

Antidiabetic Activity of *Averrhoa bilimbi* L. Fruit Extracts and the Identification of Active Compounds Using LC-MS and *In silico* Methods

Diana Widiastuti^{1*}, Siska Elisahbet Sinaga², Siti Warnasih¹, Yulian Syahputri¹, Nurjanah Bella Saputri¹, Sustiprijatno³ and Wira Eka Putra⁴.

1. Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Jl. Pakuan, RT.02/RW.06, Tegallega, Bogor 16129, Indonesia
2. Department of Nutritional Science, Universitas Widya Nusantara, Jl. Tondo, Palu 94148, Indonesia.
3. Research Center for Plant Conservation, Botanic Gardens, and Forestry, National Research and Innovation Agency, Jl. Raya Jakarta-Bogor Km 46, Cibinong-Bogor, West Java 16911, Indonesia
4. Biotechnology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jl. Cakrawala, Malang 65145, Indonesia

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*Corresponding author
Diana Widiastuti

Email:
dianawidi25@unpak.ac.id

ABSTRACT

Diabetes mellitus patients are typically in need of drugs with minimal long-term side effects, leading to an increasing interest in herbal medicine sourced from medicinal plants. Among the available medicinal plants, the fruit of *Averrhoa bilimbi* L., commonly known as belimbing wuluh, has been recognized for its therapeutic potential and is often used as an alternative medicine for diabetes mellitus. Therefore, this study aims to determine antidiabetic activity of the active fraction of *A. bilimbi* L. fruit using *in vitro* and *in silico* methods and to determine the active compounds contained in the fruit fraction (*A. bilimbi* L.) based on Liquid Chromatography Mass Spectrometer (LC-MS). LC-MS analysis results showed the presence of 16 organic compounds in the *n*-hexane fraction, which had the highest IC₅₀ (7.03 µg/mL) antidiabetic activity compared to the ethyl acetate (102.77 µg/mL) and *n*-butanol (181.65 µg/mL) fractions. *In silico* molecular docking, analysis was performed for all identified compounds to further support antidiabetic characteristics. Computational predictions showed that 2 *A. bilimbi* fruit compounds with the greatest RT value, namely 2,3,4,5,6-pentaphenylbenzazocine (-16.0 kcal/mol) and Thraustochytrioside A (-6.2 kcal/mol), exhibited higher binding affinity values compared to miglitol (-5.4 kcal/mol). This showed that both compounds had the potential to be developed as α -glucosidase inhibitor alternatives for treating diabetic disorders.

Keywords: Antidiabetic activity; *Averrhoa bilimbi* L.; *in vitro*; *in silico*, LC-MS.

INTRODUCTION

Several studies have shown that diabetes mellitus patients often require lifelong treatment to mitigate and prevent the progression of symptoms toward more serious outcomes. However, the drugs typically used in the management of the condition have the potential to cause side effects due to long-term use. According to the International Diabetes Federation in 2019, approximately 463 million individuals, constituting 9.3% of the global population aged 20-79 years, were affected by the condition (Karuranga *et al.*, 2022). In Indonesia, Riskesdas 2018 data showed a

diabetes mellitus prevalence of 2% among individuals aged ≥ 15 years who were diagnosed by healthcare professionals, marking an increase from 1.5% in 2013. The prevalence derived from blood sugar assessment in 2018 also amounted to 8.5%, showing an increase from 6.9% in 2013 (Kulzer *et al.*, 2021; Pradono *et al.*, 2020; Saeedi *et al.*, 2019; Shurrab & Arafa, 2020).

The management of diabetes mellitus comprises various methods, including the use of oral antidiabetic drugs, insulin injections, and the inhibition of α -glucosidase enzyme (Abbas *et al.*, 2019). Several studies have reported that the

inhibition of α -glucosidase enzyme serves as an alternative therapy for type 2 diabetes mellitus, as it does not augment insulin secretion. In addition to the conventional treatment methods, there is a growing interest in exploring alternative antidiabetic options sourced from medicinal plants. *Averrhoa bilimbi* L, commonly known as belimbing wuluh, represents one such plant with promising therapeutic potential. This tropical species is valued for its diverse biological contribution, spanning the fruit, flower, leaf, stem, and root parts. *A. bilimbi* has also showed efficacy in addressing various health concerns, including asthma, diabetes, cough, fever, and throat soreness (Khoo *et al.*, 2016). In line with previous studies, belimbing wuluh is a promising candidate for use as a functional food raw material due to its rich content of beneficial active compounds, including antioxidants, hypoallergenic, antibacterial, antidiabetic, anti-larvicidal, antifungal, and anti-inflammatory components (Octaviani & Fadila, 2018). A comprehensive review study by Lakmal showed that the ethanol extract of its leaves could reduce blood glucose in streptozotocin (STZ)-induced diabetic rats at a dose of 125 mg/kg BW. Further supporting its potential antidiabetic effects, Setyawan *et al.*, 2021 showed that administration of belimbing juice at a dose of 2mL/200g BW could affect blood glucose levels in rats experiencing hyperglycemia (Muthu *et al.*, 2016).

A phytochemical study by Kurup *et al.* (Kurup & Mini, 2017) showed the presence of various bioactive components in belimbing extract, including phenolic compounds, alkaloids, flavonoids, saponins, terpenoids, and tannins. In addition, its ethanol extract was found to contain flavonoid compounds and exhibited antidiabetic activity (Setyawan *et al.*, 2021). Despite this potential, the fruit parts of belimbing wuluh have received limited attention and have been primarily used as antidiabetic agent in previous reports (Kurup & Mini, 2017). The study conducted by Widiastuti *et al.* (2023) presented significant results regarding the ethyl acetate extract derived from belimbing flowers. The results showed the presence of several compounds with good antidiabetic activity based on LC-MS analysis through both *in silico* and *in vivo* methods. Therefore, this study aims to identify the active compounds of belimbing fruit using LC-MS analysis and their antidiabetic activity using *in vitro* and *in silico* methods.

MATERIALS AND METHODS

Tools and Materials

The tools used included a blender, glass bottles, vials, funnels, Whatman 41 filter paper, round bottom flasks, rotary evaporator vacuum (BUCHI), analytical balances (OHAUS), glassware, separating funnels, separating funnel supports, UV-Vis spectrophotometer (Thermo Fisher Scientific), Brand® cuvette (Sigma-Aldrich), refrigerator, micropipette, incubator, pH meter, water bath, desiccator, millipore filter, micro syringe, and Liquid Chromatography Mass Spectrometry (LC-MS, GeneCraft Labs). Meanwhile, the materials used included the fruit of *A. bilimbi* L. obtained from Parungpanjang Village, Parungpanjang District, Bogor Regency, 96% ethanol (technical, Smart Lab), *n*-hexane (technical, Smart Lab), ethyl acetate (technical, Smart Lab), *n*-butanol (technical, Smart Lab), distilled water (Agarindo Biological Company), α -glucosidase (Sigma-Aldrich), quercetin (Sigma-Aldrich), dimethyl sulfoxide (DMSO) (Sigma-Aldrich), phosphate buffer, *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG)(Merck), sodium carbonate (Merck), acetonitrile (gradient grade, Merck), and formic acid (Reagent grade, Sigma-Aldrich).

Extraction and Fractionation

Belimbing wuluh fruit obtained from Parungpanjang, Bogor (500 g) was macerated using 96% technical ethanol solvent with a 1:1(v/w) ratio for 3 x 24 hours at room temperature and concentrated using a rotary evaporator at 47°C. Subsequently, 10 g of the ethanol extracts obtained were fractionated using *n*-hexane, ethyl acetate, and *n*-butanol solvents. The ethanol extracts were dissolved in water at a ratio of 1:10 (v/w) and then partitioned with *n*-hexane with a volume ratio of 1:1(v/b). The same process was used to yield ethyl acetate and *n*-butanol extracts. The *n*-hexane, ethyl acetate, and *n*-butanol phases were evaporated using a rotary vacuum evaporator with a temperature of 40°C for the *n*-hexane and ethyl acetate fractions, while *n*-butanol had 55°C (Sinaga, *et al.*, 2022; Widiastuti *et al.*, 2023).

α -glucosidase Enzyme Inhibition Test

α -glucosidase enzyme inhibition test referred to the method used by Barajas *et al.* 2020 and was carried out on *n*-hexane, ethyl acetate, and *n*-butanol fraction extracts. The mother liquor was prepared with DMSO using 4 mg of each extract fraction dissolved in 100 μ l DMSO.

Furthermore, dilution was carried out using DMSO with various concentrations, and sample testing was carried out with and without enzymes.

A total of 10 μ l of the sample was added into a test tube, followed by 250 μ l p-NPG 5 mM and 495 μ l phosphate buffer pH 7, 0.1 M, with preincubation for 5 min at 37°C. Furthermore, 250 μ l of α -glucosidase enzyme solution was added, and in the absence of the enzyme, it was replaced with 250 μ l of phosphate buffer pH 7, followed by incubation for 15 minutes at 37°C. The reaction was stopped by adding 1 ml of 0.2 M Na₂CO₃, and the test was carried out with 4 different concentrations. Enzyme activity was measured based on the amount of *p*-nitrophenol formed in a UV-Vis spectrophotometer at a wavelength of 400 nm (Magaña-Barajas *et al.*, 2021).

The blank was tested with and without enzymes from a mixture of DMSO solution, phosphate buffer, *p*-NPG, and Na₂CO₃ without adding extracts using the same process as sample testing. Comparison testing as a positive control using quercetin was carried out with and without enzymes of various concentrations, namely 1, 2.5, 5, and 10 μ g/ml. The percentage of inhibition was then measured using the following equation 1:

$$\% \text{ Inhibition} = \frac{(B - S)}{B} \times 100 \quad (1)$$

B is the absorbance of the blank; S is the absorbance of extract (the difference between the absorbance with and without the enzyme).

The IC₅₀ value was obtained from the linear regression equation $y = a + bx$. The x-axis was the sample's concentration, and the y-axis was the % inhibition. Furthermore, the IC₅₀ value could be calculated using the following equation (2):

$$IC_{50} = \frac{(50 - a)}{b} \quad (2)$$

a is the intercept; b is the slope.

The Identification of Active Compounds by LC-MS

A total of 1.4 mg of the *n*-hexane fraction of belimbing fruit was dissolved with nitrile acetone and then filtered using a millipore filter. The sample filtrate (0.5 μ l) was then injected into the instrument system. Furthermore, LC-MS analysis was performed with an Electro-spray Ionization (ESI) source connected to a Quadrupole Time-of-Flight (QTOF) mass spectrometer with positive ionization mode. The ESI parameters included the

capillary temperature of 100°C, fogging gas of 600L/h, source voltage of 1.5kV, sample cone voltage of 38 V, Desolvation T: 300°C, Source T: 100°C, Desolvation gas: of 500 L/h. Full scan mode from 100-1000 m/z was carried out with a source temperature of 110 °C. The eluents were H₂O 0.1% formic acid (A) and nitrile acetone (B), and the elution system was performed with gradient elution at 0-1 minute. The ratio of solvent was 70% A and 30% solvent B at 6-18 min, as well as 5% solvent A and 95% B %. At 19-20 min, the solvent was linear gradient elution 70% A and 30% solvent B. Furthermore, data processing was performed using MassLynx software.

Molecular Docking, Biological Activity, and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) Prediction

Using the previously described method, a molecular docked analysis was carried out in study on 2 substances with the highest RT values, namely 2,3,4,5,6-pentaphenylbenzazocine and Thraustochytrioside A and alpha-glucosidase as protein targets (Hidayatullah *et al.*, 2022, 2023). The 2D structures of Thraustochytrioside A (CID. 21593887) and 2,3,4,5,6-pentaphenylbenzazocine (CID. 12206448) were found in the PubChem database (<https://pubchem.ncbi.nih.gov/>). Furthermore, antidiabetic drug Miglitol (CID: 441314) was used in this study as a control (Jokiaho *et al.*, 2022; Kashtoh & Baek, 2022). Furthermore, the UniProt database was used to retrieve the alpha-glucosidase protein sequence (P10253), and the SWISS-MODEL (<https://expasy.org/swissmodel>) was used to carry out homology modeling. For molecular docking, the program PyRx (<https://pyrx.sourceforge.io>) was used. PkCSM ADMET (<https://biosig.lab.uq.edu.au/pkcsmadmet>) and Pass Online (<http://way2drug.com/passonline/index.php>) were used to calculate the biological activity and ADMET profile of the 2 drugs. The comparison was made by comparing the biological activities of the 2 drugs and other factors.

RESULTS AND DISCUSSION

This study began by identifying the fruit of *A. bilimbi* L used by the Herbarium Bogoriense (LIPI), Bogor. The results showed that the fruit was part of the starfruit plant. The macerate obtained was followed by multilevel fractionation using a separating funnel with *n*-hexane, ethyl acetate, and *n*-butanol as solvents. The results of multilevel fractionation using *n*-hexane, ethyl acetate, and *n*-

butanol solvents obtained successive yields (Table I).

Table I. The yield of all extracts isolated from the fruit of *A. bilimbi* L.

Extract	Fruit Yield (%)
<i>n</i> -hexane	3.34
Ethyl acetate	6.51
<i>n</i> -butanol	15.97

The yield results showed the presence of active compounds extracted from these fractions. As Mayanti reported, the high active compounds present in a sample was showed by the high yield produced (Mayanti *et al.*, 2022). Differences in extract levels also showed that the content of extractive substances differed between various solvents and from the same sample (Sinaga *et al.*, 2023).

Results of α -Glucosidase Enzyme Inhibition Activity

Antidiabetic activity test with α -glucosidase enzyme inhibition was carried out on 3 fractions of liquid-liquid partition results, including *n*-hexane, ethyl acetate, and *n*-butanol from fruit extract. Each fraction was made in 4 concentration variations, namely 12.5, 25, 50, and 100 μ g/mL. Furthermore, quercetin was a positive control because it could inhibit α -glucosidase activity. α -glucosidase used in this study was obtained from *Saccharomyces cerevisiae* because using acarbose as a comparison was less sensitive in inhibiting α -glucosidase enzyme. This was because acarbose was more active in inhibiting α -glucosidase derived from mammals than those from bacteria and yeast. In the IC₅₀ values and inhibitory activity of all fractions isolated from the fruit extracts were presented (Table II).

Table II. The IC₅₀ values and inhibitory activity of all fractions isolated from the fruit extracts.

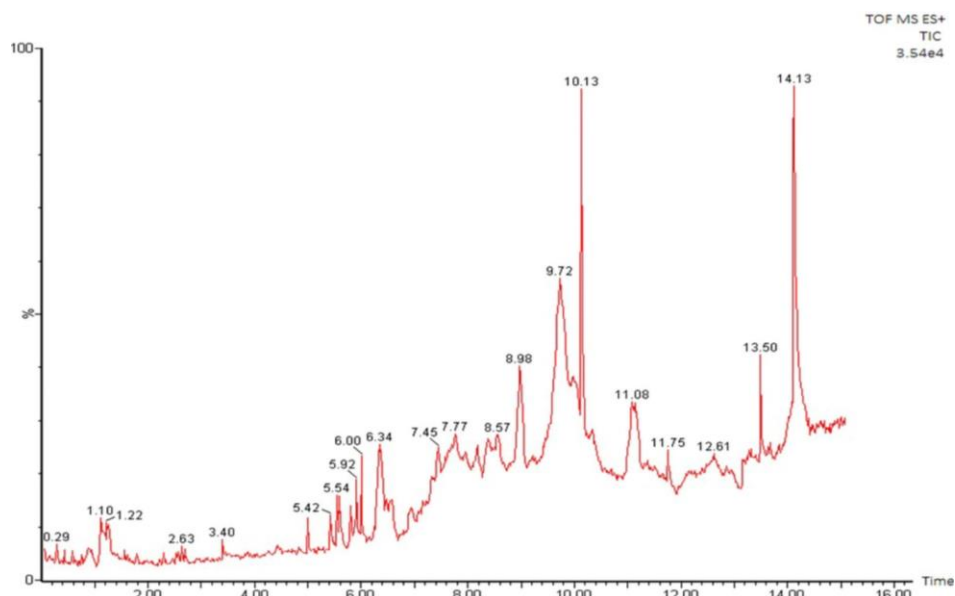
Extracts	Fruit	
	IC ₅₀ (μ g/mL)	Antidiabetic activity
<i>n</i> -Hexane	7.03	Very strong
Ethyl Acetate	102.77	Moderate
<i>n</i> -Butanol	181.65	Weak
Quercetin (control)	2.47	Very strong

Antidiabetic activity from the Belimbing Wuluh fraction in Table 2 showed that the IC₅₀ value for each fraction was different. Extract fractions with an IC₅₀ value of \leq 50 μ g/mL, 50-100 μ g/mL, 101-150 μ g/mL, and \geq 151 μ g/mL was classified as very strong, strong, moderate, and weak, respectively (Culas *et al.*, 2021).

The results showed that the IC₅₀ value for each fraction was different, and antidiabetic activity was also distinct for the 3 fractions. The IC₅₀ values for the *n*-hexane, ethyl acetate, and *n*-butanol fractions were 7.03 μ g/mL, 102.77 μ g/mL, and 181.65 μ g/mL, respectively. The IC₅₀ value of each fraction was compared with the quercetin IC₅₀ value, and the results showed that quercetin had a very active value of 2.47 μ g/mL. Compared with the 3 starfruit extract fractions, the *n*-hexane fraction was slightly different from quercetin, which had a very active inhibitory ability compared to the ethyl acetate and *n*-butanol fractions. The results showed that the levels and physical forms of the 3 types of fractions were different, showing the presence of variations in the type of solvents and components (Erugur *et al.*, 2019; Sinaga, *et al.*, 2022). The active compound type exhibited greater prominence in the *n*-hexane fraction, which showed an affinity for secondary metabolite compounds characterized by the highest solubility in non-polar environments, including terpenoids and steroids. (Artanti *et al.*, 2019).

The Identification of Compounds in the Most Active Fractions with LC-MS

The identification of compounds with LC-MS was carried out 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 *n*-hexane fraction isolated from the fruit of Belimbing. The *n*-hexane fraction was identified using LC-MS to obtain chromatograms and mass spectra data, as shown in Figure 1. Furthermore, the identification results suggested that there were 16 compounds in *A. bilimbi*. These identified compounds had different activities against several diseases, such as diabetes mellitus (Table III). The peak chromatogram at retention time was 9.72, 8.98, 7.77, and 3.40, showing the presence of compounds with antidiabetic activity. Based on LC-MS results, compound with the highest intensity in antidiabetic activity was tri(1-adamantyl) methane, showing its high ability to inhibit enzymes or prevent increased glucose levels.

Figure 1. Chromatogram of *n*-hexane fraction extract by LC-MSTable III. The results of component analysis using LC-MS on the *n*-hexane fraction of the fruit extracts.

RT	M/Z	Compound	class
0.289	183.0141	Dihydroxytartaric acid	Ascorbic acid
1.105	173.0387	3-Dehydroshikimat	Phenol
1.224	216.9984	Malonate Carbon	Phenolic
3.400	185.1406	Chamazulene	Monoterpene
5.423	357.2601	Lagochilin	Terpene
5.542	307.2484	2,5,8,11-Tetramethyl-3,6,9,12-tetraoxahexadecan-1-ol	Terpene
5.916	279.2688	2-pentadecylfuran	Terpene
6.001	295.2546	Methyl Linoleate	Sesquiterpene
6.341	561.5414	1,1'-Methylenebis (4-tetradecylbenzene)	Phenolic
7.446	311.2950	Hexadecyl methacrylate	terpenoids
7.769	593.3267	19-Tr-andrographolid	Terpene
8.534	607.3482	Tautomycetin	Polyketide
9.976	391.3236	Methyl lithocholate	Steroids
9.724	419.3680	tri(1-adamantyl)methane	cycloalkane
10.131	710.5571	Thraustochytroside A	Alkaloids
14.134	536.2362	2,3,4,5,6-pentaphenylbenzazocine	Alkaloids

Antidiabetic activity of the tri(1-adamantyl) methane compound in vitro inhibited the aldose reductase and aldehyde reductase enzymes with IC₅₀ values ranging from 1.37 to 38.4, showing active antidiabetic activity. In addition, in vivo, diabetic rats induced by streptozotocin at a dose of 20 mg/kg showed a hypoglycemic effect and succeeded in reducing blood glucose levels (Al-Abdullah *et al.*, 2015). Compound methyl lithocholate was reported to exhibit similar effects on diabetic rats at a dose of 15 nmol/kg per day for 4

weeks by reducing blood glucose levels (Chae *et al.*, 2010).

The 19-Tr-andrographolide compound had antidiabetic activity in a study of streptozotocin-diabetic rats fed high fructose fat, showing a hypoglycemic effect that reduced blood glucose levels (Nugroho *et al.*, 2012). The chamazulene compound also had similar activity in streptozotocin rats at 0.3 mg/kg, which lasted ±12 h and possessed antihyperglycemic solid antidiabetic function (Bakun *et al.*, 2021).

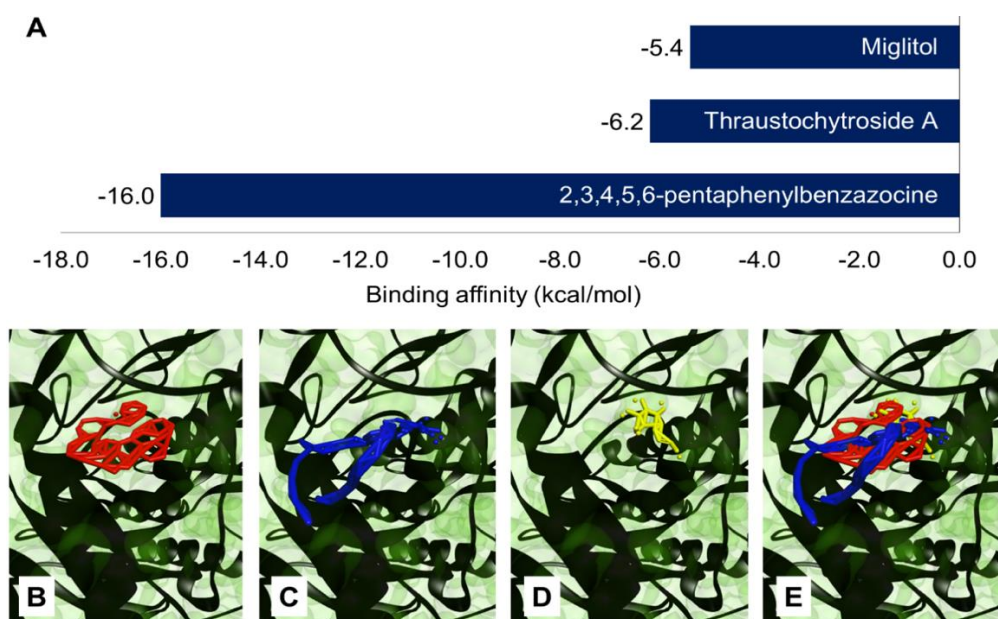


Figure 2. Molecular docking of 2,3,4,5,6-pentaphenylbenzazocine and Thraustochytrside A against the α -glucosidase. A). Binding affinity values, B). 3D visualization of 2,3,4,5,6-pentaphenylbenzazocine– target protein complex, C). Thraustochytrside A – target protein complex, D). Miglitol – target protein complex, and E). All ligands – target protein complexes.

The results of identifying the components of the *n*-hexane fraction extract compounds in *A. bilimbi* L (Table III). The *n*-hexane fraction of belimbing showed the presence of 16 secondary metabolites, including terpenoids, monoterpenes, sesquiterpenes, diterpenoids, steroids, alkaloids, phenolics, and polyketides. The components of these compounds had interesting bioactivities, such as antibacterial, antioxidant, anticancer, and antiviral.

A significant method that could be used in the management of diabetic patients was known as the inhibition of the alpha-glucosidase enzyme's activity (Bhuyan *et al.*, 2022; Hedrington & Davis, 2019). Consumption of miglitol had become the most prevalent treatment for diabetes mellitus and was regarded as one of the standard medications for the condition (Khwaja & Arunagirinathan, 2021; Wang *et al.*, 2020). Computational predictions in this investigation showed that 2 *A. bilimbi* fruit compounds with the greatest RT value, namely 2,3,4,5,6-pentaphenylbenzazocine (-16.0 kcal/mol) and Thraustochytrside A (-6.2 kcal/mol), exhibited higher binding affinity values compared to miglitol (-5.4 kcal/mol). This showed

that both compounds had potential to be developed as α -glucosidase inhibitor alternatives for treating diabetic disorders. According to previous studies, the capacity to interact with the target protein increased in direct proportion to the negative value of the binding affinity (Xue *et al.*, 2022) (Figure 2).

A three-dimensional image of the protein-ligand complex showed that the ligands occupied a position comparable to the binding site (Figure 2). Since this showed that 2,3,4,5,6-pentaphenylbenzazocine and Thraustochytrside A were competitors for the control drug miglitol, their continued development as antidiabetic treatments were highly probable. In addition, various biological effects, such as beta-adrenergic receptor kinase inhibitor, glucan endo-1,3-beta-D-glucosidase inhibitor, G-protein-coupled receptor kinase inhibitor, immunosuppressant, maltose-transporting ATPase inhibitor, mannose isomerase inhibitor, sugar-phosphatase inhibitor, and TP53 expression enhancer, were found. The drug development criteria were also presented, including absorption, distribution, metabolism, excretion, and toxicity (Figure 3).

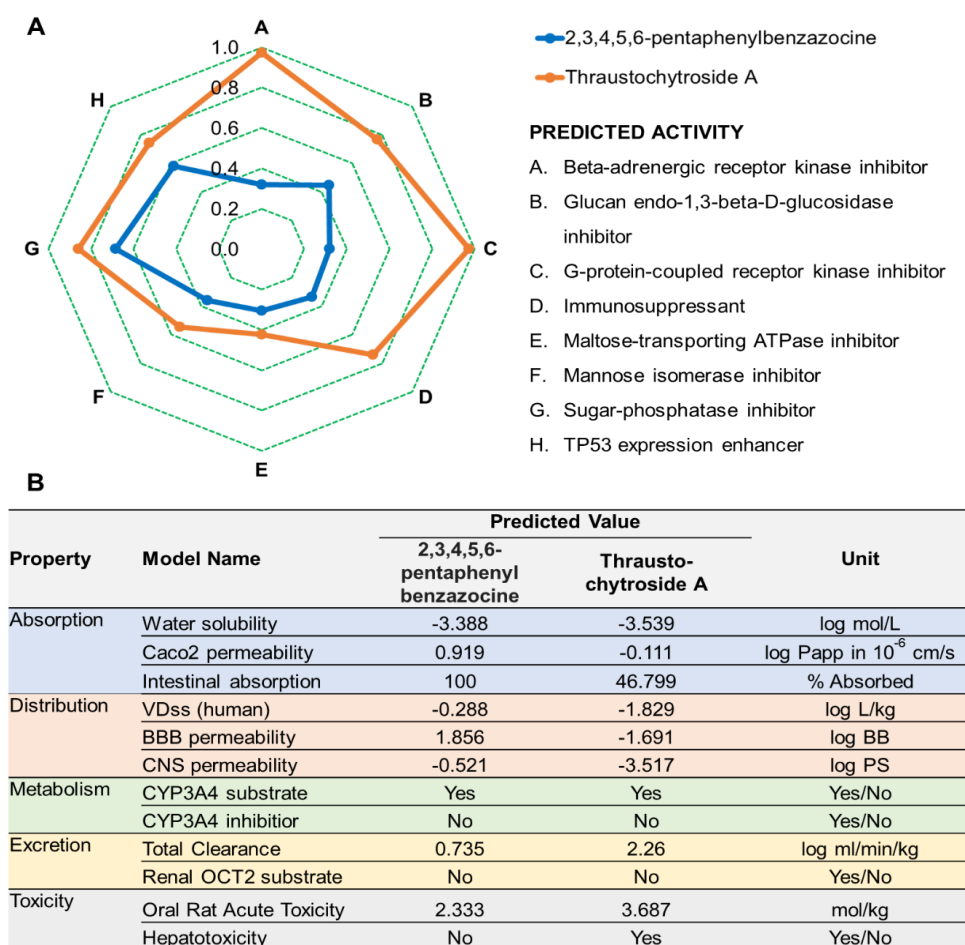


Figure 3. 2,3,4,5,6-pentaphenylbenzazocine and Thraustochytride A biological activity prediction (A), absorption, distribution, metabolism, excretion, and toxicity parameters (B).

According to the data, 2,3,4,5,6-pentaphenylbenzazocine had favourable absorption properties compared to Thraustochytride A. The results also showed that 2,3,4,5,6-pentaphenylbenzazocine had a better chance of passing through the blood-brain barrier and central nervous system (log BB value > 0.3 and log PS > -2). In addition, both compounds showed the positive CYP3A4 substrate, which suggested their significant role in metabolism. For the excretion category, Thraustochytride A had fewer half-life to be eliminated by the liver as excretion system. 2,3,4,5,6-pentaphenylbenzazocine was considered to have safer properties compared to Thraustochytride A with hepatotoxicity properties (Pires *et al.*, 2015) Several activities, including beta-adrenergic receptor kinase, glucan endo-1,3-beta-D-glucosidase, maltose-transporting ATPase, mannose isomerase, and sugar-

phosphatase inhibitors, were shown to be associated with glucose control in the body. These results suggested that 2,3,4,5,6-pentaphenylbenzazocine and Thraustochytride A had a high potential for development as antidiabetic drugs through alpha-glucosidase inhibition. According to previous data, *Averrhoa carambola* L. root extracts significantly reduced serum levels of blood sugar, total cholesterol, triglycerides, and free fatty acids while increasing the amount of serum insulin (Xu *et al.*, 2014). *A. bilimbi ethanolic leaves extract* had been shown to stimulate brown fat differentiation, which was an important step in the fight against diet-induced obesity (Lau *et al.*, 2019).

CONCLUSION

In conclusion, the IC₅₀ values obtained for the *n*-hexane fraction, ethyl acetate fraction, and *n*-

butanol fraction of *A. bilimbi* L. showed varying degrees of antidiabetic activity, with values of 7.03 µg/mL, 102.77 µg/mL, and 181.65 µg/mL, respectively. The *n*-hexane fraction exhibited high antidiabetic activity when compared to the ethyl acetate and *n*-butanol fractions. Furthermore, the use of LC-MS analysis in the examination of the *n*-hexane fraction of *A. bilimbi* led to the identification of 16 distinct compounds. Computational predictions conducted in this study showed significant results, particularly in terms of the retention time (RT) values. The results showed that 2,3,4,5,6-pentaphenylbenzazocine had an RT value of -16.0 kcal/mol, while -6.2 kcal/mol was recorded for Thraustochytride A. These results suggested that both compounds had potential to be developed as alpha-glucosidase inhibitors, which could be explored further for the treatment of diabetic conditions. Future studies were advised to focus on the isolation and comprehensive characterization of *A. bilimbi* extracts. This was to identify and isolate pure compounds with antidiabetic activity, as well as explore their potential bioactivities beyond diabetes management. The results were expected to provide in-depth understanding of the therapeutic potential of starfruit compounds and their applications in addressing various health-related concerns.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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