

Identification of Active Compounds from *Averrhoa bilimbi* L. (Belimbing Wuluh) Flower using LC-MS and Antidiabetic Activity Test using *in vitro* and *in silico* Approaches

Diana Widiastuti^{1,*}, Siska Elisahbet Sinaga², Siti Warnasih¹,
Erna Pujiyawati¹, Supriatno Salam³ and Wira Eka Putra⁴

¹Department of Chemistry, Faculty of Mathematics and Natural Science, Universitas Pakuan, West Java, Indonesia

²Department of Nutritional Science, Universitas Widya Nusantara, Palu, Indonesia

³Department of Pharmacy, Faculty of Pharmacy, Universitas Mulawarman, Kalimantan, Indonesia

⁴Biotechnology Study Program, Department of Applied Sciences, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, East Java, Indonesia

(*Corresponding author's e-mail: dianawidi25@unpak.ac.id)

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Abstract

Averrhoa bilimbi L., (Belimbing wuluh), is traditionally used for various antidiabetic, anti-larvicidal, antioxidant, hypoallergenic, antibacterial, antifungal, and anti-inflammatory treatments. This study evaluated 3 extracts (*n*-hexane, ethyl acetate, and *n*-butanol) isolated from starfruit flowers for antidiabetic effects using *in vitro* and *in silico* methods. Ethyl acetate extract showed the highest activity, followed by *n*-hexane and *n*-butanol extracts in inhibiting α -glucosidase. Each extract has an IC₅₀ value of 89.84, 52.88, and 114.86 μ g/mL. The active compounds contained in the ethyl acetate extract were determined using LC-MS, and the compounds with the highest retention time were identified as tautomycetin (8.585) and lithocholic acid (8.993). Tautomycetin and lithocholic acid showed promising results in the *in silico* test. The computational predictions showed that tautomycetin (-6.2 kcal/mol) and lithocholic acid (-10.0 kcal/mol) have better binding affinity values than miglitol (-5.4 kcal/mol). This is the first time the antidiabetic test of starfruit flower extract has been carried out.

Keywords: Antidiabetic test, *Averrhoa bilimbi* L, *In silico*, Lithocholic acid, LC-MS, Tautomycetin

Introduction

The prevalence of diabetes patients in Indonesia has reached 10.7 million people, which places Indonesia in 7th place among the ten countries with the highest number of sufferers in 2019. To prevent the severity that occurs, many oral diabetes drugs are available in the community to treat diabetes, such as metformin, acarbose, and miglitol. Many researchers use local food, for example, *Manihot esculenta*, and *L. domesticum*, as medicinal plants containing active compounds, thus attracting attention to research other local foods that can be used as new antidiabetic agents, which are relatively easy to obtain. One of the plants included in the local food is the belimbing wuluh plant (*Averrhoa bilimbi* L.), famous for its use as herbal medicine and found in West Java Province in Indonesia [1-4].

In general, belimbing wuluh commonly called with starfruit can be used to treat asthma, diabetes, cough, sore throat, canker sores, and fever [5-7]. Parts of the belimbing wuluh used for treatment include flowers, leaves, fruit, and stems. Belimbing Wuluh contains active compounds that are antioxidants, hypoallergenic, antibacterial, antidiabetic, anti-larvicidal, antifungal, and anti-inflammatory, and can also be used as a functional food raw material. Ethanol extract 125 mg/kg Body Weight (BW) of belimbing wuluh leaves could reduce blood glucose in streptozotocin (STZ)-induced diabetic rats [8]. Rikhana demonstrated that administration of belimbing juice at a dose of 2 mL/200 g BW could affect blood glucose levels in rats experiencing hyperglycemia, thereby strengthening the suspicion that belimbing wuluh has antidiabetic activity. Kurup *et al.* [8] discovered phenolic compounds, alkaloids, flavanoids, saponins, terpenoids, and tannins in belimbing extract in a phytochemical study. The ethanol extract of belimbing wuluh flower contained flavonoid compounds and had antidiabetic activity [6]. Based on the explanation of the various benefits of belimbing wuluh, it is necessary to do activity test research on diabetes using belimbing wuluh flowers, considering there is still a lack of research on this plant flower. Therefore, this study will use various fractions of starfruit flower extract to determine its antidiabetic activity [6,7].

This research has focused primarily on the flower parts of the belimbing wuluh plant, which have been used as an antidiabetic agent [9]. All active compounds were identified using LC-MS, and their antidiabetic activity was assessed *in vitro* and *in silico*.

Materials and methods

Tools and materials

The tools used in this study namely, Whatman 41 filter paper, round bottom flasks, a blender, glass bottles, vials, funnels, rotary evaporator vacuum (BUCHI), analytical balances (OHAUS), glassware, separating funnels, separating funnel supports, UV-Vis spectrophotometer, cuvette, refrigerator, micropipette, incubator, pH meter, water bath, desiccator, millipore filter, micro syringe, and LC-MS. The materials used included: The flowers of *Averrhoa bilimbi* L. obtained from Parungpanjang Village, Parungpanjang District, Bogor Regency, 96 % technical ethanol, *n*-hexane, ethyl acetate, *n*-butanol, distilled water, α -glucosidase enzymes, quercetin, dimethyl sulfoxide (DMSO), phosphate buffer, *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG), sodium carbonate, acetonitrile, and formic acid.

Extraction and fractionation

Belimbing wuluh flower samples (145 g) obtained from Parungpanjang Village, Parungpanjang District, Bogor were macerated in 96 % ethanol at a ratio of 1:1(w/v) for 3×24 h at room temperature before being concentrated in a rotary evaporator at 47 °C. The crude extract was dissolved in water at a 1:10 (w/v) ratio and then partitioned by liquid-liquid extraction by *n*-hexane at a volume ratio of 1:1 (v/v). The same method was used to extract ethyl acetate and *n*-butanol. The *n*-hexane, ethyl acetate, and *n*-butanol phases were evaporated in a rotary vacuum evaporator at temperatures of 40 and 55 °C.

α -glucosidase inhibition test

The 250 μ L of 5 mM *p*-nitrophenyl- β -D-glucoside and 0.1 M phosphate buffer, pH 7.0 (495/490 μ L) were added to a test tube containing 5 μ L (standard solution) / 10 μ L (sample solution) in DMSO with various concentrations as above. After the homogeneous solution was preincubated for 5 min at 37 °C, the reaction was started by adding 250 μ L of the α -glucosidase solution; incubation was continued for 15 min. The reaction was stopped by adding 1 mL of 0.2 M Na₂CO₃. Enzyme activity was measured based on the absorption reading of the *p*-nitrophenol formed at λ 400 nm. Quercetin was used as a reference solution. The percentage of inhibition was measured using the following equation:

$$\% \text{ Inhibition} = \frac{(B - S)}{B} \times 100$$

Given B is the absorbance of the blank and S is the absorbance of the extract (the difference between the absorbance with and without the enzyme). The IC₅₀ value is obtained from the linear regression equation $y = a + bx$. The x-axis is the sample's concentration, and the y-axis is the % inhibition. Then the IC₅₀ value can be calculated by the following equation:

$$IC_{50} = (50 - a)/b$$

Given a is the intercept and b is the slope.

Identification of active compounds by liquid chromatography mass spectrometry (LC-MS)

Ethyl acetate extract (1.4 mg) was dissolved in 100 mL methanol. The solution was filtered using a 0.2 μ m GHP filter and injected into the UPLC system. The LC-MS system used is (Xevo-ToF-1) equipped with a C-18 column (Particle dimensions 1.8 μ m, 2.1×100 mm²), and MS with Xevo G2-S resolution QTOF acquisition mode ESI (-) and MSE. The eluent consists of 0.1 % formic acid in distilled water (A) and 0.1 % formic acid in acetonitrile (B). The total running time is 20 min with a temperature of 100 °C. The elution system was run with gradient elution at 0 - 1 min, the ratio of solvent A was 70 %, solvent B was 30 %, at 6 - 18 min, solvent A was 5 % solvent, B was 95 %, at 19 - 20 min, solvent was linear gradient elution A 70 % solvent B 30 %. Furthermore, data processing is done using MassLynx software.

Molecular docking, biological activity, and ADMET (absorption, distribution, metabolism, excretion, and toxicity) prediction

In this study, a molecular docking analysis was conducted on 2 compounds with the greatest RT values, namely tautomycetin and lithocholic acid, and α -glucosidase as protein targets, using the prior procedure [10,11]. Tautomycetin (CID: 6439037) and lithocholic acid (CID: 9903) 2D structures were retrieved from the PubChem database (<https://pubchem.ncbi.nih.gov/>). In addition, Miglitol (CID: 441314), an antidiabetic medication, was employed as a control in this work [12,13]. The protein sequence α -glucosidase (P10253) was obtained from UNIPROT (<https://www.uniprot.org/>), then homology modeling was performed using the SWISS MODEL (<https://expasy.org/swissmodel>). The software PyRx (<https://pyrx.sourceforge.io/>) was utilized for molecular docking. In addition, Pass Online (<http://way2drug.com/passonline/index.php>) and PkCSM ADMET (<https://biosig.lab.uq.edu.au/pkcsmdmet>) were utilized to estimate the biological activity and ADMET profile of the 2 compounds. The analysis was conducted by analyzing the similarities between the 2 substances' biological activity as well as other parameters.

Results and discussion

This research began by identifying the flowers of *Averrhoa bilimbi* L used in this study by the Herbarium Bogoriense (LIPI), Bogor. The results show that the flowers part of belimbing wuluh. The resulting macerate was followed by multilevel fractionation using a separating funnel with *n*-hexane, ethyl acetate, and *n*-butanol as solvents. The results of multilevel fractionations obtained successive yields, as shown in **Table 1**.

Table 1 The yield of *n*-hexane, ethyl acetate, and *n*-butanol extracts isolated from the flower of *Averrhoa bilimbi* L.

Extract	Flower Yield (%)
<i>n</i> -Hexane	18.84
Ethyl acetate	3.09
<i>n</i> -Butanol	18.64

Results of α -glucosidase inhibition activity

Antidiabetic activity test with α -glucosidase inhibition was carried out on 3 fractions of liquid-liquid partition results: *n*-hexane, ethyl acetate, and *n*-butanol. Each extract was made in 4 concentration variations, including 12.5; 25; 50; 100 μ g/mL. Quercetin is a positive control because quercetin can inhibit α -glucosidase activity, and because the α -glucosidase enzyme used in this study comes from *Saccharomyces cerevisiae*, using acarbose as a comparison will be less sensitive in inhibiting the α -glucosidase enzyme, this is because acarbose is more active in inhibiting α -glucosidase enzymes derived from mammals than those derived from bacteria and yeast [14].

The percentage of inhibition was measured using the % inhibition equation. The correlation between the rate of inhibition and the extract concentration was plotted, and the (IC₅₀) value was calculated through the regression equation. Antidiabetic activity is known from the IC₅₀ value. The IC₅₀ value is defined as the concentration of an inhibitor to inhibit 50 % of the activity of the α -glucosidase enzyme under test conditions, so the lower the IC₅₀ value indicates, the higher the antidiabetic activity of the extract (**Table 2**).

Table 2 The IC₅₀ values and inhibitory activity of each fraction separated from flower extract.

Extracts	Flower	
	IC ₅₀ (μ g/mL)	Antidiabetic activity
<i>n</i> -Hexane	89.84	Strong
Ethyl Acetate	52.88	Strong
<i>n</i> -Butanol	114.86	Moderate
Quercetin (control)	2.47	Very Strong

The ethyl acetate extract was the most potent extract to inhibit the α -glucosidase, followed by the *n*-hexane extract and *n*-butanol extract, which belonged to the moderate category. The differences in ethyl acetate, *n*-hexane, and *n*-butanol to inhibit α -glucosidase indicated differences in the antidiabetic activity of the 3 extracts. This difference can be mentioned due to differences in the active antidiabetic compounds contained in the extracts. The ethyl acetate fraction of belimbing wuluh has the highest antidiabetic activity compared to other extracts because it has the lowest IC₅₀ value. It is suspected that the active antidiabetic compounds from the flavonoid, terpenoid/steroid, and phenolic compounds contained in starfruit flowers are more soluble in ethyl acetate [15].

The previous study showed that the ethyl acetate extract of Pandan Wangi leaves (*Pandanus amaryllifolius* Roxb) contains terpenoid and steroid compounds that have potential as antidiabetic *in vitro* with an inhibitory power of 0.79 % at a concentration of 3.17 ppm [16]. Terpenoids/steroids can be found in the form of glycosides which have the potential for antidiabetic activity. This glycoside bond causes inhibition of α -glucosidase activity because this terpenoid/steroid glycoside structure is similar to the structure of the α -glucosidase substrate [17]. In addition to terpenoid/steroid compounds, phenolic compounds can also provide inhibition which is a reversible mixture, meaning that it does not have a similar structure to the substrate. Still, the inhibitor will bind to the free enzyme and the enzyme-substrate complex [18].

Several studies have proven the role of phenolic compounds, including *Averrhoa bilimbi* fruit polyphenols [8], *Psidium guajava* polyphenols, and *Syzygium cumini* [19] as inhibitors of antidiabetic activity. Changes in the structure of phenolic compounds also affect the ability to inhibit α -glucosidase. The gradual decrease in α -glucosidase inhibition from *trans-N*-(*p*-cumoryl) tyramine, *trans-N*-feruloyl tyramine from *cis-N*-feruloyl tyramine. These data show that methylation of the hydroxyl group on phenethyl cyanamide will weaken the ability of α -glucosidase inhibition. This is in line with the results of the previous research which was done by Zhou *et al.* [20] who suggested that the *cis* structure had a lower inhibitory ability against α -glucosidase compared to the *trans* structure.

Identification of compounds in the most active fractions with LC-MS

Identification of compounds with LC-MS was made to determine the peak areas, molecular weights, and possible structures of compounds present in the fractions. The most active fraction in inhibiting α -glucosidase activity was the ethyl acetate fraction. The ethyl acetate fraction was identified using LC-MS to obtain data from chromatograms and mass spectra. The chromatogram of this fraction is shown in **Figure 1** and compounds suspected to be identified at the peak of the spectrum are presented in **Table 3**. The mass spectrum of the ethyl acetate fraction is shown in **Figure 2**.

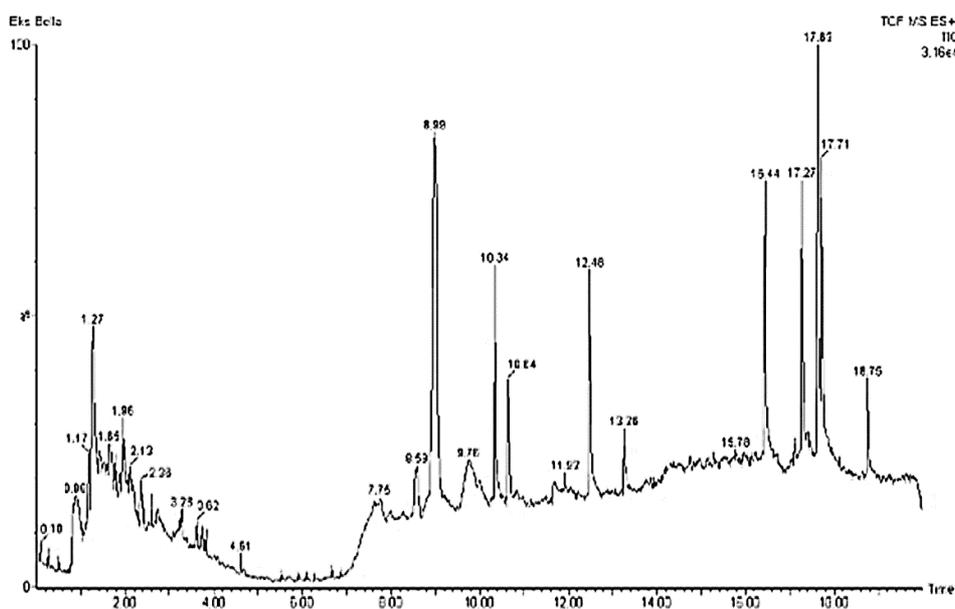
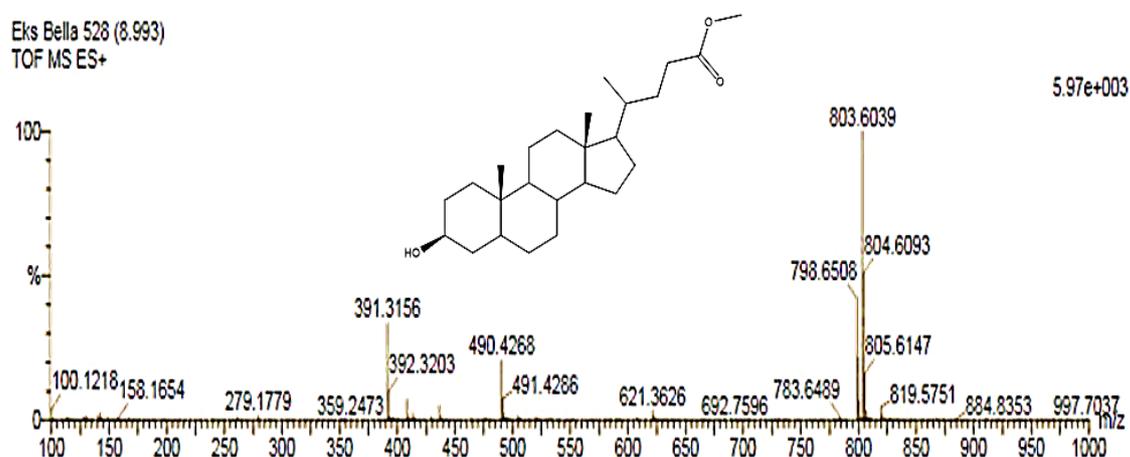


Figure 1 Chromatogram of the ethyl acetate fraction of belimbing wuluh flowers using LC-MS.

Table 3 Alleged compounds resulting from LC-MS of the ethyl acetate fraction of belimbing wuluh flowers-

No.	Compound	RT (Minute)	Chemical formula	Class
1.	<i>Isosakuranin</i>	1.173	C ₂₂ H ₂₄ O ₁₀	Flavonoid Glycosides
2.	<i>Asperulosidic acid</i>	1.275	C ₁₈ H ₂₄ O ₁₂	Terpenoids (Iridoids)
3.	<i>Umbelliferon</i>	2.125	C ₉ H ₆ O ₃	Phenol
4.	(±)- <i>lyoniresinol</i>	2.363	C ₂₂ H ₂₈ O ₈	Polyphenols
5.	<i>Chrysomycin B</i>	3.281	C ₂₇ H ₂₈ O ₉	Glycosides
6.	<i>Etil umbelliferone -3-karboksilat-β-D- glucopyranoside</i>	3.621	C ₁₈ H ₂₀ O ₁₀	Phenol Glycosides
7.	<i>β-Apopicropodophyllin</i>	3.706	C ₂₂ H ₂₀ O ₇	Phenols
8.	<i>Tautomycetin</i>	8.585	C ₃₃ H ₅₀ O ₁₀	Polyketide
9.	<i>Lithocholic acid</i>	8.993	C ₂₅ H ₄₂ O ₃	Steroids

The retention time of 8.993 is shown in the spectrum (**Figure 2**). There is a compound that is suspected of having antidiabetic activity, namely lithocholic acid (C₂₅H₄₂O₃) (**9**), with a molecular weight of [M⁺H]⁺ 391.3156 m/z. In a previous study, Lithocholic Acid (LCA) was modified with an exendin-4 derivative on a lysine residue to produce Lys²⁷-LCA-exendin 4 (LCA-M1). Then investigated the biological characteristics of diabetic rats *in vivo*, which showed that injection of LCA-M1 (10 nmol/kg) was very effective in controlling low blood glucose at 12 h (4.1 mM). LCA-M1 also showed potent activity in stimulating insulin with a value of 104.1 ng/mL/2 h. The lithocholic acid compound was found in the methanol extract of the leaves of the bitter melon (*Trichosanthes anguina* L.) in a retention time of 24.58 with an area of 1.15 % [21].

**Figure 2** The LCMS spectrum of the ethyl acetate fraction at a retention time of 8.993.

The retention time of 1.273 is shown in the spectrum (**Figure 3**). There is a compound that is suspected of having antidiabetic activity, namely asperulosidic Acid (C₁₈H₂₄O₁₂) (**2**), with a molecular weight of [M⁺H]⁺ 433.1440 m/z. The asperulosidic acid compound was found in the research of Milella *et al.* [22], which isolated *Arcytophyllum thymifolium* and produced new compounds from the coumarin, penylated flavanone, and terpenoid (iridoid) groups. These compounds were reported to have the potential to inhibit α-glucosidase and α-amylase. Asperulosidic acid in the terpenoid (iridoid) class showed the most vital activity as an α-amylase inhibitor with IC₅₀ = 69.4 μM and the roots of the noni plant (*Morinda citrifolia*) found an asperulosidic acid compound of 0.49 % of the dry root.

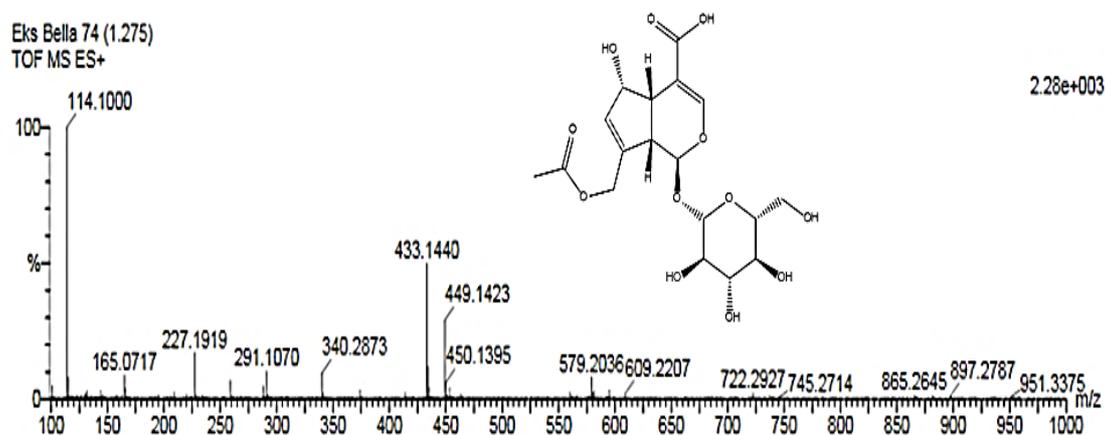


Figure 3 The LCMS spectrum of the ethyl acetate fraction at a retention time of 1.275.

The retention time of 2.125 is shown in the spectrum (**Figure 4**). There is a compound suspected of having antidiabetic activity, namely umbelliferone ($C_9H_6O_3$) (**3**), with a molecular weight of $[M^+H]^+$ 163.1460 m/z. Ramu *et al.* [23] showed that Umbelliferone and lupeol were identified from banana flowers' ethanol extract (*Musa sp var. Nanjangud rasa bale*). The umbelliferone compound was tested for the inhibitory effect of α -glucosidase *in vitro* to produce a strong effect with an IC_{50} 7.08 μ g/mL compared to quercetin with an IC_{50} of 9.68 μ g/mL. As a phenol group, umbelliferone compounds are also found in star fruit *Averrhoa bilimbi* L. extracts.

Inhibiting the activity of the α -glucosidase is 1 strategy that can be employed in the treatment of diabetes [24,25]. Miglitol consumption is currently the most popular treatment for diabetes mellitus and is considered to be one of the standard therapies for the disease [26]. In this study, computational predictions showed that 2 *Averrhoa bilimbi* flower compounds which have the highest RT value, specifically tautomycetin (-6.2 kcal/mol) and lithocholic acid (-10.0 kcal/mol), showed better binding affinity values than miglitol (-5.4 kcal/mol).

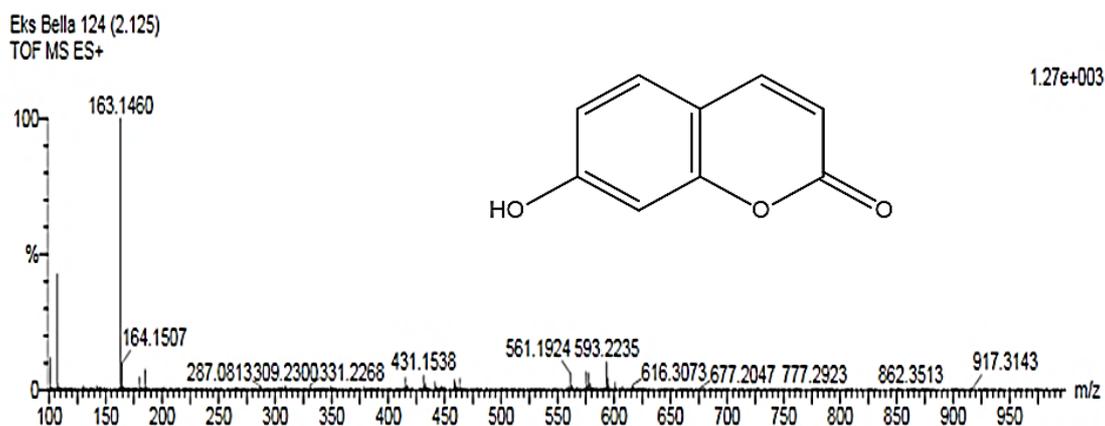


Figure 4 The LCMS spectrum of the ethyl acetate fraction at a retention time of 2.125.

This demonstrates that these drugs have favorable prospects for advancement as α -glucosidase inhibitor options in the management of diabetes. According to the findings of other research, the ability to interact with the target protein improves in direct proportion to the binding affinity value's negative value [27]. In addition, a three-dimensional visualization of the protein-ligand complex reveals that the ligands occupy a location that is analogous to that of the binding site (**Figure 5**). Due to the fact that this demonstrates that tautomycetin and lithocholic acid are rivals for the control drug miglitol, it is redundant that it will be further developed as an antidiabetic medication.

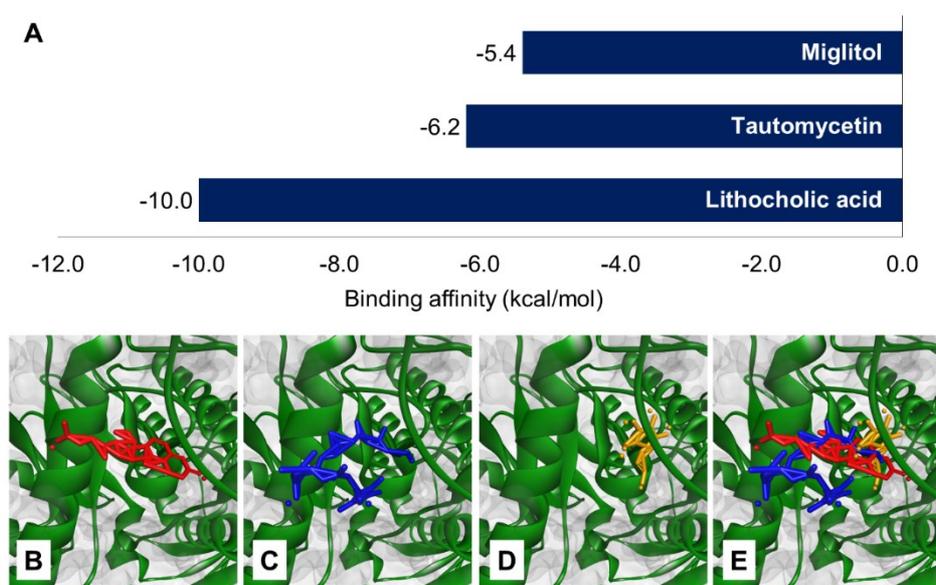


Figure 5 Molecular docking of tautomycetin and lithocholic acid against the α -glucosidase. A) Binding affinity values, B) 3D visualization of lithocholic acid - target protein complex, C) Tautomycetin - target protein complex, D) Miglitol - target protein complex, and E) All ligands - target protein complex.

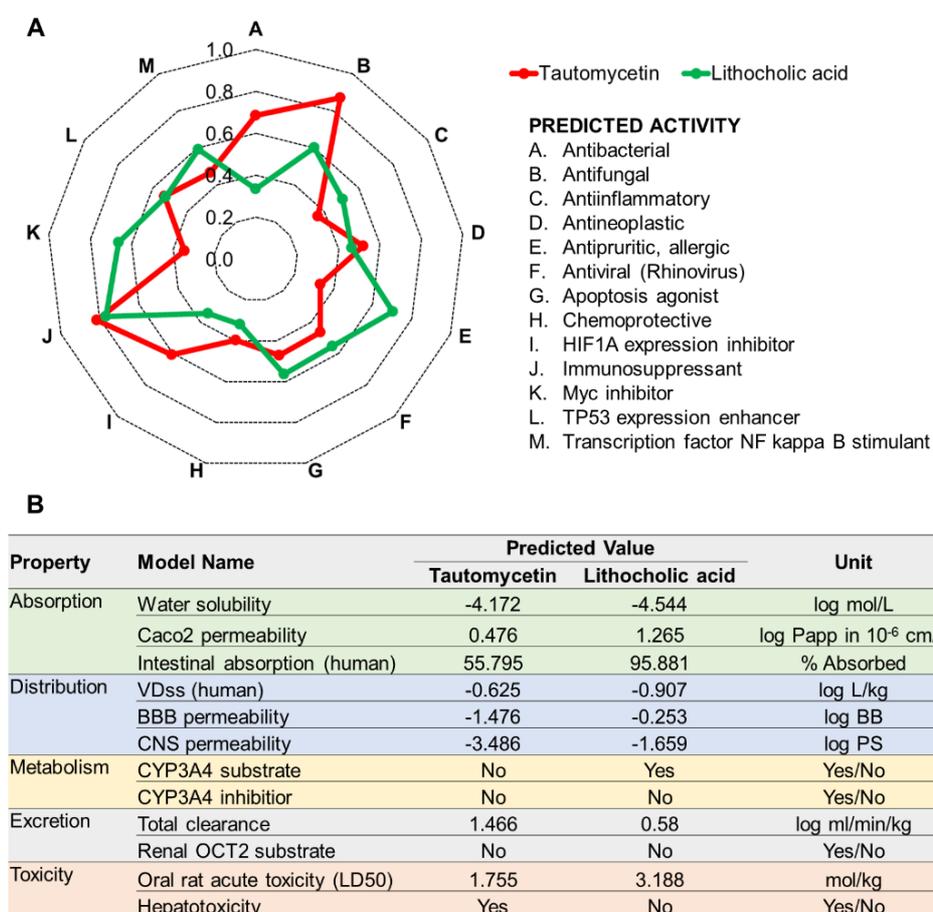


Figure 6 a) Tautomycetin and lithocholic acid biological activity prediction, and B) absorption, distribution, metabolism, excretion, and toxicity parameters.

Additionally, a wide range of biological effects, including antibacterial, antifungal, antiinflammatory, antineoplastic, antipruritic, allergic, antiviral (Rhinovirus), apoptotic agonist, chemoprotective, HIF1A expression inhibitor, immunosuppressant, Myc inhibitor, TP53 expression enhancer, and transcription factor NF kappa B stimulant, was found based on the prediction of the biological activity of the tautomycetin and lithocholic acid. Furthermore, the drug development criteria including the absorption, distribution, metabolism, excretion, and toxicity was provided (**Figure 6**). Despite the fact that these predictions did not result in the discovery of any direct antidiabetic activity, other activities in the form of an anti-inflammatory and immunosuppressant could be indirectly associated to the regulation of diabetes mellitus.

Inflammation, as shown by a significant number of studies, appears to play a role in the development of diabetes mellitus [28]. It has been revealed that chronic inflammation is a factor in the development of long-term complications associated with diabetes, such as retinopathy, cardiovascular disease, non-alcoholic fatty liver disease, and nephropathy. Chronic inflammation may also be the driving force behind the correlation between type 2 diabetes and other disorder conditions, such as Alzheimer's disease, polycystic ovarian syndrome, and rheumatoid arthritis [29]. Overall, it has been demonstrated that the *Averrhoa bilimbi* plant possesses antidiabetic properties, including the ability to treat hyperglycemia, promote glucose metabolism through glycolysis, and limit hepatic endogenous glucose synthesis through gluconeogenesis [8,9].

Conclusions

The extract of the ethyl acetate fraction of belimbing wuluh flowers was the most potent in terms of antidiabetic activity *in vitro* through inhibition of α -glucosidase with an IC_{50} value of 52.88 $\mu\text{g/mL}$, followed by *n*-hexane extract (89.84 $\mu\text{g/mL}$) and *n*-butanol (114.86 $\mu\text{g/mL}$). The active compounds extracted from the ethyl acetate fraction, which have the potential as antidiabetics from the flowers of starfruit (*Averrhoa bilimbi* L.), were determined by the LCMS method with the highest RT values to be lithocholic acid, tautomycetin, and umbelliferone. The conclusion that can be drawn from the results of the *in-silico* approach is that the lithocholic acid and tautomycetin compounds show a better bond affinity value than umbelliferone. Although this prediction did not result in the discovery of direct antidiabetic activity, other activities, such as anti-inflammatory and immunosuppressive, may be related to diabetes mellitus self-regulation so that the description of new drug discovery can be addressed not only as an antidiabetic but other activity as well.

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