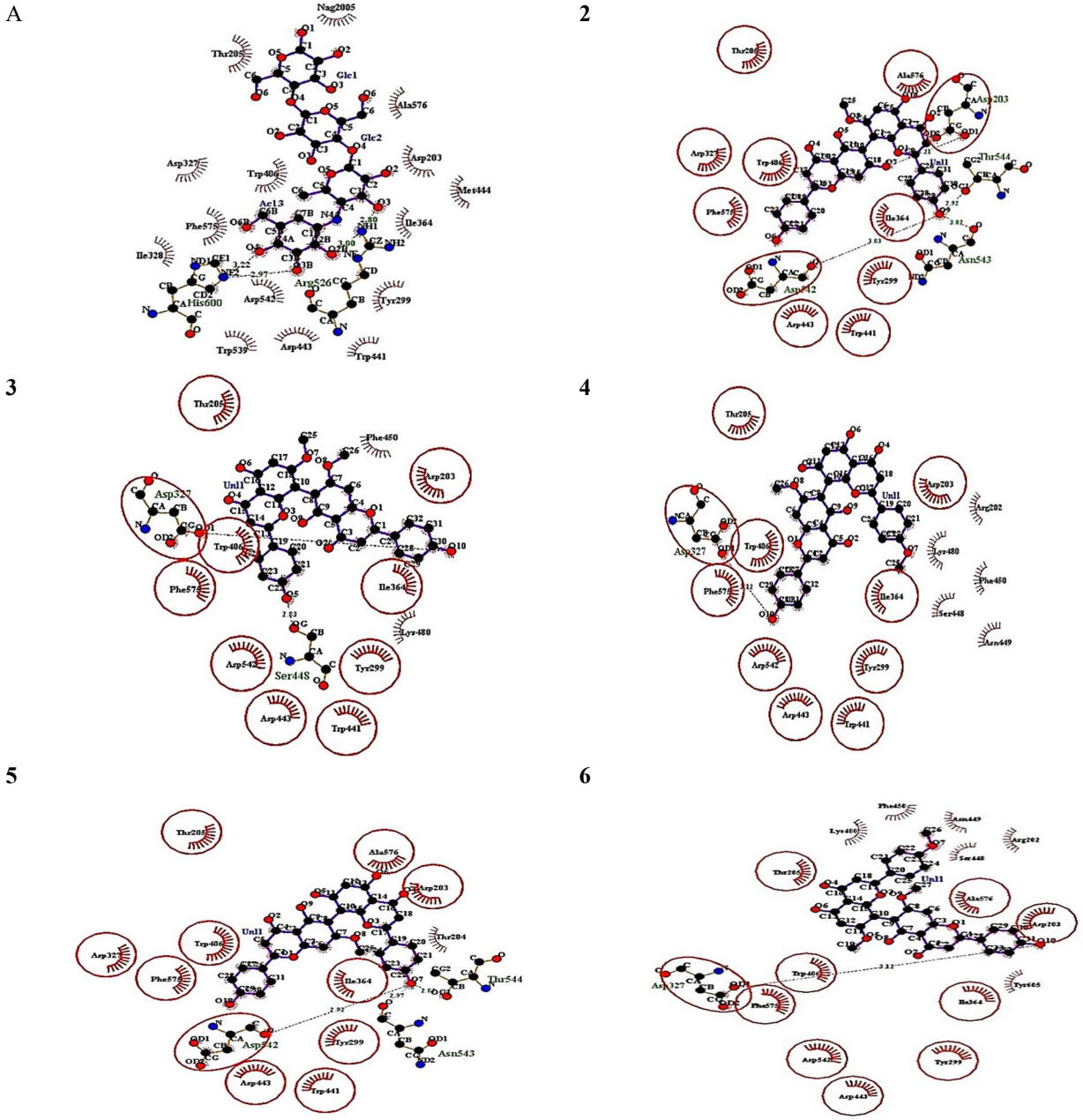


Fig.-2: binding interactions standard acarbose (A) and compounds (**2-6**) against maltase-glucoamylase (PDB 2QMJ, 1.90 resolution). Pink lines suggest hydrophobic interactions, while green dashed lines represent hydrogen bonds between substances and amino acids.

The study of how drugs move through the body, including their absorption, distribution, metabolism, and excretion, is known as "pharmacokinetics" (ADME). The structures of acarbose and compounds **1** to **26** were exported to the format SMILES using the Open Babel tool to estimate their pharmacokinetic characteristics (drug-likeness qualities) through *in-silico* following "Lipinski's rule of five." A particular organic molecule's ability to exhibit traits typical of an orally active drug is assessed using the concept of medication likeness.[26]

We used Lipinski's rule. For compounds meeting two or more of the following criteria: low molecular mass (MW < 500 Dalton), high lipophilicity (log P < 5), hydrogen bond donors < 5, hydrogen bond acceptors < 10, and a molar refractive range of 40–130 could be predicted the likelihood of success or failure due to a high drug-likeness.[27] Toxicity refers to a compound's or drug candidate's toxic effect in a biological system, and now, its analysis can be done computationally through numerous web servers. This research conducted toxicity analysis using the admetSAR website, yielding reactivity data and confidence scores. Roman *et al.*[28] reported the confidence value of the chance of a drug being active or inactive against a parameter. As an example of the explanation in Table-4, ligand **2** has a confidence value of 0.9356 on the carcinogenicity parameter with a passive category, which means that the ligand has a 93.56 percent chance of being inactive on that parameter. Compounds are categorized into four classes based on their acute oral toxicity (ADMET prediction profile). Compounds in Category I have $LD_{50} \leq 50$ mg/kg; Category II have a range of 50 mg/kg < LD_{50} < 500 mg/kg; Category III have in the range of 500 mg/kg < LD_{50} < 5000 mg/kg, and Category IV have LD_{50} > 5000 mg/kg.[29] The pharmacokinetics and physicochemical characteristics of acarbose and all biflavonoids, summarized in Table-4, give a quantitative account of what happens to a substance administered to humans.

Table-4: Pharmacokinetics and Toxicity Prediction of the Acarbose and Compounds **1-26**

Ligand	Pharmacokinetics						Toxicity Prediction					
							hERG Inhibition		Carcinogenicity		Acute oral toxicity	
	MW (g/mol)	NHA	NHD	Log P (cLogP)	MR	Lipinski's Rule of Five Violations	C	S	C	S	C	S
2	552.48	10	4	0.44	151.44	2	weak inhibitor	0.9494	-	0.9356	III	0.6544
3	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9613	-	0.9165	III	0.6241
4	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505
25	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505
5	552.48	10	5	0.44	151.44	2	weak inhibitor	0.9494	-	0.9356	III	0.6544
6	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
7	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
8	594.56	10	2	1	164.85	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
9	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505
11	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
12	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9593	-	0.9092	III	0.5810
13	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505
14	552.48	10	5	0.44	151.44	2	weak inhibitor	0.9494	-	0.9356	III	0.6544
15	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9613	-	0.9165	III	0.6241
16	594.56	10	2	1	164.85	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
17	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505

19	552.48	10	5	0.44	151.44	2	weak inhibitor	0.9494	-	0.9356	III	0.6544
20	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9613	-	0.9165	III	0.6241
21	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
22	566.51	10	4	0.63	155.91	2	weak inhibitor	0.9624	-	0.9248	III	0.6505
23	594.56	10	2	1	164.85	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
24	580.54	10	3	0.81	160.38	2	weak inhibitor	0.9581	-	0.8987	III	0.5395
26	538.46	10	5	0.52	146.03	2	weak inhibitor	0.9789	-	0.9367	II	0.4709
1	538.46	10	6	0.25	146.97	3	weak inhibitor	0.9545	-	0.9307	II	0.6295
10	538.46	10	6	0.25	146.97	3	weak inhibitor	0.9545	-	0.9307	II	0.6295
18	538.46	10	6	0.25	146.97	3	weak inhibitor	0.9545	-	0.9307	II	0.6295
Acarbose	645.6	19	14	-6.94	136.69	4	weak inhibitor	0.8586	-	0.9670	IV	0.6165

Note: MW (molecular weight); NHA (number of hydrogen acceptors); NHD (number of hydrogendonorsr); MR (molar refractivity); hERG (human ether-á-go-go-related gene); C (category) and S (confidence scores)

Most biflavonoids meet the requirements for orally active medicines, except for biflavonoids **1**, **10**, **18**, and **26**, according to Lipinski's rule of five (RO5), toxicity analysis, and acute toxicity prediction. These biflavonoids were expected to be neither irritating nor carcinogenic. Therefore, they could be considered candidates for anti α-glucosidase drugs. According to Zhang *et al.*[30] carcinogenic compounds may contribute to the development of cancer cells in the body. The pore-forming subunit of ion channels required for cardiac repolarization is encoded by the human ether-á-go-go-related gene (hERG). Inhibition of hERG activity can cause electrocardiographic abnormalities that raise the risk of Long QT syndrome and even sudden death.[31] In light of the ADMET prediction study, all biflavonoids compounds, except for compounds **1**, **10**, **18**, and **26**, may make good candidates for this investigation.

In-vitro α-Glucosidase Inhibitory Studies

Our research team has previously shown that biflavonoids are active against cancer MCF-7 and HeLa cells *in-vitro*. Seven biflavonoids were taken out of the acetone-extracted leaves of *A. hunsteinii* using various chromatographic methods. Five compounds of them were identified as methyl ether derivatives of C-C type of biflavonoids, such as 7,7"-di-*O*-metilagathisflavone (**3**); 4"',7,7"-tri-*O*-metilagathisflavone (**6**); 4',4"'-di-*O*-metilamentoflavone (**13**); 7-*O*-metilcupressuflavone (**19**); and 4"',7-di-*O*-methylcupresuflavone (**25**). Compound **25** was the first time isolated from *A. hunsteinii* and the Araucaria genus.[14] For biflavonoids **3**, **6**, **13**, and **19** have ever been isolated from other species of Aracauria, such as *A. columnaris* (G. Forst) Hook, *A. rulei* F. Muell, *A. araucana* (molina) K. Koch, *A. cunninghamii* Mudie, and *A. bidwilli* Hook.[32] These biflavonoids had a binding affinity and BSS percent ranging (-7,4 to -8,6) kcal/mol and (62-69)%, respectively. In the course of our investigation, the substances' ability to inhibit α-glucosidase was assessed. All experiments used acarbose as a standard inhibitor with an IC_{50} of 0.57 ± 0.33 μM at a micromolar concentration. On the other hand, Proenca *et al.*[22] reported that standard acarbose had IC_{50} values of 607 ± 56 μM, whereas Shah *et al.*[33] revealed IC_{50} of 840 ± 1.7 μM for α-glucosidase inhibiting. The results of the five biflavonoids isolated from *A. hunsteinii* leaves are summarized in Table-5. All the substances showed inhibitory activity against -glucosidase, with IC_{50} values ranging from 78.32 to 12282.03 μM. The blifavonoids **3**, **13**, **19**, and **25** were found to possess high activity compared to Proenca *et al.*[22] and Shah *et al*.[33] However, these compounds displayed low inhibiting compared to the acarbose from this research. The ability of the compounds to inhibit α-glucosidase was in the following order: biflavonoids **19** > **25** > **3** > **13.**

Biflavonoids showed potential α-glucosidase inhibitory efficacy compared to conventional acarbose during *in-vitro* enzymatic activity. Laishram *et al.*[34] reported that amentoflavone (10) had IC_{50} values of 8,09±0,023 μM, while Wu *et al.*[35] revealed compound **10** and its derivatives 4'-*O*-methylamentoflavone,

17, **13**, and **12** showed IC_{50} values of 3.28; 2.16; 1.79; 4.69, and 8.29 µM, respectively. According to *in vivo* experiments by Tchimene *et al.* [9], colaviron, a biflavonoid from *Garcinia kola*, can reduce blood glucose levels from the initial 14.6 ± 0.98 mmol/L to 8.5 ± 3.02 mmol/L after 6 hours. Cane *et al.*[10] reported the effect of treatment (macrophylloflavone with varying doses of 6, 7, and 8 (µg/kg body weight) compared to negative and positive controls in diabetic rats, afforded a significantly different value in blood glucose levels at the posttreatment. The blood glucose levels (mean SD mg/dL) for the negative and positive controls, in contrast to the initial blood glucose levels of 300 mg/dL in diabetic rats, were 412.20 ± 7.76 and 98.00 ± 1.67, respectively. In the meantime, diabetic rats receive treatment with macrophylloflavone, a biflavonoid molecule found in *Garcinia macrophylla* Mart.

Table-5: Inhibition Potency of Biflavonoids Against Glucosidase

Biflavonoids		IC_{50} (mean±std) µM
No.	Name	
3	7,7"-di-*O*-methylagathisflavone	388.39±0.68
6	4'",7,7"-tri-*O*-methylagathisflavone	12282.04±196.55
13	4',4'"-di-*O*-methylamentoflavone	389.76±1.54
19	7-*O*-methylcupressuflavone	78.32±0.52
25	7,4'"-di-*O*- methylcupressuflavone*	537.98±2.35
	Acarbose (Positive control) in this research	0.57±0.03
	Acarbose [22]	607±56
	Acarbose [33]	840±1.73

* The first time isolated from *A. hunsteinii* and the Araucaria genus.[14]

showed the ability to lower blood glucose levels from a level of 300 mg/dL to 171.00 ± 3.81 (dose 6 g/kg body weight), 138.00 ± 1.87 (dose 7 g/kg body weight), and 108.40 ± 3.21 (an amount of 8 g/kg body). The results of *in-silico* docking studies confirm that biflavonoids could be a promising remedy for DM disease. These compounds can be developed into α-glucosidase inhibitors, anti-diabetic drugs used to treat type-II diabetes post-prandial hyperglycemia. This enzyme converts the α-1-4 bond linkage in starch or oligosaccharides into monosaccharides like glucose.[36]

The correlation between structure and their activity (SAR) has been investigated to comprehend the impacts of substituents on the hydroxyl/methoxy functionality concerning the inhibitory action of α-glucosidase. Studies employing molecular modeling have demonstrated how active drugs interact with enzyme binding sites. Biflavonoids of **2**, **3**, **4**, **5**, **13**, **19**, and **25** have been selected as a possible lead compound in the treatment of DM disease after their α-glucosidase inhibitory profiles were examined using a combination of *in-vitro* and *in-silico* techniques. These compounds were classified into three structures, agathisflavone (**2** to **5**), cupressuflavone (**19** and **25**), and amentoflavone (**13**) (Tables-1 and 5). The number and position of hydroxyl functionality groups on its structures were one of causing the different activities of biflavones.[35] The methylation on C4'" of cupressuflavone **25** caused its activity against inhibition α-glucosidase a decrease, compared to **19**. This case was also found on agathisflavone **6**, whose activity was lower than **3**. Meanwhile, the methylation on C4' and/or C4'" of amentoflavone **13** also reduces its activity, compared to 10.[34] Monomeric forms of flavonoids, such as those extracted from *Tinospora crispa* leaves, their inhibition activity against α-glucosidase have been demonstrated to be impacted by the 4′-hydroxyl group on the B ring of biflavones.[37] The IC_{50} of myricetin is lower than quercetin, kaempferol, and apigenin. This case was observed due to the hydroxyl groups of myricetin being more than quercetin, kaempferol, and apigenin, consequently, the better the α-glucosidase activity.

The hydroxyl groups of these flavonoids may be directly related to their function as enzyme α-glucosidase inhibitors. Interestingly, these 4′-hydroxy groups-containing flavone dimers may interact favorably with the glucosidase enzyme residues. This *in-silico* and *in-vitro* studies could create more powerful α-glucosidase inhibitors for treating DM disease.

The result of docking orientations and parameters strengthen the experiment of laboratories results and is consistent between *in-vitro* and *in-silico* investigations. Therefore, to enrich our group effort, we will pursue to further consider biflavonoids as the "lead compound" for antidiabetics and must be developed to become competitive candidates for drug discovery.

CONCLUSION

The *in-silico* research and *in-vitro* study's findings highlight the biflavonoids' derivatives α-glucosidase inhibitory ability succeeded. All biflavonoids could be developed as orally active drugs to inhibit α-glucosidase, except compounds **1**, **10**, **18**, and **26** due to toxicity. *In-silico* prediction combined with *in-vitro* assay aligned as shown by all isolated biflavonoids from *A. hunsteinii*, 7-*O*-methylcupresuflavone (**19**); 4'",7-di-*O*-methylcupresuflavone (**25**); 7,7"-di-*O*-methylagathisflavone (**3**); 4',4'"-di-*O*-methyl-amentoflavone (**13**) and 4'",7,7"-tri-*O*-methylagathisflavone (**6**). The research is interesting and merits further investigation. More study on these substances as lead compounds for upcoming and *in vivo* studies is necessary to create therapeutically relevant α-glucosidase inhibitors in preventing and treating diabetes.

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CONFLICT OF INTERESTS

There is no conflict of interest between any authors in this research.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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