Inhibitory Activity of Pancreatic Lipase by Wungu (*Graptophyllum pictum* (L.) Griff.) Leaf Extract

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Abstract. Obesity is a problem in many parts of the world, and its prevalence is increasing rapidly. Inhibition of pancreatic lipase to inhibit triglyceride hydrolysis can prevent obesity and reduce its prevalence, so drugs that can inhibit pancreatic lipase are very useful as anti-obesity. Orlistat is the single most successful approved pancreatic lipase inhibitor compound, but its use is still limited due to its relatively high price and several side effects. A safe and effective alternative to pancreatic lipase inhibitors is urgently needed. Metabolites in wungu leaves (*Graptophyllum pictum* (L.) Griff.), which is a traditional Indonesian plant, have potential as an anti-obesity. This study aims to determine the effect of wungu leaf metabolites on lipase as an anti-obesity. This study began with an in vitro test, which tested lipase activity with the addition of wungu leaf extract. The best extract samples were then tested with LC-MS to determine the profile of secondary metabolites. Furthermore, an in silico approach was carried out by paying attention to the LIPINSKI and ADMET rules, so as to obtain potential medicinal compounds. It can be concluded that wungu leaf extract has the potential to be developed as an alternative anti-obesity active ingredient derived from archipelago herbs.

1 Introduction

Obesity is a problem in various parts of the world, and its prevalence continues to increase rapidly, especially in developed countries and in developing countries. Obesity is a state of increased body weight above 20% of normal limits [3]. In general, obesity can be caused by a calorie imbalance, where energy intake exceeds the body's needs, resulting in excess energy that is stored in the form of fat tissue in the body.

Lipase is an enzyme that can catalyze triacylglycerol into free fatty acids and glycerol [5]. Triacylglycerol that has been converted into free fatty acids and glycerol can be absorbed by the body, causing obesity. Inhibition of lipase enzymes to inhibit triglyceride hydrolysis can reduce and prevent the prevalence of obesity, so drugs that can inhibit pancreatic lipase enzymes in digestion are very useful to be used as anti-obesity. This makes pancreatic lipase an important target in the treatment of obesity. Currently, one of the most common ways to

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treat obesity is by using drugs containing orlistat, but the use of orlistat is still limited. because it is relatively expensive and has several side effects on the digestive tract, kidney function, and liver [27]. An alternative solution that can be used to overcome obesity, besides using synthetic drugs such as orlistat, is to use herbal plants, which are possibly safer and can minimize side effects due to the chemical content in synthetic drugs.

Wungu is a type of plant that has medicinal properties, so it can be used as a medicinal plant. The results of phytochemical test analysis conducted by Manoi [10] showed that secondary metabolites contained in wungu leaves are non-toxic alkaloids, steroidal glycosides, saponins, gallic tannins, anthocyanins, leukoanthocyanins, protocatechuic acids, and flavonoids. This research aims to determine the activity of pancreatic lipase enzyme inhibition by wungu leaf extract (*Graptophyllum pictum* (L.) Griff) as an anti-obesity agent.

2 Methods

2.1 Simplisia preparation

Fresh wungu (*Graptophyllum pictum* (L.) Griff) leaves were washed and cut into smaller sizes. After cleaning, the samples were dried using an oven at 30°C for 3 days. The dried samples were then made into simplisia by mashing and filtering using an 80 mesh filter.

2.2 Simplisia extraction

Extraction was carried out by two methods, namely maceration and infusa. Maceration was carried out in ethanol and ethyl acetate solvents, with a ratio of 1:10 to simplisia and solvent. Maceration was carried out at room temperature for 3×24 hours. Infusa is carried out with a closed glass container in distilled water solvent, namely simplisia boiled using boiling distilled water for about 15 minutes and then cooled at room temperature to then be filtered and the filtrate is taken. The filtrate resulting from the extraction process is evaporated using a rotary evaporator at 45°C. The concentrated extract of the wungu leaf sample was then calculated and used in the next test.

2.3 Enzyme activity assay

Preparation of buffers, reagent solutions, test samples and controls were carried out. Triton-X phosphate solution (0.1 M phosphate buffer saline at pH 7.2, 0.15 M NaCl and 0.5% Triton-X 100) was used as buffer. Pancreatic lipase (Lipase Cas 9001-62-1 (L 1471 OTTO)) of 0.1 mg was dissolved in 10 mL of buffer solution. 20.92 mg of p-nitrophenyl butyrate (p-NPB) substrate was dissolved in 10 mL of buffer. 13.911 mg of p-nitrophenyl butyrate (p-NP) substrate was dissolved in 10 mL of buffer. Orlistat as much as 50 mg was dissolved in 10 mL of buffer. The test sample solution was made in several concentrations with buffer as the solvent.

The reaction mixture with three replicates consisted of p-NPB (50µL), lipase (25 µL), extract (25 µL). Orlistat was used as positive control, while blank solution was used as negative control. The mixture was homogenized and incubated at 37°C with optimum incubation time (15 minutes). Absorbance was determined using Spektrostar Nano UV-VIS at a predetermined optimum wavelength (401 nm). The data obtained were then processed and visualized with respect to biochemical parameters using Microsoft Excel and GraphPad Prism9.

2.4 UPLC-MS QToF analysis

V Prior to UPLC-QToF-MS analysis, reverse-phase SPE (Solid Phase Extraction) was performed. A strong polar to weak polar solvent gradient is used to ensure impurities are completely removed from the sample to be tested. UPLC-MS QToF (ultra performance liquid chromatography-quadrupole time of flight-mass spectrometry) analysis used a UPLC-MS system with QToF as the analyzer and positive electrospray ionization as the ionization source with an Acquity C18 1.8 µm; 2.1 × 150 mm column.

The eluents used were a mixture of (A) Water (HPLC grade)/formic acid (Merck, Darmstadt, Germany) 99.9/0.1 [v/v]; (B) Acetonitrile (Merck, Darmstadt, Germany)/formic acid 99.9/0.1 [v/v] and a gradient elution system. The source temperature was 100°C and the desolvation temperature was 350°C. A 10 mg sample of extract was dissolved in a 10 ml volumetric flask with absolute methanol, then a volume of 5 µl was injected into the UPLC-MS system. From the chromatogram data, the area was calculated in percentage. Parameters for analysis were set using positive ion mode with spectra obtained over the mass range from m/z 120 to 1,000. Chromatograms in .raw format were converted into .abf format using the Reifycs file converter. MS-Dial version 4.70 windows software was used to process the converted chromatograms. Component identification was based on the m/z ratios measured in MS-Dial, MSP spectral kit files (https://systemsomicslab.github.io/compms/msdial/main.html#MSP), inhouse.txt files based on literature studies, and PubChem (https://pubchem.ncbi.nlm.nih.gov/). The data obtained were then re-selected using Ms. Excel.

2.5 In Silico approach

The 3D structures of compounds obtained from the LCMS analysis of Wungu leaves in *SDF format were downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) and processed using a Python script to add hydrogen atoms and Gasteiger charges to each structure to improve the accuracy of the docking calculations. The protein structure of *Sus scrofa* pancreatic lipase was downloaded from the RCSB website (https://www.rcsb.org/) with PDB code 1LPB. This structure was chosen according to the type of lipase used in the in vitro assay of this study. The protein structure was also pretreated with AutoDockTools v1.5.7, where the crystallographic ligand (MUP) and water structures were removed, while the Ca^{2+} cofactor was retained. The protein structure was also given the addition of hydrogen atoms and Kollman charges before finally becoming a receptor structure ready for ligand docking.

Ligand docking simulations were carried out using the GNINA open-source library, run using Google Colab. The MUP structure in the 1LPB crystallographic structure was taken as the reference size of the docking area (grid box) (11.435999, 5.882, 16.630001) and the center of the docking area (9.81899999, 23.48983351, 50.86733309), each represented by x, y, and z coordinates. The selected scoring function parameter was "vina" with exhaustiveness set at 64. Evaluation of the docking quality for each ligand was seen from the total binding energy produced; the lower the binding energy produced in kcal/mol, the better the binding energy. The best pose of each protein-ligand complex was then selected for further analysis.

Some of the ligands were selected for further study of their oral bioavailability potential include ligands with the best binding strength in molecular docking simulations, ligands with high abundance based on UPLC-MS results, and orlistat as a comparator. The evaluation parameters were taken based on the Lipinski Rules of Five reference, including the molecular weight of the compound, the number of H atoms donor and acceptor, rotatable bonds, logP, and the total polar area of the compound. The test was performed using the ADMETLab3

website (https://admetlab3.scbdd.com/server/evaluation). Results were then saved for comparison and further evaluation.

3 Results and Discussion

3.1 Wungu leaf extract

Extraction in this study was carried out using distilled water, ethanol, and ethyl acetate solvents. The extraction results were obtained in the form of crust-shaped extracts whose yield values were calculated (Table 1). The distilled water extract had the highest yield value of 2.45 ± 0.05%, followed by ethanol extract 2.12 ± 0.74%, and ethyl acetate extract 1.34 ± 0.28%.

Table 1. Yield of simplisia extract

Solvent	Extract yield (%)
Distilled water	2.45 ± 0.05
Ethanol	2.12 ± 0.74
Ethyl acetate	1.34 ± 0.28

Extraction is a method of separating a component from a mixture based on the type, physical properties, and chemical properties of a sample using a solvent. The difference between the pressure inside and outside the cell causes the sample submerged in the solvent during the extraction process to break the cell wall and membrane, which causes the solvent to enter the cell and dissolve the secondary metabolite content contained in the cell [17].

Maceration is a fairly simple extraction process or method without a heating system or known as cold extraction, which is during this process the sample and solvent do not undergo a heating process, so that it can be used to extract compounds that are not heat resistant. The maceration technique is carried out by putting the sample in the form of simplisia into a solvent according to the level of solubility of the sample. The maceration process is carried out in an inert container that is tightly closed and stored at room temperature with several times stirring or shaking.

Infusa is a traditional extraction method that has been widely used in traditional medicine methods by most ethnicities in the world. This method is used to extract natural ingredients that are thermolabile or natural ingredients with essential oil content [21]. Infusa is carried out in a tightly closed container, and after cooling, filtering is carried out, this is to ensures that the compounds extracted from the simplisia do not evaporate, but are condensed again.

The yield value is the percentage ratio of the final weight of the extract produced to the initial weight of the sample used. The difference in yield value can be caused by differences in the form of the sample (still in the form of thick extract or has reached the form of crust). Thick extracts certainly have a larger gram compared to crust-shaped extracts because there is still solvent left in the sample and measured during the final weighing, even though it has gone through the evaporation stage with a rotary evaporator. The type of solvent used in the extraction process greatly affects the active compounds that will be extracted. Based on the principle of like dissolves like, non-polar solvents will attract active compounds that are non-polar, polar solvents will attract active compounds that are polar, and semi-polar solvents will attract active compounds that are semi-polar as well [24].

3.2 Enzyme activity and kinetics

Most fatty acids from the diet are ingested as triglycerides, and their availability in the body will depend on the hydrolysis of ester bonds on the glycerol backbone. An increase in the amount of fatty acids that are re-esterified for storage when the body is in a state of energy surplus (excess calories) results in fat accumulation that contributes to an increase in fat mass, which will lead to overweight and obesity. Therefore, pancreatic lipase is a key enzyme in the digestion and absorption of lipids and the hydrolysis of triacylglycerols into monoacylglycerols and fatty acids in the duodenum. Inhibition of lipase activity in breaking down fats results in fats from the diet not being optimally absorbed by the body, so that they will be directly excreted through the feces. This approach is most effective for discovering new agents in the treatment of metabolic disorders. So far, orlistat, which is a hydrogenated derivative of lipstatin, has been the single most successful approved pancreatic lipase inhibitor compound [27].

Enzyme kinetics is an important part of biochemistry that studies the interaction of enzymes with their substrates to produce products. In the study of enzyme kinetics, understanding the action of enzymes and the various factors that affect their activity is very important. For this reason, enzyme kinetics analysis can be interpreted through various curves that describe the relationship between substrate concentration, product, inhibitor, reaction speed, and so on. Enzyme reactions are characterized by the formation of products that increase over time (Figure 1a). At the beginning of the reaction, the rate of product formation is high because the substrate is still abundant. As time increases, the reaction slows down due to substrate depletion, product inhibition effects, or the reaction has entered a saturation phase. This curve is important for understanding the dynamics of enzymatic reactions. This curve is also in line with enzyme activity, which is increasing activity until it reaches optimal conditions, then decreases when the substrate begins to run out of reaction. Michaelis-Menten explains that product concentration and substrate concentration are interrelated (Figure 1). (Figure 1b) Over time, substrate is converted to product, so [P] increases as [S] decreases [5].

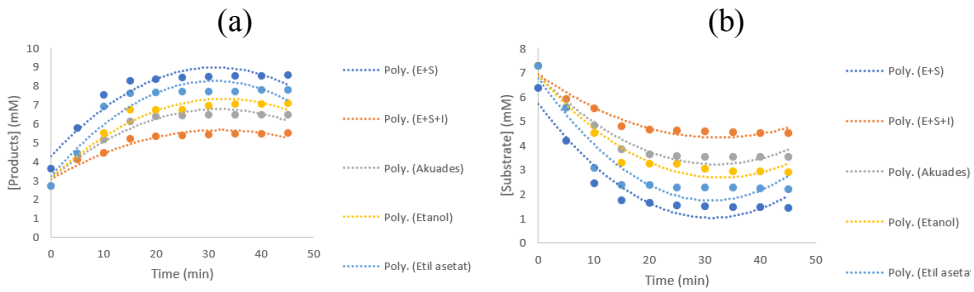


Fig 1. (a) Product formation; (b) Residual substrate

The polynomial equation of the relationship between [P] and time (Figure 1) gives an overview of the reaction rate value (Figure 2) that can categorize the type of inhibition that occurs in the reaction. Based on Figure 2, the inhibition that may occur is mixed inhibition, which is a combination of competitive and non-competitive inhibition. This is indicated by a change in reaction speed due to the presence of the inhibitor and a decrease in the concentration of the product produced.

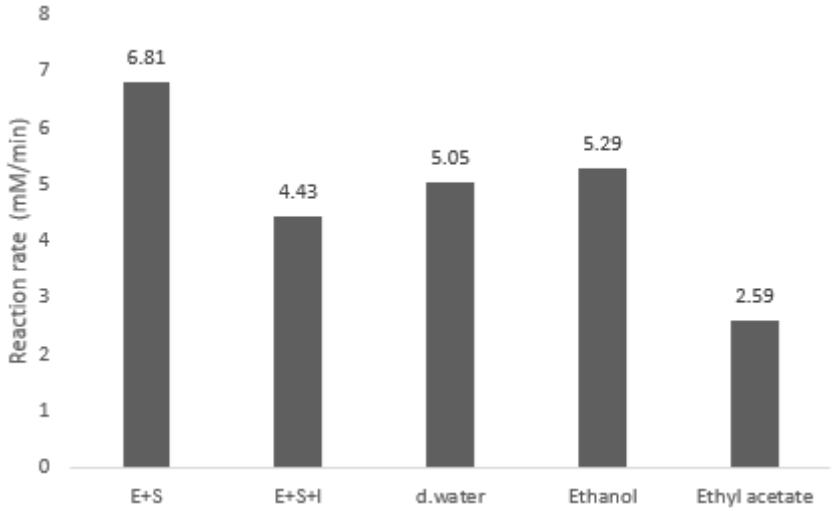


Fig 2. Lipase reaction rate at 15 min

The inhibition value describes the amount of inhibition the sample has on enzyme activity, so the greater the percent inhibition, the greater the inhibition of the sample in forming the product. Orlistat in millimolar (mM) concentration units, as a comparative inhibitor compound, has an inhibition value of 98.93% at a concentration of 500 ppm, which means that this compound is quite potent as a pancreatic lipase inhibitor. The extract of wungu leaf simplisia has a good inhibition value (Figure 4) (≥ 90), so that if directly compared with orlistat, it will be classified as an effective compound in lipase inhibition. The wungu leaf extract that produced the largest inhibition value was the extract with distilled water solvent (98.73%) at a concentration of 500 ppm. The superiority of the distilled water sample in the inhibition power can be caused by the abundance of compounds that are suitable in the mechanism of lipase inhibition compared to ethanol and ethyl acetate samples. For this reason, secondary metabolite profiling was carried out with UPLC-MS QToF to determine more specific compounds in the sample.

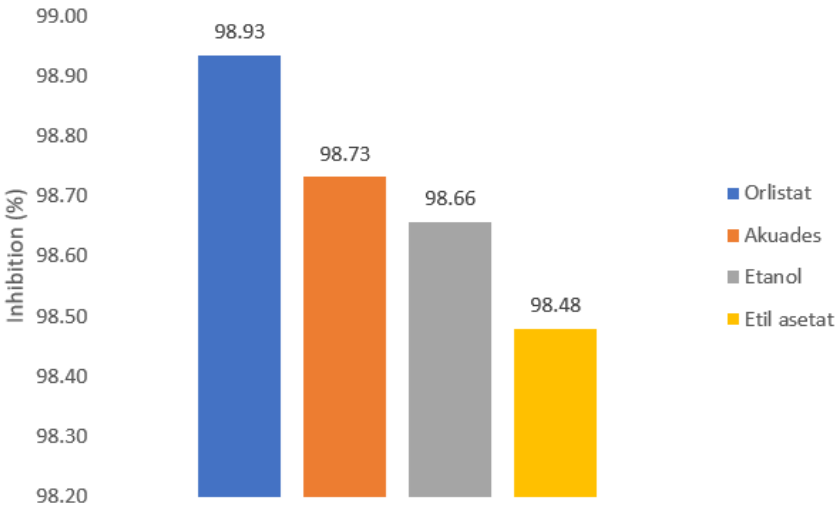


Fig 3. Lipase inhibition value with 500 ppm concentration

3.3 Metabolite profile

Chromatograms were processed by integrating the MassLynx Raw Data Reader Interface Library (Waters Corporation, Milford, MA) to produce 104 compounds that were further filtered based on quality parameters, namely retention time and mass (m/z) matching against the reference, high identification score (total score ≥ 90), high retention time similarity with the reference (RT similarity ≥ 90), and good signal to noise (S/N average ≥ 20). This process resulted in a total of 47 confident compounds, then sorted based on the abundance of compounds in the sample to produce a metabolite profile (Table 2) of wungu leaves with distilled water solvent. Betaine and L-phenylalanine were also found in ethanol and hexane solvents [29], so that these two compounds can be categorized as semi-polar. Several previous studies [14,30] conducted GC-MS testing on wungu leaves, but none of the compounds were the same as those obtained in this study.

Table 2. Top 10 of 47 compounds from the metabolite identification results of wungu leaf extract using UPLC-MS QToF method

No	Compound	Class	TA	MF	MW	RT
1	Phenylacetylglycine	Carboxylic acids and derivatives	7579130.67	C10H11NO3	193.20	1.78
2	Isoshaftoside	Flavonoids	1987043.67	C26H28O14	564.49	4.44
3	2`-Methylacetanilide	Benzene and substituted derivatives	695848.00	C9H11NO	149.19	1.88
4	L-Tyrosine	Carboxylic acids and derivatives	595579.67	C9H11NO3	181.19	1.41
5	Betaine	Carboxylic acids and derivatives	575286.67	C5H11NO2	117.15	1.27
6	Quinolone	Quinolines and derivatives	539635.67	C9H7NO	145.16	3.87
7	3-Indoleacetic acid	Indoles and derivatives	208247.33	C10H9NO2	175.18	1.73
8	L-Phenylalanine	Amino acids and derivatives	181404.67	C9H11NO2	165.19	2.86
9	N-Nitrosopiperidine	Piperidines	172826.67	C5H10N2O	114.15	3.24
10	Phenylacetaldehyde	Benzene and substituted derivatives	140741.33	C8H8O	120.15	1.87

TA : Total area

MF : Molecule form

MW : Molecule weight (g/mol)

RT : Retention time (minutes)

3.4 In silico approach

A virtual screening process was conducted to identify potential ligands from a total of 47 test ligands, which consisted of secondary metabolites derived from Wungu leaf. Ligands were docked to the initial MUP docking site at 1LPB in a predetermined grid box and then subjected to empirical and ensemble convolutional neural network (CNN) scoring functions to obtain accurate binding energies [31]. The ligands were then evaluated based on their binding energy value, obtained from a molecular docking simulation in the lipase 1LPB structure. The binding energy values provide insight into protein interactions that are precisely regulated based on cellular function [32]. In total, 2 ligands based on their order of abundance, and 1 ligand with the best binding energy were selected. The binding energies of the docked ligands are shown in Table 3.

Table 3. Selected ligands' binding energy and bioavailability

Parameters	Compounds			
	Orlistat	Lonicerin	Isoshaftoside	Phenylacetylglycine
ΔG (kcal/mol)	-6.89	-10.06	-8.75	-7.00
Molecular weight (Da)	495.39	594.16	564.15	193.07
Hydrogen acceptor	6	15	14	4
Hydrogen donor	1	9	10	2
Rotatable bonds	24	6	4	5
logP	6.82	0.84	0.23	0.52
Topological polar surface area	81.70	249.20	250.97	66.40

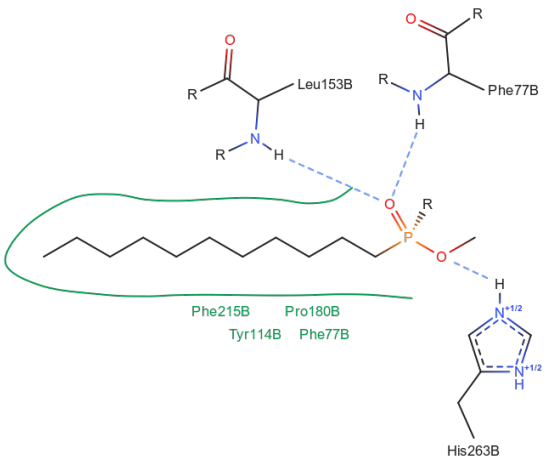
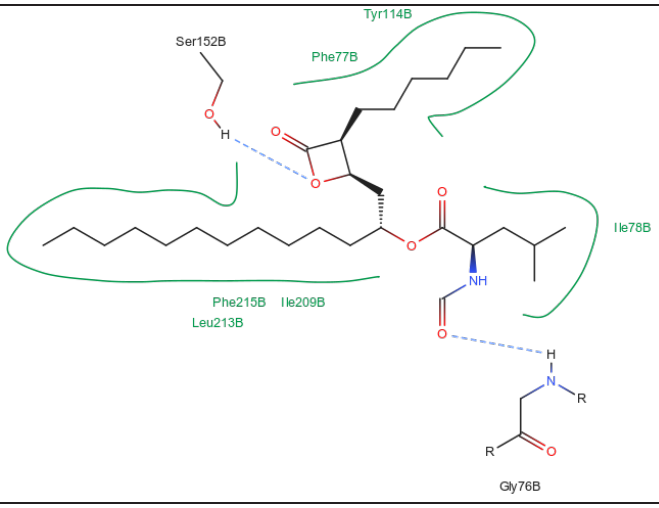
: Control drug
: Parameters violated

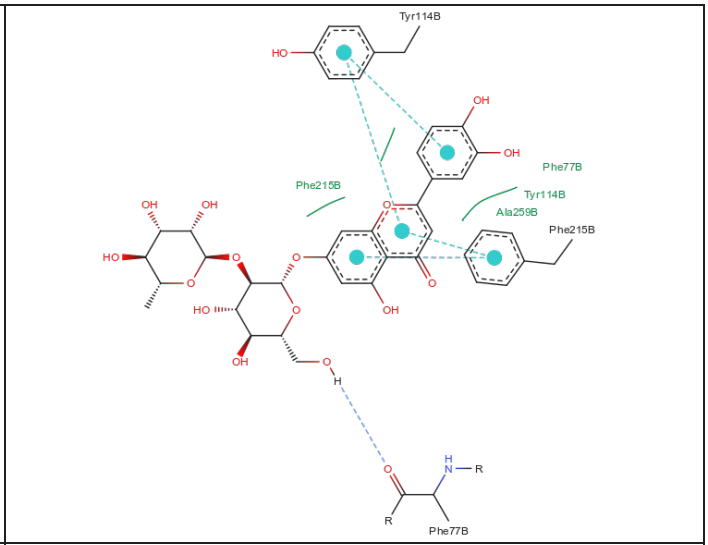
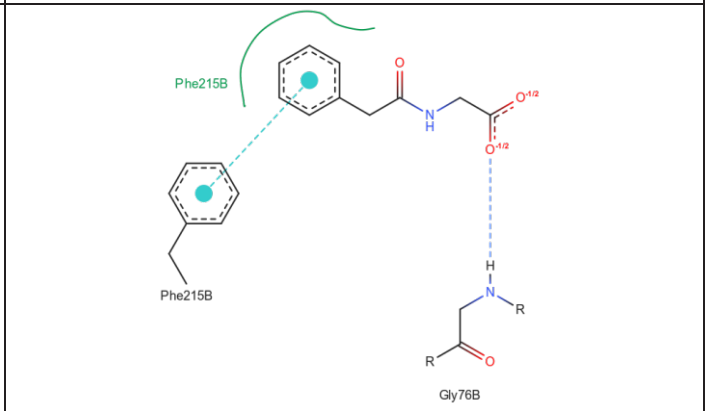
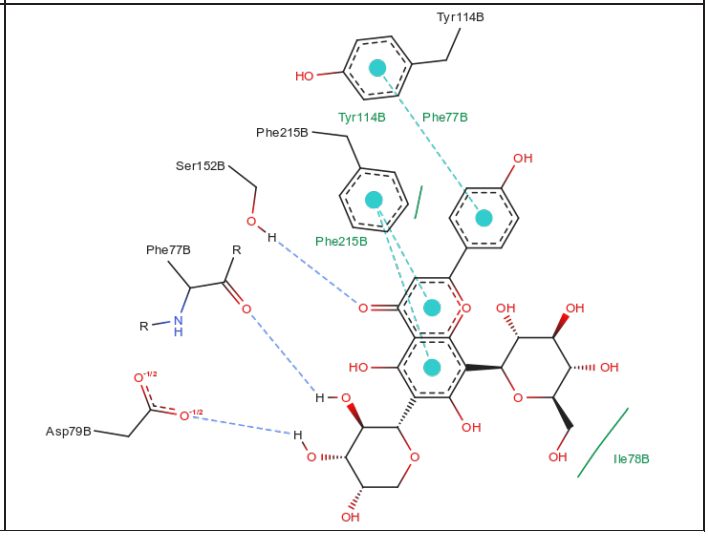
Bioavailability analysis was also conducted on those selected ligands from Wungu leaves and orlistat, as shown in Table 3. This was done to ensure that the obtained test ligands were usable and could be absorbed by the body. The selected ligands were those with no more than two violations of Lipinski's rule [33]. Some parameters used to assess the bioavailability of ligands (Table 3) are molecular weight <500 Da which facilitates the diffusion process into the body, hydrogen acceptor and donor respectively ≤10 and ≤5 because they impact passive diffusion across cell membranes, rotatable bond ≤10 to ensure the compound has optimal flexibility, logP and topological polar surface area to ensure the ease of the compound to cross the membrane [33].

The crystal ligand MUP (methoxyundecylphosphinic acid) was taken as a reference to determine the grid box size and the center of the docking area. MUP is a unique ligand docked to the 1LPB structure and is an effective pancreatic lipase inhibitor compound [37]. MUP has a binding strength of -9.88 kcal/mol, which is obtained from 3 hydrogen bonds (Leu153 and Phe77 form a hydrogen bond with 1 oxygen atom that is double bonded to the phosphorus atom; His263 forms a hydrogen bond with the oxygen atom in the phosphonate side group) 1 covalent bond (between the phosphorus atom of MUP and the OG atom at Ser152), and hydrophobic interactions (formed by Phe77, Tyr114, Pro180, and Phe215) (Table 4). Orlistat

as a control, has a higher binding energy compared to MUP, which is -6.89 kcal/mol formed from 2 hydrogen bonds (between the O atom in the betalactone side ring of orlistat with Ser152, and the oxygen atom in the amino ester side group with Gly76) and hydrophobic interactions (formed by Phe77, Ile78, Tyr114, Ile 209, Leu213, and Phe215). Lonicerin which is one of the compounds resulting from LCMS has a lower binding energy, which is -10.06 kcal/mol formed from 1 hydrogen bond (H atom bonded to Phe77) and 4 pi-pi interactions, better than the 2 compounds with the most abundance, namely phenylacetylglycine and isoshaftoside which are -7.00 kcal/mol and -8.75 kcal/mol, respectively.

Table 4. 2-dimensional interaction between ligand and docked lipase structure

Compound	2-dimensional interaction
MUP	
Orlistat	

Lonicerin	
Phenylacetylglycine	
Isoshaftoside	

Note : hydrogen bond ionic interaction metal interaction cation-pi interaction pi-pi interactic hydrophobic c

This difference in binding strength results from the number of interactions formed, such as hydrogen bonds and hydrophobic interactions, as well as the resulting interaction distance [38]. In the case of stronger binding energy with fewer interactions, this can be due to the optimal interaction distance between the ligand and protein atoms [38]. This explains why fewer hydrogen interactions can result in stronger binding energies, as in the lonicerin interactions and phenylacetylglycine interactions.

Despite its strong binding energy, lonicerin exhibits four violations of Lipinski's rule, thus failing to be one of the best candidate compounds from Wungu leaves. Phenylacetylglycine as the most abundant compound, on the other hand, has a good binding energy (-7.00 kcal/mol), and does not show any violations. These results indicate its potential use as a single compound that is even safer and more effective in inhibiting lipase activity compared to orlistat as a positive control, which has 2 violations of Lipinski's rule.

4 Conclusions

Wungu leaf extract samples with distilled water solvent showed greater potential in inhibiting pancreatic lipase activity. Based on an in silico approach to the LCMS results of wungu leaf compounds with distilled water extracts, phenylacetylglycine in wungu leaves has potential as an alternative anti-obesity compound.

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