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In silico study of Sambiloto (*Andrographis paniculata*) compounds from GC-MS and LC-MS/MS as alpha-glucosidase and DPP-4 enzyme inhibitor

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ABSTRACT

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia, impaired insulin secretion, and insulin action. To overcome this disease, some people treat it with natural ingredients. Sambiloto (*Andrographis paniculata*) is reported to have a wide range of pharmacological activities, one of which is anti-diabetic. Sambiloto showed activity in lowering blood glucose which has the potential as an antidiabetic. Computational methods, such as molecular docking, can increase the effectiveness and reduce the cost of searching for new active compounds. The purpose of this study was to determine the component compounds contained in the ethanol extract of Sambiloto and obtain the potential compounds to inhibit the alpha-glucosidase and DPP-4 enzymes as anti-diabetics with molecular docking method. Sambiloto leaves were macerated for 3 x 24 hours using ethanol 96% as a solvent and concentrated with an evaporator. Sambiloto extract was analyzed using LC-MS, and GC-MS. In-silico analysis includes geometry optimization and molecular docking methods. Preparation of the test ligands was carried out by the ChemBioDraw Ultra and ChemBio3D applications, then optimization by Gaussian 09 application. The crystal structures of the target proteins used were those with PDB ID 5NN8 for alpha-glucosidase and 2QOE for DPP-4. Molecular docking was performed using Autodock 4.2.3 application. From analysis with LC- MS/MS and GC-MS methods, 18 compounds were identified. Molecular docking was performed on the identified compounds. The results of molecular docking showed that the compound S17 (*11-(P- Bromoanilino)-5H-Dibenzo [B,E] [1,4] Diazepine*), S1 (*andrographolide*) and S2 (*andrographanin*) have the potential to inhibit the activity of alpha-glucosidase enzyme; on the other hand S17 (*11-(P-Bromoanilino)-5H-Dibenzo [B,E][1,4]Diazepine*) and *S5* (*andrographolactone*) have the potential to inhibit the activity of DPP-4 enzyme. These compounds have the potential to inhibit alpha- glucosidase and DPP-4 enzymes which act as antidiabetics.

Keywords: alfa-glucosidase, diabetes mellitus, DPP-4, *molecular docking*, Sambiloto (A*ndrographis paniculata*)

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INTRODUCTION

Metabolic disorder is one of the main health problems that occur among Indonesians. Diabetes mellitus is a disease caused by a disorder in the secretion of insulin, glucagon, and other hormones resulting in impaired carbohydrate and fat metabolism. When glucose from food is not metabolized normally by the body, the accumulation of glucose increases in the blood, called hyperglycemia (Dipiro 2020). According to the International Diabetes Federation (IDF), Indonesia is ranked 7th among 10 countries with a total of 10.7 million sufferers (Kementrian Kesehatan Republik Indonesia, 2020). The WHO organization estimates that the number of people with type 2 diabetes will increase significantly over the next few years (Perkeni, 2019).

The main pathophysiology of type 2 diabetes mellitus is genetic insulin resistance and pancreatic beta cell dysfunction. In individuals under normal conditions, the pancreas can function properly and can adjust insulin secretion to maintain plasma glucose levels. If insulin release is no longer sufficient to normalize plasma glucose, dysglycemia, including prediabetes and diabetes, may occur. Pancreatic beta cells cannot maintain adequate insulin secretion and release less insulin as glucose levels increase (Dipiro 2020).

Diabetes Mellitus is influenced by several proteins, one of which is the alpha-glucosidase enzyme. An alpha-glucosidase enzyme is a key enzyme in the absorption of sugar in the intestine, and its activity is closely related to blood glucose levels (Perkeni, 2019). The mechanism of alpha-glucosidase enzyme can inhibit the breakdown of sucrose and complex carbohydrates in the small intestine. Diabetes drugs with this mechanism work competitively by inhibiting maltase, isomaltase, sucrose, and glucoamylase enzymes in the small intestine (Dipiro 2020).

Another hormone that plays a role in regulating glucose in the blood is the hormone incretin. Incretin is a peptide secreted in the small intestine in response to food in the intestine. Two types of incretin peptides affect glucose metabolism, namely GLP-1 (Glucagon Like Peptide-1) and GIP (Glucose Dependent Insulinotropic Peptide). Some people who have type 2 diabetes also have GLP-1 deficiency which is resistant to GIP hormone. GLP-1 plays a role in increasing insulin secretion, especially in phase 1 insulin secretion, by stimulating glucose in beta cells and also by inhibiting glucagon secretion. Both lead to a decrease in blood glucose levels. GLP-1 enters the bloodstream and actively works, but GLP-1 does not last long in the blood, which is only about 1-2 minutes because it is immediately destroyed by the enzyme DPP-4 (dipeptidyl peptidase- 4). One of the efforts to keep GLP-1 in the blood longer is to inhibit the DPP-4 enzyme (Dipiro, 2020).

One of the efforts to overcome diabetes mellitus is by utilizing herbs that work to help reduce blood sugar levels that have been used in traditional medicine for generations by the people of Indonesia (Safitri et al. 2015). One of the medicinal plants that have a lot of secondary metabolite content and are widely used in the community to overcome diabetes mellitus is the leaves of Sambiloto (*Andrographis paniculata*). Sambiloto is proven to show anti-hyperglycemic activity and inhibit tissue damage through oxidative stress in diabetic rat models induced by streptozotocin. Sambiloto ethanol extract showed activity to reduce blood glucose (Sivakumar and Rajeshkumar 2016). The content of chemical compounds in the sambiloto plant are diterpenoid and flavonoid compounds, including andrographolide, isoandrographolide, neoandrographolide, andrographolidegraphosterin, 14-deoxy-11, 14-deoxyandrographolide- 19- β-D-glucoside, 12-didehydroandrographolide,14-deoxyandrographolide, homoandrographolide, andrographolidegraphan, and stigmasterol (Chao and Lin 2010; Dai et al. 2019). Andrographolide is the main compound that can reduce glucose levels by inhibiting the alpha-glucosidase enzyme (Dai et al., 2019; Jarukamjorn & Nemoto, 2008; Nugroho et al., 2012). In this study, analysis compound was also carried out with GC-MS and LC-MS/MS methods and compared with previous research, to further determine in silico mechanism of action in inhibiting 2 enzymes, alpha-glucosidase and DPP4 enzyme which play a role in lowering blood sugar.

In the development of new drugs, especially in finding active compounds from herbs, the use of several computational methods has been developed, one of which is molecular docking (Knoll et al.,

2006). Drug design development can be done with Computer-Assisted Drug Design (CADD). Molecular docking is one of the most frequently used methods in Structure-Based Drug Design (SBDD). It can predict the approximate position of the ligand in the target receptor with a high degree of accuracy. Molecular docking is a computational method to predict the interaction between a receptor such as a protein or nucleic acid molecule (DNA or RNA) and its ligand, as well as to assess the binding affinity and positioning of the ligand within receptor (Morris et al. 2008). The complex interaction of the ligand with protein is identified using a docking program to predict the interaction of two molecules. The interaction can be seen from the binding site of the macromolecular target (Kalyaanamoorthy & Chen, 2011; Meng et al., 2011). Molecular docking has advantages such as shorter time and lower cost compared to in vitro tests (Cosconati et al., 2010).

In this study, we focused on herbal plant sambiloto ((*Andrographis paniculata*) and conducted in silico study with its phytoconstituents analyzed by LC-MS/MS and GC-MS. The interactions of its phytoconstituents with enzyme alpha-glucosidase protein (PBD ID 5NN8) and enzyme DPP-4 (PBB ID 2QOE) were reported by molecular docking studies. Alpha-glucosidase and DPP-4 enzymes were chosen as targets in this study. The alpha-glucosidase enzyme is an enzyme that is responsible for the process of breaking down complex carbohydrates into glucose and absorbing glucose into the circulation. Inhibition of this enzyme will prevent excess glucose intake from food. Meanwhile, the DPP-4 enzyme is staged. Our results from the present study have established the strong candidate of phytoconstituents of sambiloto to be used as a drug-like molecule for preventing diabetes mellitus.

MATERIALS AND METHODS

Materials

Sambiloto plants were obtained from the Installation of Research and Assessment of Agricultural Technology (IP2TP) Cicurug Sukabumi - West Java. The results of the determination carried out at the Jatinangor Herbarium, Faculty of Mathematics and Natural Sciences Padjadjaran University showed that the sambiloto plant used in the study is a species of *Andrographis paniculata* which belongs to the Acanthaceae family (No. 38/HB/12/2021). This plant in Indonesia is known by the common name sambiloto.

The hardware used was an Asus M509BA laptop, AMD A4-9125 Radeon R3 processor, 4 compute cores 2C+2G, 2300, Mhz, 2 Core(s). Storage SSD (Solid State Drive) 1TB, RAM 8.00 gigabytes. The software used was Masslynx V4.1, ChemBioOffice 2014 (ChemBioDraw Ultra and ChemBio3D Ultra), Discovery Studio Visualizer 2016, GaussianView 5.0, and AutoDock 1.5.6.

Methods

Extraction

80 grams of sambiloto leaves were macerated with 96% ethanol solvent for 3x24 hours, then the extract was concentrated with a rotary evaporator.

Compound analysis

Analysis of compounds in the extract was carried out by LC-MS/MS, and GC-MS. Analysis using a Liquid Chromatography-Mass Spectrometer (LC-MS/MS) instrument was conducted at Bogor Forensic Laboratory Center with UPLC (Ultra Performance Liquid Chromatography), UPLC Column HSS (High strength silica), and Two-Generation Quadrupole time-of-flight mass spectrometry. The results obtained are chromatograms and mass spectra that will be checked using Masslynx software. The mass spectrum pattern will be matched with external literature such as journals, mass bank databases, PubChem, and ChemSpider. The results of the mass spectra that have been matched with the literature will be taken for the preparation of test ligands.

For GC-MS analysis using Shimadzu-GC 2010 with the mass selective detector, carrier gas using helium. The optimization process was carried out by programming the interface temperature of 250°C, injector 150°C, and initial column temperature programmed 50°C increased to 100°C with a temperature increase set at 10°C/minute (held for 3 minutes). Then increased back to 200°C, increased

back to 250°C. Components were identified by comparing the mass spectra of the samples with the internal Willey Library7 (Rohloff, 2015).

In silico testing

The in-silico process includes ligand preparation, protein preparation, validation of molecular docking method, simulation of molecular docking of test ligand with protein, and interpretation of results.

Test ligand preparation

The test ligand used was the result of determining components of the sambiloto extract which were analyzed using LC-MS/MS and GC-MS instruments. The compounds obtained were checked with the PubChem database and made 2D and 3D compound re-structures using the ChemBioDraw Ultra and ChemBio3D applications. Then geometry optimization was carried out using the Gaussian application.

Protein target and natural ligand preparation

The proteins used are alpha-glucosidase enzyme and DPP-4 enzyme, downloaded on the Protein Data Bank website https://www.rcsb.org/structure/ (PDB ID 5NN8 for alpha-glucosidase enzyme) and (PDB ID 2QOE for DPP-4 enzyme). Preparation of the target protein and natural ligand was done by separating the protein structure and natural ligand and removing water molecules. Using the Visual Discovery Studio application.

Validation of molecular docking method

Validation of molecular docking was carried out using the AutoDock version 4.2.3 application (Morris et al., 2008). The proteins used were the alpha-glucosidase enzyme and the DPP- 4 enzyme that had been prepared. The natural ligands that had been separated from the proteins using the Discovery Studio application were then reattached to the target proteins. The grid center was placed approximately to the center of the ligand, covering all binding site residues. Determination of the Gridbox is done by setting the central region of the natural ligand and docking calculation with the number of GA runs as many as 100 maximum numbers of evals and the algorithm method used is Lamarckian Genetic Algorithm (LGA). Validation is declared valid if the Root Mean Square Deviation (RMSD) value ≤ 2Å (López-Camacho et al. 2016).

Molecular docking simulation

Simulation of molecular docking of test compounds to alpha-glucosidase and DPP-4 target proteins using the AutoDock application version 4.2.3. Gridbox settings according to the size and area that have been obtained from the validation results. Furthermore, the interpretation of molecular docking results is carried out by looking at the value of ΔG (binding free energy), and inhibition constant. Visualization using the Discovery Studio Visualizer 2016 application to explore the bond interaction formed between the test ligand with alpha-glucosidase and DPP-4 enzyme (López-Camacho et al. 2016). Visualization of docking results draws the interaction of ligands with proteins that form intermolecular bonds with amino acid residues enzyme (López-Camacho et al. 2016).

RESULT AND DISCUSSION

Analysis of compounds from Sambiloto extract by LC-MS/MS

Analysis of compounds in the extract with LC-MS/MS instrument identified some chromatographic peaks (Figure 1).

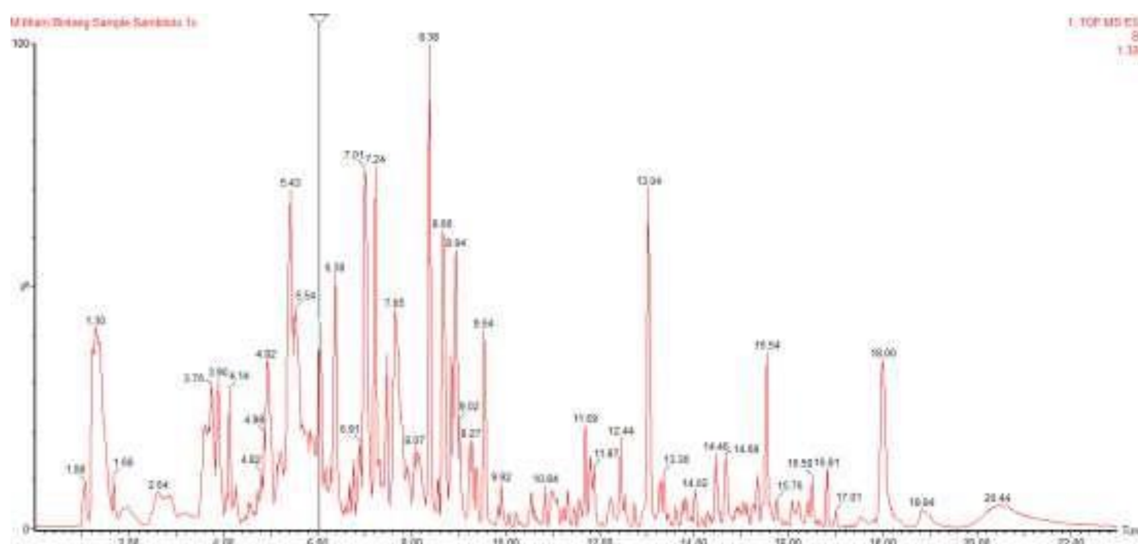

Figure 1. Chromatogram profile of LC-MS/MS analysis results of sambiloto ethanol extracts

Characterization of the structure of chromatogram peaks is based based on accurate mass, fragmentation patterns, literature data, molecular weights, and chemical formulas, matched with external literature from the form of mass spectra of compounds compared with mass bank databases, PubChemm, ChemSpider, and journals. Analysis using LC-MS/MS showed the presence of 22 peaks (Table 1), but only 16 compounds had their chemical structures identified. Other peaks whose structures cannot be carried out in silico studies. Of the 16 peaks, as listed in Table 1 were identified from the alkaloid, diterpenoid, and flavonoid compound classes including Andrographolide, Andrographanin, Bisandrographolide, 14-Deoxyandrographolide, Andrographolactone, 14-Deoxy-11,12-didehydroandrographolide, Lambertianic acid, Cevadine, Caffeoylquinic acid and 5,7-dihydroxy-3-(4-hydroxyphenyl)-66-methoxy-4H-chrome-4-one4',55,7-Trihydroxy-6-methoxy isoflavone. Several diterpenoids including 14-deoxy-11, 12-di- dehydroandrographide, andrographolide, neoandrographolide and isoandrographolide have been reported previously from *A. paniculata* (Chao & Lin, 2010).

Alkaloid compounds were detected in this extract which confirmed the presence of aromatic and oxidized nitrogen functional groups. Previous studies have confirmed the presence of hydroxyl, aromatics, alkanes, esters, amines, ethers, nitro aromatics, phenolics, and phosphine groups present in the extract of sambiloto leaf plants (Dwivedi et al. 2021). Alkaloids were detected at retention time 7.65 with m/z value 592.3492, mass number 591.7, and molecular formula $C_{32}H_{49}NO_9$. The compound detected was Cevadine, the results of the fragmentation pattern were compared with external literature contained in the mass bank database. Cevadine was detected at m/z 592 with product ions 389 and 289.

Analysis of compounds from Sambiloto extract by GC-MS

The results of GC-MS analysis identified compounds successfully extracted from ethanol solvents and derivatized by adding Trimethylsilyl in methanol. Derivatization is intended to improve the volatility of a compound so that it can be analyzed by gas chromatography. Analysis of sambiloto ethanol extract with derivatization showed more peaks (Mushtaq et al. 2014).

Further identification of each peak was carried out using mass spectra based on the database Similarity Index (SSI). Analysis of the extract using the GC-MS method identified the presence of 8 compounds. The identified metabolites will be bound to TMS which has functional groups such as amino acids (-COOH, -NH2, -OH) and monosaccharides. The results of the 8 main peaks consisted of

peak number 1 in the Willey7 internal library so the compounds were identified as 1-(3-hydroxycyclopent-2-en-1-yl)propane-2-one, furan-2,5-dicarboxylic acid, 2,4-Di-Tert-Butyl-6-Cyanoaniline, 3-hydroxy-3-(4-hydroxyphenyl)propanoic acid, 1-(2-hydroxyphenyl)ethan-1-one, propyl 4-hydroxybenzoate, 11-(P-Bromoanilino)-5H- Dibenzo[B, E][1,4]Diazepine, and 3-butyl-3,5,5-trimethyl cyclo hex- 1-en-1-ol.

Table 1. LC-MS/MS analysis of sambiloto ethanol extracts

Peak	Rt (min)	MW (g/mol)	(m/z)	Molecular formula	Compound classes
1.	1.08	167	167.013	$C_6H_3N_2O_4$	Nitrobenzenes
2.	1.30	117.15	118.146	$C_5H_{11}NO_2$	Amino acid
3.	1.68	268.10	268.104	$C_{10}H_{14}N_5O_4$	Nucleotides
4.	2.64	165.19	166.086	$C_9H_{11}NO_2$	Amino acid
5.	3.76	205.0	205.097	$C_{11}H_{12}N_2O_2$	Alkaloids
6.	3.90	355.1	355.105	$C_{16}H_{18}O_9$ (Caffeoyl quinic acid)	Phenolic acid
7.	4.14	595.5	595.167	$C_{27}H_{31}O_{15}$	Flavonoids
8.	4.92	334.4	335.220	$C_{20}H_{28}O_4$ (14-Deoxyandrographolide)	Diterpenoids
9.	5.43	332.4	333.203	$C_{20}H_{28}O_4$ (Andrographolactone)	Diterpenoids
10.	6.03	333.4	333.205	$C_{20}H_{28}O_4$ (14-Deoxy-11,12-didehydroandrographolide)	Diterpenoids
11.	6.38	350.4	351.219	$C_{20}H_{30}O_5$ (Andrographolide)	Diterpenoids
12.	7.01	350.4	701.431	$C_{20}H_{30}O_5$ (Andrographolide)	Diterpenoids
13.	7.24	318.4	319.219	$C_{20}H_{30}O_3$ (Andrograpanin)	Diterpenoids
14.	7.47	318.4	319.228	$C_{20}H_{30}O_3$ (Andrograpanin)	Diterpenoids
15.	7.65	591.7	592.349	$C_{32}H_{49}NO_9$ (Cevadine)	Alkaloids
16.	8.07	332.4	333.208	$C_{20}H_{28}O_4$ (14-Deoxy-11,12-didehydroandrographolide)	Diterpenoids
17.	8.38	318.4	319.226	$C_{20}H_{30}O_3$ (Andrograpanin)	Diterpenoids
18.	8.66	318.4	319.226	$C_{20}H_{30}O_3$ (Andrograpanin)	Diterpenoids
19.	8.94	316.4	317.210	$C_{20}H_{28}O_3$ (lambertianic acid)	Diterpenoids
20.	9.27	300.26	301.216	$C_{16}H_{12}O_6$ (5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H- chromen-4-one 4',5,7- trihydroxy-6-methoxyisoflavone)	Flavonoids
21.	9.54	316.4	317.204	$C_{20}H_{28}O_3$ (lambertianic acid)	Diterpenoids
22.	9.92	664	665.406	$C_{40}H_{56}O_8$ (Bisoandrographolde)	Diterpenoids

LC MS/MS analysis of 3 out of 7 compounds of the diterpenoid group whose fragmentation patterns have been studied in previous studies. The fragmentation pathway and diagnostic product ions are andrographolide, 14-deoxy-11, 12- didehydroandrographolide, and bis andrographolide. One of the compounds detected was the main compound of andrographolide with the molecular formula $C_{20}H_{30}O_5$, showing fragmentation patterns at m/z 351 and 702. While other diterpenoid compounds are 14-deoxy-11, 12-didehydroandrographolide ([M+ Ion H]$^+$ with at m/z 333, bisandrographolide A on MS/MS at m/z 665 (Dwivedi et al., 2021; Solomon Jeeva, 2014).

From the results of GC-MS and LC-MS/MS analysis, the chemical structure of 18 compounds was successfully determined, while the chemical structure of several other compounds could not be determined so they could not proceed to in silico studies. The identified compounds were then

analyzed for physicochemical properties based on the Lipinski rule of five with parameters of Molecular Weight < 500 g/mol, LogP < 5, number of hydrogen bond acceptors < 10, and number of hydrogen bond donors < 5 (Lipinski, 2004). The results of the physicochemical properties analysis parameters can be seen in Table 2.

Physicochemical parameters are one of the requirements that must be met, especially for peroral drug preparations (Lipinski, 2004). The molecular weight of a drug is related to the process of the drug in penetrating biological membranes. Peroral drug requirements based on Lipinski's rules are < 500 g/mol. The results of the molecular weight determination showed that 16 test ligands had BM 500 values of less than g/mol, indicating that the test ligands could pass through the cell membrane (Table 2 marked with a).

The physicochemical property parameter that determines the hydrophobic or hydrophilic nature of a ligand is the Log P value. The requirements for drugs by the peroral route based on Lipinski's rule must have a LogP value ≤ 5 (Lipinski, 2004). 16 test ligands have a LogP value ≤ 5, indicating that the test ligand has lipophilic properties and can pass through the cell membrane so that it can reach the target. as shown in Table 2, marked with b). Bond acceptors and donors are influential in the process of membrane permeability. The requirement for the number of acceptor bond donors is < 10 and the number of hydrogen donor bonds is < 5 (Lipinski, 2004). From the determination of physicochemical parameters, fourteen test ligands have met the rules as good antidiabetic candidates (Table 2 marked with c).

Preparation of test ligands

The test ligands used in this study are ligands from the ethanol extract of sambiloto that have met the requirements of the physicochemical properties parameters that are used as test ligands based on Lipinski's rules. All ligands were made two- and three-dimensional structures using ChemDraw 2D and 3D applications using Discovery Studio Visualizer (Figure 2). Furthermore, geometry optimization was carried out using the Gaussian09 application with the Semi-Empirical (AM1) method (Julaiha et al. 2019).

Protein target and natural ligand preparation

The working targets used are the Alpha-Glucosidase enzyme and the DPP-4 enzyme which were downloaded from the Protein Data Bank (PDB) site https://www.rcsb.org/ which is managed by the Research Collaboratory for Structural Biology (RCSB). The downloaded enzymes have PDB ID codes, namely 5NN8 and 2QOE. The following visualization of the work targets and natural ligands (Figure 3):

The natural ligand on the alpha-glucosidase enzyme is acarbose which is proven to have an activity to inhibit the enzyme (Julaiha et al. 2019). The natural ligand on the DPP-4 enzyme is triazolopiperazine (Kowalchick et al. 2007) which also has activity as an inhibitor (Figure 3).

The selection of the two working targets is an enzyme that affects the increase in blood glucose in type 2 DM disease and also because these enzymes can be inhibited by the test compound so that it forms a complex between the working target and the ligand in the form of an inhibitor. The organism used in modeling is the human organism (Homo sapiens). Apart from that, the modeling method used is X-ray diffraction (XRD), this method only has one post at rest and a resolution of ≤ 2 Å. Natural ligands that form complexes with action targets are medicinal compounds that have been used and proven have a mechanism of action as inhibitor (Kowalchick et al., 2007; Roig-Zamboni et al., 2017).

Table 2. Parameters of physicochemical properties

Ligand code	Ligand name	MW (g/mol)	LogP	H-bond acceptor	H-bond donor
S1	Andrographolide [abc]	350.00	1.96260	5	3
S2	Andrographanin [abc]	318.00	4.0209	3	1
S3	Bisandrographolide	664.00	5.36789	8	4
S4 .00	14-Deoxyandrographolide [abc]	334.00	2.99179	4	2
S5	Andrographolactone[abc]	296.00	4.22613	2	0
S6	14-Deoxy-11,12-didehydroandrographolide [abc]	332.00	2.76779	4	2
S7	Lambertianic acid[a]	316.00	5.07570	3	1
S8	5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one 4',5,7- Trihydroxy-6-methoxyisoflavone[abc]	300.00	1.93897	6	3
S9	Cevadine [b]	591.00	0.99457	10	6
S10	Caffeoyl quinic acid [ab]	354.00	-0.6459	9	6
S11	1-(3-hydroxycyclopent-2-en-1- yl) propane-2-one [abc]	140.00	1.81740	2	1
S12	furan-2,5-dicarboxylic acid [abc]	156.00	0.67600	5	2
S13	2,4-di-tert-Butyl-6-Cyanoaniline[abc]	230.00	3.73547	2	2
S14	3-hydroxy-3-(4-hydroxyphenyl)propanoic acid[abc]	182.00	0.90030	4	3
S15	1-(2-hydroxyphenyl)ethan-1-one [abc]	136.00	1.59480	2	1
S16	propyl 4-hydroxybenzoate [abc]	180.00	1.95900	3	1
S17	11-(P-Bromoanilino)-5H-Dibenzo[B,E][1,4]Diazepine [abc]	363.00	4.70999	3	2
S18	3-butyl-3,5,5-trimethyl cyclo hex- 1-en-1-ol [abc]	196.00	4.44479	1	1

Andrographolide (S1)

Andrographanin (S2)

14-deoxyandrographolide(S4)

Andrographolactone (S5)

14-Deoxy-11,12-didehydroandrographolide (S6)

Lambertianic acid (S7)

5,7-dihydroxy-3-(4-hydroxyphenyl)-6-methoxy-4H- chromen-4-one 4',5,7-Trihydroxy-6-methoxyisoflavone (S8)

1-(3-hydroxycyclopent-2-en-1-yl)propan-2-one (S11)

furan-2,5-dicarboxylic acid (S12)

2,4-Di-Tert-Butyl-6-Cyanoaniline (S13)

3-hydroxy-3-(4-hydroxyphenyl)propanoic acid (S14)

1--(2-hydroxyphenyl)ethan-1-one (S15)

propyl 4-hydroxybenzoate (S16)

11-(P-Bromoanilino)-5H-Dibenzo[B,E][1,4]Diazepine (S17)

3-butyl-3,5,5-trimethylcyclohex-1-en-1-ol (S18)

Figure 2. Ligand structure modelling

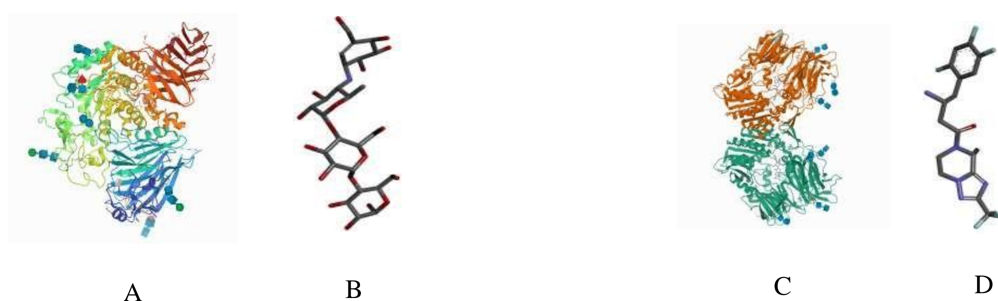

Figure 3. Target protein and natural ligands

(A) Crystallographic Structure of Alpha-Glucosidase Enzyme (B) Natural ligand Acarbose
(C) Crystallographic Structure of DPP-4 Enzyme (D) Natural ligand Triazolopiperazine
(Source: RSCB Protein Data Bank)

Besides that the enzyme can be inhibited by the test compound therefore it forms a complex with a resolution of ≤ 2 Å. Crystallographic resolution categories are divided into four categories, small (<3.00 Å), medium (2.70-2.00 Å), high (2.00-1.50 Å), and very high (<1.5 Å). The smaller the crystallographic value, the more specific the visualization of the resulting image (Riverson & Rizarullah, 2020).

Docking validation

Molecular docking was performed using Autodock version 4.2.3 (Morris et al. 2008). Validation of molecular docking is done by natural ligands docking to the test enzyme and then comparing the pose of the natural ligand before and after, known as redocking which is expressed in the form of RMSD (Root Mean Square Deviation). The purpose of redocking is to determine the validated ligand docking location in the form of the size and position of the grid box. The validation of molecular docking is declared valid if the RMSD value is ≤ 2 Å, which means that the molecular docking method provides a deviation that is not large and can be used further for the simulation of molecular docking of test ligands enzyme (López-Camacho et al. 2016).

Docking validation of alpha-glucosidase enzyme

Redocking natural ligand against alpha-glucosidase enzyme is valid with grid box (40,76,40), grid spacing 0,775A°, and grid center (-14,425; -37,292; 95,129). This redocking gives an acceptable RMSD value, which is 1,705A° with a ΔG value of -9,65 Kcal/mol. or equivalent to the inhibition constant of 84,93nM.

The interaction of the natural ligand redocking results is in accordance with the interaction in its crystal structure. The interaction occurs through the formation of 18 hydrogen bonds, 2 hydrophobic interactions, and several Van der Walls interactions. Visualization of the interactions can be seen in Figure 4.

Docking validation of DPP-4 (*Dipeptidyl Peptidase IV*) enzyme

Redocking natural ligand against DPP-4 enzyme is valid with grid box (46,44,44), grid spacing 0,375A°, and grid center (-7,646; 66,207; 39,187). This redocking gives an acceptable RMSD value at 1,878A° with an ΔG value of -8,26 Kcal/mol. or equivalent to the inhibition constant of 874,76 nM.

The interaction of the natural ligand redocking results following the interaction in its crystal structure. The interaction occurs through the formation of 6 hydrogen bonds, 3 hydrophobic interactions, and several Van der Walls interactions. Visualization of the interactions can be seen in Figure 4.

Interactions between enzymes and natural ligands can be in the form of hydrogen bonds, hydrophobic bonds, and Van der Waals interactions, which are intermolecular bonds. Intermolecular bonds are important bonds that occur between drug interactions and their targets. The conformation with the lowest binding energy shows the best interaction pose. The conformational suitability of the test ligand is compared with the native ligand to confirm the free energy value because the active ligand must bind to specific amino acid residues. The important amino acid residues responsible for activity are ALA 284, ASP 404, MET 519, ASP 616, HIS 674, ARG 600, and TRP 376 in Alpha-glucosidase enzyme while Glu 205, Glu 206 in DPP-4 enzyme (Kowalchick et al., 2007; Roig-Zamboni et al., 2017). The results of re-bonding natural ligands have interactions with amino acid residues by forming hydrogen bonds, proving the validation of molecular docking has been valid (Julaiha et al. 2019).

Molecular docking on alpha-glucosidase enzyme

Molecular docking is a computational technique used to predict the interaction between a small molecule, such as a drug candidate, and a target protein, usually an enzyme. It helps in understanding the binding affinity and the orientation of the ligand within the enzyme's active site, which is crucial

for designing effective inhibitors. A molecular docking simulation for the test ligand was carried out to obtain the interaction and affinity of the test ligand to the active side of the Alpha-glucosidase enzyme, according to the previously validated docking parameters. This simulation allows for the identification of key interactions between the ligand and the enzyme, providing insights into the potential efficacy of the ligand as an enzyme inhibitor. (Julaiha et al. 2019)

The ΔG value of the test ligands compared to the natural ligand is -9.65 kcal/mol (Table 3), where ligands S1, S1, S4, S5, S17, and S18 are predicted to have a better affinity to the enzyme. Meanwhile, the inhibition constant value of the test ligands compared to the natural ligand is 84.93 nM (Table 3), where ligands S1, S2, S4, S5, S4, S6, S17, and S18 showed less than 100 uM and are predicted as strong inhibitors (Zheng and Polli 2010). Interaction of the test ligand with the alpha-glucosidase enzyme, S1 showed the best interaction because it interacts with important amino acid residues which are responsible for the activity of ALA 284 and ASP 404 (Julaiha et al. 2019), and based on ΔG value, inhibition constant, and interaction with Alpha- glucosidase enzyme showed that ligand S17 (11-(P-Bromoanilino)-5H-Dibenzo [B,E][1,4] Diazepine), S1(andrographolide) and S2 (andrographanin) have potential as inhibitors on alpha-glucosidase enzyme (Morris et al. 2008).

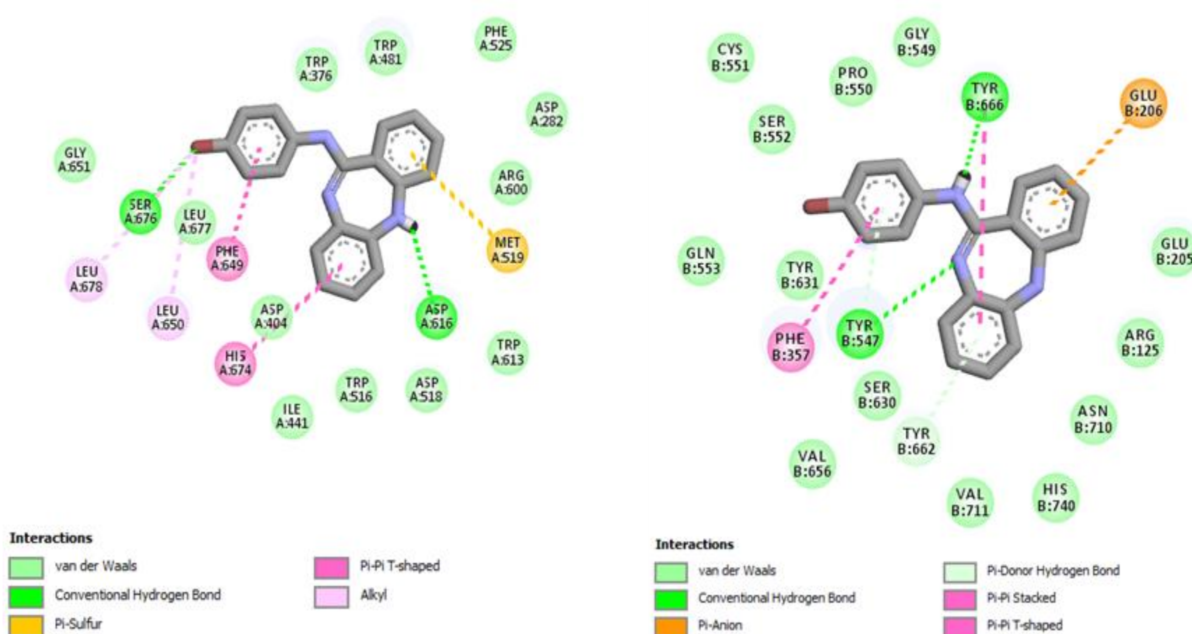

Figure 4. Visualization of alpha-glucosidase enzyme-natural ligand acarbose interaction (A) DPP-4 enzyme interaction with triazolopiperazine natural ligand(B)

Molecular docking on DPP4 enzyme

A molecular docking simulation for the test ligands was conducted to obtain the interaction and affinity of the test ligands towards the active side on the DPP-4 enzyme, under the previously validated docking parameters. This approach allows for the assessment of how well the ligands fit within the enzyme's active site, providing valuable insights into their potential to inhibit enzyme activity. By evaluating the binding energy and key interactions, the simulation helps identify the most promising candidates for further in vitro and in vivo studies, ultimately contributing to the development of more effective therapeutic agents.

The ΔG values of the test ligands (Table 4) were compared with the ΔG value of the natural ligand of -8.26 kcal/mol, where ligands S17 and S5 had more negative ΔG values than the natural ligand, so

they were predicted to have better affinity to the DPP-4 enzyme. While ligands S17 and S5 showed lower inhibition constant values compared to the natural ligand (Table 4). Value less than 100 uM are considered strong inhibitors (Zheng and Polli 2010). From the interaction of the test ligand with the DPP-4 enzyme, S17 has the best interaction because it interacts with amino acid residues GLU 205 and GLU 206 which is responsible for the activity. The best molecular docking results based on ΔG, Ki values and interactions with the DPP-4 enzyme are the S17 ligand (11-(P-Bromoanilino)-5H-Dibenzo [B,E][1,4]Diazepine) and S5 (andrographolactone), which have potential to act as an inhibitor of the DPP-4 enzyme and predicted that these ligand will have a better affinity in interacting with the enzyme (Morris et al. 2008).

Table 3. Molecular docking on alpha-glucosidase enzyme

Ligand code	ΔG (kcal/mol)	Inhibition Constant
Acarbose	-9.65	84.93 nM
S1	-7.47	3.34 uM
S2	-7.72	2.18 uM
S4	-7.05	6.84 uM
S5	-6.99	7.55 uM
S6	-7.09	6.40 uM
S7	-5.67	70.12 uM
S8	-6.05	36.50 uM
S11	-5.23	146.78 uM
S12	-0.88	227.88 mM
S13	-6.64	13.67 uM
S14	-2.75	9.66 mM
S15	-5.21	151.86 uM
S16	-5.59	80.11 uM
S17	-8.18	1.02 uM
S18	-6.92	8.43 uM

Table 4. Molecular docking on DPP-4 enzyme

Ligand code	ΔG (kcal/mol)	Inhibition constant
Triazolopiperazine	-8.26	874.76 nM
S1	-7.70	2.28 uM
S2	-8.15	1.06 uM
S4	-7.87	1.70 uM
S5	-8.28	851.87 nM
S6	-7.99	1.40 uM
S7	-6.81	10.22 uM
S8	-7.08	6.41 uM
S11	-5.33	123.74 uM
S12	-1.47	84.28 mM
S13	-6.51	16.90 uM
S14	-4.21	821.99 uM
S15	-5.53	88.91 uM
S16	-5.31	127.56 uM
S17	-9.35	140.32 nM
S18	-7.16	5.64 uM

CONCLUSION

Based on LC-MS / MS and GC-MS analysis obtained 18 identified compounds. These compounds come from alkaloids, flavonoids, and terpenoids. The results of molecular docking showed that the compound S17 (*11-(P- Bromoanilino)- 5H-Dibenzo[B,E][1,4]Diazepine*), S1 (*andrographolide*) and

S2 (*andrographanin*) have the potential to inhibit the activity of alpha-glucosidase enzyme; on the other hand, S17 (*11-(P- Bromoanilino)-5H-Dibenzo[B,E][1,4]Diazepine*) and *S5* (*andrographolactone*) have the potential to inhibit the activity of DPP-4 enzyme. These compounds have the potential to inhibit alpha- glucosidase and DPP-4 enzymes which act as antidiabetics.

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