Jurnal Jamu Indonesia (2026) 11(4): 317–327
https://jamu-journal.ipb.ac.id/index.php/JJI/index

ISSN 2407-7178
eISSN 2407-7763

Research Article
Doi: https://doi.org/10.29244/jji.v11i4.535

LC-HRMS Metabolite Profiling, Antioxidant, and α-Glucosidase Inhibitory Activities of *Averrhoa bilimbi* L. Fruit Extracts by Maceration and Ultrasound-Assisted Extraction

Profil Metabolit LC-HRMS, Aktivitas Antioksidan, dan Penghambatan α-Glukosidase Ekstrak Buah *Averrhoa bilimbi* L. Hasil Maserasi dan Ekstraksi Berbantuan Ultrasonik

Lilik Sulastri*, Siti Nurul Habibah, Ema Hermawati, Rikkit

Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Pakuan University, Bogor 16143, West Java, Indonesia

ARTICLE INFO

Article history
Received on: 2026-06-10
Revised on: 2026-08-04
Accepted on: 2026-08-04

Keyword:
α-Glucosidase inhibition;
Antioxidant activity;
Averrhoa bilimbi;
LC-HRMS;
Ultrasound-assisted extraction

Kata kunci:
Aktivitas antioksidan;
Averrhoa bilimbi;
Ekstraksi berbantuan ultrasonik;
LC-HRMS;
Penghambatan α-glukosidase

ABSTRACT

Bilimbi fruit (*Averrhoa bilimbi* L.) contains bioactive compounds with antioxidant and α-glucosidase inhibitory potential. This study compared 70% ethanolic extracts prepared by maceration and ultrasound-assisted extraction (UAE) in terms of yield, total phenolic content (TPC), total flavonoid content (TFC), DPPH radical-scavenging activity, α-glucosidase inhibition, and LC-HRMS metabolite profiles. Maceration produced a higher yield than UAE (16.23% vs. 11.16%), whereas TPC and TFC were comparable. UAE showed lower DPPH IC_{50} (37.06 ± 0.27 vs. 51.59 ± 0.21 µg/mL) and α-glucosidase IC_{50} (23.94 vs. 33.45 µg/mL) than maceration. LC-HRMS yielded 24 tentative or probable metabolite annotations, of which seven were selected for focused interpretation. Because each extraction method was performed once and analytical measurements were technical replicates, between-method differences are descriptive. LC-HRMS findings were qualitative and do not establish quantitative enrichment or compound-specific causality.

ABSTRAK

Buah bilimbi (*Averrhoa bilimbi* L.) mengandung senyawa bioaktif yang berpotensi memiliki aktivitas antioksidan dan penghambatan α-glukosidase. Penelitian ini membandingkan ekstrak etanol 70% yang diperoleh melalui maserasi dan ekstraksi berbantuan ultrasonik (UAE) berdasarkan rendemen, kadar fenolik total, kadar flavonoid total, aktivitas penangkapan radikal DPPH, penghambatan α-glukosidase, dan profil metabolit LC-HRMS. Maserasi menghasilkan rendemen lebih tinggi daripada UAE (16,23% vs. 11,16%), sedangkan kadar fenolik dan flavonoid total relatif sebanding. UAE menunjukkan IC_{50} DPPH lebih rendah (37,06 ± 0,27 vs. 51,59 ± 0,21 µg/mL) dan IC_{50} penghambatan α-glukosidase lebih rendah (23,94 vs. 33,45 µg/mL) dibandingkan maserasi. LC-HRMS menghasilkan 24 anotasi metabolit pada tingkat tentatif atau probable structure, tujuh di antaranya dipilih untuk interpretasi lebih lanjut. Karena setiap metode ekstraksi dilakukan satu kali dan pengukuran merupakan replikasi teknis, perbedaan antarmetode bersifat deskriptif. Data LC-HRMS bersifat kualitatif dan tidak menunjukkan pengayaan kuantitatif maupun kausalitas senyawa tertentu.

**Corresponding author:*
Lilik Sulastri (liliks2881@gmail.com)

Citation: Sulastri, L., Habibah, S. N., Hermawati, E., & Rikkit. (2026). LC-HRMS Metabolite Profiling, Antioxidant, and α-Glucosidase Inhibitory Activities of Averrhoa bilimbi L. Fruit Extracts by Maceration and Ultrasound-Assisted Extraction. Jurnal Jamu Indonesia, 11(4), 317–327. https://doi.org/10.29244/jji.v11i4.535

© 2026 Authors. This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License (http://creativecommons.org/licenses/by-nc-sa/4.0/)

1. INTRODUCTION

Diabetes mellitus (DM) is a major and growing global public health challenge. Approximately 589 million adults were estimated to be living with diabetes in 2024, with the global burden projected to increase substantially by 2050 (Genitsaridi et al., 2026). Persistent postprandial hyperglycemia contributes to diabetes-related complications, making regulation of carbohydrate digestion through α-glucosidase inhibition an important therapeutic approach. Acarbose and other α-glucosidase inhibitors can reduce postprandial glucose excursions but are frequently associated with gastrointestinal adverse effects (Dirir et al., 2022). Oxidative stress also contributes to the progression of diabetes through excessive reactive oxygen species production, oxidative cellular damage, pancreatic β-cell dysfunction, and insulin resistance (Arambas & Vidakovic, 2022). These complementary mechanisms have encouraged investigation of plant-derived compounds with both antioxidant and α-glucosidase inhibitory properties (Kashtoh & Baek, 2022).

Bilimbi (*Averrhoa bilimbi* L.) is a tropical species widely distributed in Indonesia and other parts of Southeast Asia and has attracted attention as a source of potentially bioactive phytochemicals (Alhassan & Ahmed, 2016). Previous studies have reported phenolic compounds, flavonoids, tannins, saponins, terpenoids, and organic acids in *A. bilimbi* fruit and related preparations (Setyawan et al., 2021). Bilimbi extracts have also demonstrated antioxidant and α-glucosidase inhibitory activities *in vitro*, although reported activities vary with extraction conditions, solvent composition, and fractionation procedures (Kumar & Berlin, 2017; Widiastuti et al., 2024). These findings support further investigation of the relationship between extraction conditions, phytochemical composition, and biological activity rather than assuming uniform activity across bilimbi preparations.

Extraction technique is an important determinant of the chemical composition and biological properties of plant extracts. Conventional maceration is widely used because of its operational simplicity but requires prolonged solvent-material contact. Ultrasound-assisted extraction (UAE), in contrast, uses acoustic cavitation to enhance solvent penetration, disrupt plant tissues, and accelerate mass transfer, potentially reducing extraction time while altering recovery of individual metabolites (Chemat et al., 2012; Liao et al., 2021). Despite previous studies on the antioxidant and α-glucosidase inhibitory properties of *A. bilimbi*, direct comparisons between conventional maceration and UAE using the same plant material, solvent composition, and analytical framework remain limited.

Accordingly, this study aimed to compare 70% ethanolic extracts of *A. bilimbi* fruit obtained by conventional maceration and UAE in terms of extraction yield, total phenolic and flavonoid contents, DPPH radical-scavenging activity, α-glucosidase inhibitory activity, and LC-HRMS metabolite profiles. Metabolite profiling was further used to characterize qualitative differences in detected compounds that could provide a basis for future mechanistic investigation, without assuming quantitative metabolite enrichment or compound-specific causality.

2. METHODS

2.1. Equipment and Materials

The equipment used in this study included an analytical balance (Ohaus Pioneer PA214, Ohaus Corporation, Parsippany, NJ, USA), an ultrasonic bath (Elmasonic S 40 H, Elma Schmidbauer GmbH, Singen, Germany), a rotary evaporator (Büchi Rotavapor R-210, Büchi Labortechnik AG, Flawil, Switzerland), a UV-Visible spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan), a refrigerated centrifuge (Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany), a microplate reader (BioTek Epoch™ 2 Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA), and an LC-HRMS system equipped with a Thermo Scientific Orbitrap Exploris™ 120 mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chemicals and reagents included α-glucosidase from *Saccharomyces cerevisiae*, p-nitrophenyl-α-D-glucopyranoside (pNPG), acarbose, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and quercetin from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), Folin-Ciocalteu reagent, sodium acetate, phosphate buffers, sodium hydroxide (NaOH), and other analytical-grade reagents were obtained from Merck KGaA (Darmstadt, Germany).

2.2. Sample Preparation

Fresh fruits of *A. bilimbi* were collected from Tenjolaya, Bogor Regency, West Java, Indonesia, in September 2025. The plant material was botanically authenticated at the Center for Medicinal Plant Information and Development, Dr. Sardjito General Hospital, Ministry of Health, Yogyakarta, Indonesia. The fruits were washed thoroughly under running water and dried in a hot-air oven at 50 °C until constant weight. The dried material was ground into a fine powder, sieved to obtain a homogeneous particle size, stored in airtight containers at room temperature, and protected from light until extraction.

2.3. Extraction Process

For maceration, 300 g of powdered *A. bilimbi* fruit was extracted with 70% ethanol at a solid-to-solvent ratio of 1:10 (w/v), corresponding to a cumulative solvent volume of 3 L. The powdered material was placed in a dark, airtight container and subjected to three successive extraction cycles.

During each maceration cycle, the material was immersed in 1 L of 70% ethanol, stirred occasionally for the first 6 h, and then left to macerate statically for an additional 18 h at room temperature. The mixture was filtered after each 24-h cycle, and the residue was re-extracted twice with 1 L of fresh solvent per cycle. The total extraction time was 72 h (Chemat et al., 2012).

Ultrasound-assisted extraction (UAE) was performed using a modified procedure based on Kumar et al. (2021). A 300-g portion of powdered *A. bilimbi* fruit was mixed with 3 L of 70% ethanol in

a 5-L Erlenmeyer flask, maintaining a solid-to-solvent ratio of 1:10 (w/v). Extraction was performed in an ultrasonic bath at 40 kHz for 37 min at room temperature.

Extracts obtained by both methods were filtered through Whatman No. 1 filter paper. The filtrates were concentrated under reduced pressure using a rotary evaporator at 50 ˚C until viscous crude extracts were obtained. Extraction yield was calculated using the equation below:

$$\text{Extraction Yield (\%)} = \frac{\text{Weight of crude extract (g)}}{\text{Weight of dried powdered sample (g)}} \times 100$$

2.4. Determination of Moisture and Total Ash Contents

Moisture and total ash contents of the crude drug and extracts were determined according to the World Health Organization guidelines and the Indonesian Herbal Pharmacopoeia. Moisture content was determined gravimetrically by drying samples at 105 ˚C until constant weight. Total ash content was determined by incinerating samples in a muffle furnace at 600 ˚C until a grayish-white residue was obtained. Results were expressed as percentages of the initial sample weight (% w/w) (World Health Organization, 1998; Ministry of Health of the Republic of Indonesia, 2022).

2.5. Phytochemical Screening

Qualitative phytochemical screening was performed to evaluate the presence of major classes of secondary metabolites, including alkaloids, flavonoids, phenolic compounds, tannins, saponins, and steroids/triterpenoids. Standard phytochemical tests and corresponding reagents were applied according to established procedures (Harborne, 1998; Shaikh et al., 2020). Characteristic color changes or precipitate formation were used as qualitative indicators of the presence of each phytochemical class.

2.6. Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined using the Folin-Ciocalteu colorimetric method with minor modifications (Suleria et al., 2020). An aliquot of 2.5 mL of extract solution (1,000 µg/mL) was mixed with 0.2 mL of Folin-Ciocalteu reagent and allowed to stand for 5 min at room temperature. Subsequently, 1.5 mL of 7% (w/v) Na_2CO_3 solution was added. The mixture was incubated at 37 ˚C for 90 min, and absorbance was measured at 640 nm using a UV-Visible spectrophotometer. Gallic acid solutions at 10, 20, 30, 40, and 50 µg/mL were used to construct the calibration curve. TPC was calculated from the calibration curve and expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g extract).

2.7. Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method with minor modifications (Chang et al., 2002; Aryal et al., 2019). An aliquot of 1.0 mL of extract solution was mixed with 0.2 mL of 10% (w/v) $AlCl_3$ solution and 0.2 mL of 1 M sodium acetate solution. The mixture was vortexed and incubated at 37 ˚C for 30 min. Absorbance was measured at 430 nm using a UV-Visible spectrophotometer. Quercetin solutions at 25, 50, 75, 100, and 125 µg/mL were used to construct the calibration curve. TFC was calculated from the calibration curve and expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g extract).

2.8. DPPH Radical-Scavenging Assay

Antioxidant activity was evaluated using the DPPH radical-scavenging assay with slight modifications from previously reported methods (Brand-Williams et al., 1995; Gülçin & Alwasel, 2023). A DPPH solution was prepared in methanol at 60 µg/mL. Extract solutions were prepared in methanol at concentrations of 5, 10, 25, 50, 100, and 200 µg/mL. Ascorbic acid was used as the positive control over the same concentration range. For each measurement, 2.0 mL of extract solution was mixed with 2.0 mL of DPPH solution and incubated in the dark at 37 ˚C for 30 min. Absorbance was measured at 515 nm using a UV-Visible spectrophotometer. The assay included a DPPH control containing DPPH without extract, a sample blank containing extract without DPPH to correct for sample background absorbance, and a solvent blank containing methanol alone. Ascorbic acid was used as the positive control. DPPH radical-scavenging activity was calculated using the following equation:

$$\text{\% Radical Scavenging Activity} = \frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}} \times 100$$

where $A_{(control)}$ is the absorbance of the DPPH control, $A_{(sample)}$ is the absorbance of the reaction mixture containing extract and DPPH, and $A_{(blank)}$ is the absorbance of the sample blank.

The IC_{50} value was estimated from the concentration-response relationship and expressed as the concentration required to scavenge 50% of DPPH radicals. Measurements were performed in technical triplicate, and values are reported as mean ± SD.

2.9. α-Glucosidase Inhibition Assay

α-Glucosidase inhibitory activity was evaluated using the p-nitrophenyl-α-D-glucopyranoside (pNPG) substrate assay with slight modifications from Sulastri et al. (2024). α-Glucosidase from *S. cerevisiae* (EC 3.2.1.20, Type I, ≥10 U/mg protein; Sigma-Aldrich, St. Louis, MO, USA; Cat. No. G5003-100UN) was used as the enzyme source. A fresh enzyme working solution of 0.45 U/mL was prepared in 0.1 M sodium phosphate buffer (pH 6.8). The substrate consisted of 20 mM pNPG prepared in the same buffer. Extracts were dissolved in 10% (v/v) dimethyl sulfoxide (DMSO), with the final DMSO concentration maintained below 1% (v/v) in the reaction mixture. For each assay, 50 µL of sample solution was mixed with 25 µL of enzyme solution and pre-incubated at 37 ˚C for 5 min. Subsequently, 25 µL of 20 mM pNPG was added, followed by incubation at 37 ˚C for 30 min. The reaction was terminated with 100 µL of 0.2 M Na_2CO_3. Absorbance of the released p-nitrophenol was measured at 405 nm using a microplate reader. The final reaction volume was 200 µL. Acarbose was used as the positive control. The negative control contained enzyme, substrate, and solvent without extract, whereas the blank contained substrate and buffer without enzyme.

Percentage inhibition of α-glucosidase activity was calculated using the following equation:

$$\%\text{Inhibition} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where $A_{(control)}$ is the absorbance of the negative control and $A_{(sample)}$ is the absorbance of the reaction mixture containing the sample extract. IC_{50} values were estimated by fitting percentage inhibition against $\log_{10}$ concentration using a four-parameter logistic nonlinear regression model. IC_{50} was defined as the concentration required to inhibit 50% of α-glucosidase activity. Measurements were performed in technical triplicate (Pan et al., 2024).

2.10. LC-HRMS Metabolite Profiling

Secondary metabolite profiling was performed by liquid chromatography-high-resolution mass spectrometry (LC-HRMS) using a Thermo Scientific Orbitrap Exploris™ 120 mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive-ion mode. Chromatographic separation was achieved using a ZORBAX Eclipse Plus C18 RRHD column (2.1 × 100 mm, 1.8 µm) with mobile phase A consisting of 0.1% formic acid in water and mobile phase B consisting of 0.1% formic acid in acetonitrile. The gradient program was 95% A at 0-1 min, 95-5% A at 1-16 min, 5% A at 16-20 min, 5-95% A at 20-25 min, followed by equilibration at 25-30 min. The injection volume was 10 µL and the total run time was 30 min.

Mass spectrometric acquisition was performed over m/z 100-1500 with a sheath gas flow of 15 arbitrary units, auxiliary gas flow of 5 arbitrary units, ion transfer tube temperature of 320 °C, full-scan resolution of 120,000, data-dependent MS^2 ($ddMS^2$) resolution of 60,000, intensity threshold of 5.0×10^3, and stepped higher-energy collisional dissociation energies of 18%, 35%, and 53%. Metabolite annotations were generated by comparing accurate-mass and MS/MS data with mzCloud and ChemSpider database information and relevant literature (Yuan et al., 2024). Candidate annotations were evaluated using retention time, accurate mass, predicted molecular formula, mass error, and spectral-library matching where available. Because authentic reference standards were not analyzed, compounds are reported as tentative or probable metabolite annotations rather than confirmed identifications.

2.11. Statistical Analysis

Analytical measurements were performed in technical triplicate and are reported as mean ± standard deviation (SD) to describe measurement precision. The triplicates represented repeated measurements from the same extract preparation rather than independent extraction replicates. Each extraction method, maceration and UAE, was performed once. Consequently, comparisons between extraction methods were interpreted descriptively and were not treated as inferential tests of independent experimental groups. Concentration-response data for the α-glucosidase inhibition assay were fitted using a four-parameter logistic nonlinear regression model to estimate IC_{50} values. DPPH IC_{50} values were estimated from the corresponding concentration-response data as described above.

3. RESULTS AND DISCUSSION

3.1. Characteristics of *Averrhoa bilimbi* L. Fruit, Crude Drug, and Extract

Fresh *A. bilimbi* fruits were processed into dried crude drug and concentrated extracts before phytochemical and biological evaluation. The fresh fruits were green and succulent, whereas the dried material was yellowish-brown, brittle, and retained a characteristic sour aroma. A total of 6.2 kg of fresh fruit yielded 750 g of dried material with a final moisture content of 8.82%. The concentrated ethanolic extracts were viscous and brownish in appearance. The morphological characteristics of the fresh fruit, dried material, powdered crude drug, and concentrated extracts are shown in **Figures 1 and 2**.



Figure 1. Morphological appearance of (a) fresh bilimbi fruit, (b) dried crude drug, and (c) powdered crude drug.

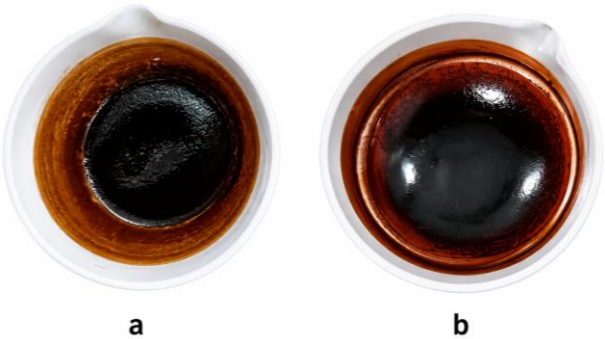

Figure 2. Seventy-percent ethanolic extracts of bilimbi fruit obtained by (a) maceration and (b) ultrasound-assisted extraction.

Extraction with 70% ethanol produced yields of 16.23% by maceration and 11.16% by ultrasound-assisted extraction (UAE). The higher yield obtained by maceration may be associated with its substantially longer solvent-material contact time, which can facilitate extraction of a broader range of soluble constituents. Nevertheless, extraction yield alone does not indicate the concentration or biological relevance of specific constituents. UAE, despite producing a lower overall yield, may alter the recovery profile of individual metabolites through ultrasound-induced cavitation and enhanced mass transfer (Chemat et al., 2012).

Moisture contents were 6.51 ± 1.38% for the maceration extract and 7.66 ± 1.44% for the UAE extract, whereas total ash contents were 6.80 ± 0.68% and 7.51 ± 0.73%, respectively (**Table 1**). These measurements provide descriptive physicochemical characteristics of the two extract preparations. Because independent extraction batches were not analyzed, the small numerical differences between methods should not be interpreted as evidence of reproducible method-dependent effects.

Qualitative phytochemical screening indicated the presence of phenolic compounds, flavonoids, alkaloids, and terpenoids in the ethanolic extracts, whereas tannins, saponins, and steroids were not detected under the applied test conditions (**Table 2**).

Table 1. Yield, moisture content, and total ash content of *Averrhoa bilimbi* ethanolic extracts obtained by maceration and ultrasound-assisted extraction

Extraction Method	Yield (%)	Moisture Content (%)	Total ash content (%)
Maceration	16.23	6.51 ± 1.38	6.80 ± 0.68
UAE	11.16	7.66 ± 1.44	7.51 ± 0.73

Note: Moisture and ash values are mean ± SD of technical measurements (n = 3). Extraction yield represents the single extraction run performed for each method.

Table 2. Qualitative phytochemical screening of *Averrhoa bilimbi* ethanolic extracts

Phytochemical compound	Qualitative screening
Phenolics	+
Flavonoids	+
Tannins	-
Saponins	-
Alkaloids	+
Terpenoids	+
Steroids	–

Note: (+), detected; (−), not detected under the applied qualitative test conditions.

3.2. Total Phenolic and Flavonoid Contents

The maceration and UAE extracts showed closely comparable total phenolic and flavonoid contents (**Table 3**). TPC values were 41.59 ± 0.124 and 41.79 ± 0.093 mg GAE/g extract for maceration and UAE, respectively, whereas TFC values were 22.25 ± 0.091 and 22.91 ± 0.092 mg QE/g extract, respectively. Given that these values were derived from technical measurements of single extraction preparations, the small numerical differences should be interpreted descriptively rather than as statistically independent between-method differences. The nearly identical TPC and TFC values indicate that the stronger bioactivities subsequently observed for the UAE preparation cannot be attributed simply to a large increase in total phenolic or total flavonoid content. Differences in individual compounds, relative composition, matrix effects, or other constituents may instead contribute to the observed activity profile. This interpretation is more consistent with the present data than assuming that UAE produced a phenolic-enriched extract.

Table 3. Total phenolic and flavonoid contents of *Averrhoa bilimbi* ethanolic extracts

Extraction Method	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid Content (mg QE/g extract)
Maceration	41.59 ± 0.124	22.25 ± 0.091
UAE	41.79 ± 0.093	22.91 ± 0.092

Note: Values are mean ± SD of technical measurements (n = 3). GAE, gallic acid equivalent; QE, quercetin equivalent.

3.3. DPPH Radical-Scavenging Activity

The DPPH assay showed an IC_{50} of 37.06 ± 0.27 µg/mL for the UAE extract and 51.59 ± 0.21 µg/mL for the maceration extract, whereas the ascorbic acid positive control had an IC_{50} of 4.82 ± 0.15 µg/mL (**Table 4**). Because a lower IC_{50} represents greater radical-scavenging activity, the UAE preparation showed approximately 1.4-fold greater DPPH potency than the maceration preparation under the tested conditions. Both extracts remained less potent than the ascorbic acid control.

Table 4. DPPH radical-scavenging activity of *Averrhoa bilimbi* ethanolic extracts

Sample	IC_{50} (µg/mL)
Ascorbic acid (positive control)	4.82 ± 0.15
UAE extract	37.06 ± 0.27
Maceration extract	51.59 ± 0.21

The DPPH assay measures the ability of antioxidant constituents to reduce the stable DPPH radical through electron- or hydrogen-donating reactions. Structural features such as phenolic hydroxyl groups and conjugated systems can influence radical stabilization and therefore contribute to differences in scavenging activity (Gülçin & Alwasel, 2023). Because TPC and TFC were very similar between the two extracts, the lower DPPH IC_{50} of the UAE extract may reflect differences in the identities or relative proportions of individual constituents rather than a substantial increase in the total phenolic or flavonoid pool.

3.4. α-Glucosidase Inhibitory Activity

α-Glucosidase inhibitory activity was evaluated using concentration-response analysis and expressed as IC_{50}. The fitted IC_{50} values were 23.66 µg/mL for acarbose, 23.94 µg/mL for the UAE extract, and 33.45 µg/mL for the maceration extract (**Figure 3**). Thus, under the tested *in vitro* conditions, the UAE extract showed a lower fitted IC_{50} than the maceration extract and a mass-based IC_{50} numerically close to that of acarbose. This numerical similarity should not be interpreted as pharmacological equivalence because acarbose is a defined compound, whereas the UAE preparation is a complex crude extract.

The lower α-glucosidase IC_{50} observed for the UAE extract cannot be explained by a substantial difference in bulk TPC or TFC because these measurements were closely comparable between the two preparations. Ultrasound-induced cavitation can disrupt plant tissues, enhance solvent penetration, and alter recovery of individual intracellular constituents (Chemat et al., 2017). Therefore, the observed difference in enzyme inhibition may

reflect qualitative or relative compositional differences rather than a simple increase in total phenolic or flavonoid content.

Phenolic compounds and flavonoids are frequently reported to interact with α-glucosidase through hydrogen bonding, hydrophobic interactions, and π-π interactions, although the precise inhibitory mechanism depends on molecular structure and cannot be inferred for a crude extract without dedicated enzyme-kinetic and compound-specific experiments (Proença et al., 2017).

The UAE preparation showed both lower DPPH and α-glucosidase IC_{50} values than the maceration preparation. This parallel pattern provides a rationale for examining whether differences in metabolite composition might contribute to the observed biological activity, but it does not establish a statistical correlation or causal relationship.

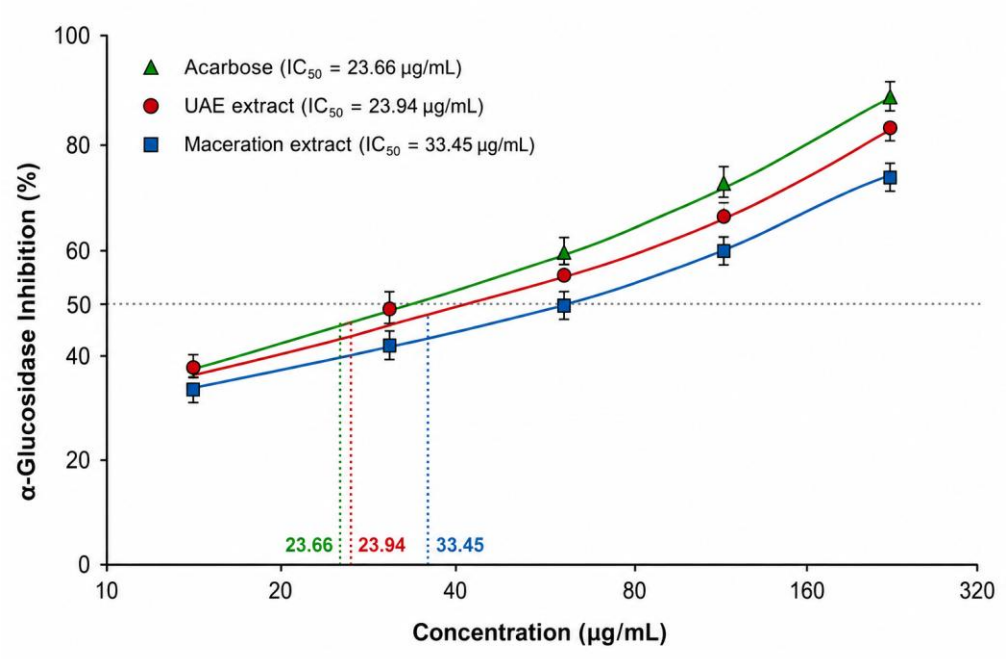

Figure 3. Four-parameter logistic concentration-response curves for α-glucosidase inhibition by acarbose and *Averrhoa bilimbi* fruit extracts obtained by maceration and ultrasound-assisted extraction (UAE). Fitted IC_{50} values were 23.66, 23.94, and 33.45 µg/mL for acarbose, UAE, and maceration, respectively. The horizontal dotted line denotes 50% inhibition, and the vertical dotted lines indicate the corresponding fitted IC_{50} values.

3.5. Secondary Metabolite Profiles of 70% Ethanolic Bilimbi Fruit Extracts

LC-HRMS was used to compare molecular features detected in the maceration and UAE extracts and to generate metabolite annotations. Because authentic reference standards were not analyzed, detected compounds are described according to annotation confidence rather than as definitively identified metabolites (Sumner et al., 2007; Alseekh et al., 2021).

The total ion chromatograms of the maceration and UAE extracts showed broadly similar chromatographic profiles (**Figure 4**). The reported normalized total ion signal was 1.00×10^6 for UAE and 9.45×10^5 for maceration, and differences in several minor signals were observed within the chromatographic window. These observations indicate differences in detected ion profiles between the two preparations; however, total ion intensity and individual LC-MS peak areas are influenced by ionization efficiency and matrix effects and should not be interpreted directly as absolute metabolite concentrations (Alseekh et al., 2021).

LC-HRMS feature detection yielded 192 molecular features in the maceration extract and 175 in the UAE extract. Seventy-three features were shared, corresponding to 24.8% of the 294 unique features detected across both preparations, while 119 and 102 features were detected only in the maceration and UAE datasets, respectively (**Figure 5**). An exploratory Wilcoxon signed-rank comparison of peak areas among shared molecular features yielded $p = 0.1204$. Because the analysis was based on features measured from single extraction preparations, this result describes the observed LC-HRMS dataset and should not be interpreted as an inferential comparison of independently replicated extraction methods. Mass errors for most detected molecular features were centered near 0 ppm and within ±5 ppm, supporting high mass accuracy of the acquired data. Nevertheless, accurate mass alone is insufficient for definitive structural identification, particularly in the absence of authentic reference standards.

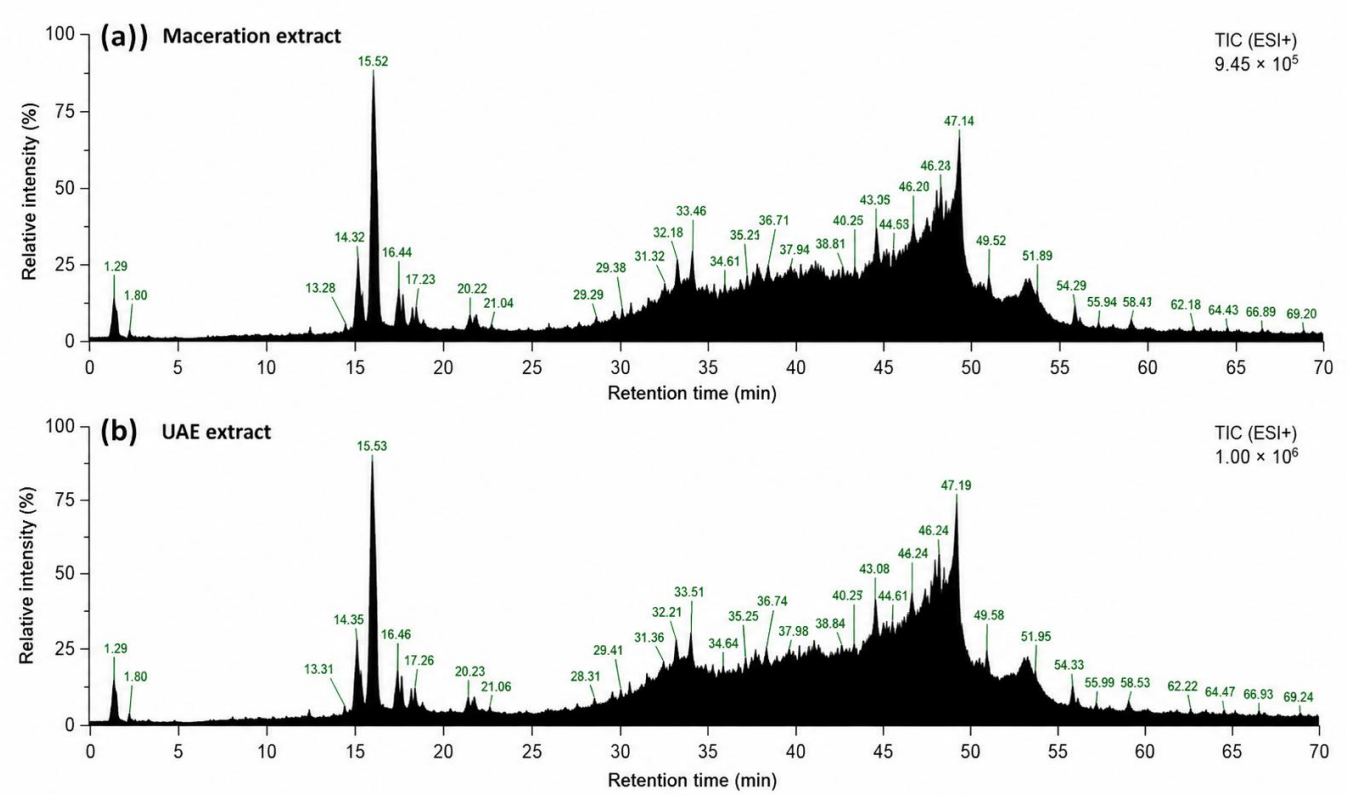

Figure 4. Total ion chromatograms of 70% ethanolic bilimbi fruit extracts obtained by (a) maceration and (b) ultrasound-assisted extraction.

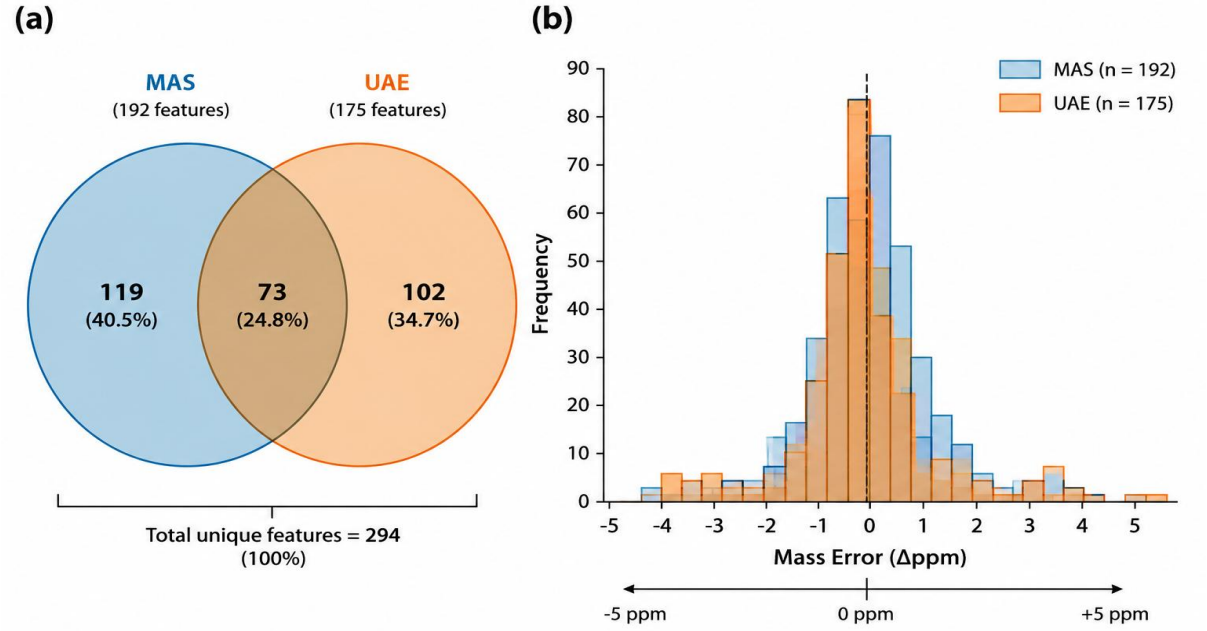

Figure 5. (a) Venn diagram of shared and method-specific molecular features detected in the maceration (MAS) and UAE extracts. (b) Mass-error (Δppm) distribution of detected molecular features. MAS denotes the maceration extract.

A total of 24 metabolite annotations were included in **Table 5**. Among these, seven compounds were selected for focused discussion because of their reported relevance to antioxidant or α-glucosidase-related activity: gallic acid, chebulic acid, quercetin 3-O-rhamnoside-7-O-glucoside, vitexin, pelargonidin, genistein 4′-O-glucuronide, and luteolin. Five of these selected metabolites showed mzCloud spectral matches of 83.1-99.6% and were classified as probable structure annotations, whereas gallic acid and chebulic acid lacked MS^2 library confirmation and remained tentative annotations. The selected annotations had mass errors below ±1 ppm.

Table 5. Tentative and probable LC-HRMS metabolite annotations in *Averrhoa bilimbi* extracts obtained by maceration and ultrasound-assisted extraction

No.	Metabolite	Formula	Obs. m/z	RT (min)	Ion	Δppm	mzCloud	Maceration	UAE	Conf.
1	Gallic acid	C7H6O5	153.01816	0.840	[M+H−H2O]+	-0.41	—	5.30×106	7.68×106	T
2	Chebulic acid	C14H12O11	357.04538	1.164	[M+H]+	0.39	—	7.73×106	8.14×106	T
3	Quercetin 3-O-rhamnoside-7-O-glucoside	C27H30O16	611.16085	6.090	[M+H]+	0.35	97.2	9.55×105	8.12×105	PS
4	Chrysin	C15H10O4	255.06523	7.637	[M+H]+	0.18	97.6	6.19×106	5.90×106	PS
5	Vitexin	C21H20O10	433.11307	6.169	[M+H]+	0.33	83.1	4.84×105	6.03×105	PS
6	Genistein 4′-O-glucuronide	C21H18O11	447.09229	6.873	[M+H]+	0.22	97.4	4.64×105	4.27×105	PS
7	Pelargonidin	C15H10O5	271.06003	6.994	[M+H]+	-0.27	99.5	5.22×106	4.74×106	PS
8	Hordenine	C10H15NO	166.12271	1.616	[M+H]+	0.43	97.1	8.75×106	1.01×107	PS
9	Salsolinol	C10H13NO2	180.10199	1.164	[M+H]+	0.47	77.7	6.54×106	8.51×106	PS
10	3-O-Ethylascorbic acid	C8H12O6	205.07077	1.675	[M+H]+	0.53	—	4.57×105	4.10×105	T
11	4,5-Dicaffeoylquinic acid	C25H24O12	517.13416	6.551	[M+H]+	0.20	93.8	—	4.46×105	PS
12	Quercetin 3-O-galactoside (Hyperoside)	C21H20O12	465.10271	6.320	[M+H]+	-0.13	94.2	—	1.13×106	PS
13	Cyanidin	C15H10O6	287.05496	8.140	[M+H]+	-0.18	98.7	—	4.92×106	PS
14	Rhodiocyanoside A	C11H17NO6	260.11292	0.800	[M+H]+	0.24	—	—	1.69×107	T
15	Wogonin	C16H12O5	285.07578	7.792	[M+H]+	0.09	97.6	—	1.37×106	PS
16	2-O-Caffeoylglucarate	C15H14O11	371.06091	3.292	[M+H]+	0.07	—	4.98×105	—	T
17	Quercetin	C15H10O7	303.05005	6.321	[M+H]+	0.40	89.6	6.61×105	—	PS
18	Quercetin-3β-D-glucoside (Isoquercitrin)	C21H20O12	465.10294	6.323	[M+H]+	0.38	95.3	1.11×106	—	PS
19	Luteolin	C15H10O6	287.05496	8.143	[M+H]+	-0.18	99.6	5.68×106	—	PS
20	Glycitein	C16H12O5	285.07581	7.792	[M+H]+	0.20	98.0	1.21×106	—	PS
21	Remerin	C17H14O7	331.08115	9.018	[M+H]+	-0.25	—	6.93×105	—	T
22	Sarmentosin	C11H17NO7	258.09732	0.784	[M+H−H2O]+	0.33	—	3.13×105	—	T
23	Genistein	C15H10O5	271.06003	8.915	[M+H]+	-0.27	98.9	1.28×106	—	PS
24	N-Methyltyramine	C9H13NO	152.10704	1.480	[M+H]+	0.33	—	4.46×106	—	T

Note: "—" indicates that the corresponding feature was not detected in that extract under the applied analytical conditions. T, tentative annotation based on accurate mass and molecular formula without an MS^2 library match; PS, probable structure annotation supported by an mzCloud MS^2 spectral match ≥70%, consistent with Metabolomics Standards Initiative confidence concepts (Sumner et al., 2007). Peak-area values are LC-MS signal responses and should not be interpreted as absolute metabolite concentrations.

Among the seven metabolites selected for focused interpretation, gallic acid and chebulic acid showed higher LC-MS peak areas in the UAE preparation than in the maceration preparation. Gallic acid and chebulic acid have previously been associated with antioxidant activity (Hadidi et al., 2024; Zhao et al., 2021), providing a plausible chemical context for the lower DPPH IC_{50} observed for the UAE extract. However, LC-MS peak areas depend on ionization response and cannot be interpreted as absolute concentrations without appropriate quantitative calibration. Therefore, the present data do not establish that these compounds were quantitatively enriched by UAE or that they caused the observed difference in antioxidant activity.

The selected flavonoids, including luteolin, vitexin, pelargonidin, genistein 4′-O-glucuronide, and quercetin 3-O-rhamnoside-7-O-glucoside, belong to structural classes associated with α-glucosidase inhibition in previous studies (Proença et al., 2017). This evidence provides a rationale for further mechanistic investigation, but the activity of the crude extracts cannot be attributed to these compounds individually on the basis of the present LC-HRMS data alone.

This study has several important limitations. First, each extraction method was performed only once, and the reported triplicate measurements represent technical rather than independent biological or extraction replicates. The reported SD values therefore describe analytical precision and do not capture variability among independent extraction batches or plant-material collections. Second, the LC-HRMS analysis was qualitative and did not use authentic reference standards or validated quantitative calibration; consequently, metabolite annotations remain tentative or probable, and peak areas cannot be interpreted as absolute concentrations. Third, associations between individual metabolites and antioxidant or α-glucosidase inhibitory activity remain inferential because compound-specific activity, targeted quantification, and enzyme-kinetic analyses were not performed. Finally, the biological assays were conducted *in vitro*, and no conclusions regarding clinical antidiabetic efficacy can be drawn from the present findings.

4. CONCLUSION

The 70% ethanolic extracts of *A. bilimbi* fruit obtained by ultrasound-assisted extraction (UAE) and conventional maceration showed comparable total phenolic and flavonoid contents but different *in vitro* bioactivity profiles. Under these conditions, UAE showed lower DPPH and α-glucosidase IC_{50} values than maceration (37.06 ± 0.27 vs. 51.59 ± 0.21 µg/mL and 23.94 vs. 33.45 µg/mL, respectively). LC-HRMS profiling yielded 24 tentative or probable metabolite annotations, seven of which were selected for focused interpretation because of their reported relevance to antioxidant or α-glucosidase inhibitory activity. These qualitative metabolite data provide a plausible chemical context for the observed bioactivity differences but do not demonstrate quantitative enrichment or compound-specific causality. Because each extraction method was performed once and replicate measurements were technical, between-method differences should be interpreted descriptively. Further studies using independent extraction replicates, targeted metabolite quantification, enzyme-kinetic experiments, and appropriate biological models are required to establish the reproducibility and biological relevance of these findings.

AUTHOR CONTRIBUTIONS

Conceptualization, L.S.; methodology, L.S. and S.N.H.; investigation, L.S., S.N.H., E.H., and R.; formal analysis, L.S. and S.N.H.; validation, L.S., E.H., and R.; data curation, S.N.H. and E.H.; visualization, S.N.H. and E.H.; resources, R.; supervision, L.S.; project administration, L.S.; funding acquisition, L.S.; writing - original draft preparation, S.N.H.; writing - review and editing, L.S. and R. All authors have read and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable. This study did not involve human participants or experimental animals and consisted entirely of *in vitro* laboratory analyses.

INFORMED CONSENT STATEMENT

Not applicable. This study did not involve human participants.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

FUNDING

This research was funded by an internal research grant from the Research and Community Service Institute (LPPM), Pakuan University, grant no. 146/KEP/REK/XI/2025.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Research and Community Service Institute (LPPM), Pakuan University, for providing financial support through the internal research grant that supported this study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ROLE OF FUNDERS

The funder had no role in the design of the study; the collection, analysis, or interpretation of the data; the preparation of the manuscript; or the decision to publish the results.

DECLARATION OF GENERATIVE ARTIFICIAL INTELLIGENCE (AI) USE

During the preparation and revision of this manuscript, the authors used ChatGPT (OpenAI) to assist with language editing, grammar, sentence structure, readability, organization, formatting, and scientific editorial refinement. The tool was also used to improve the clarity and consistency of the presentation of methods, results, interpretations, limitations, and conclusions. It was not used to generate experimental data or conduct laboratory experiments. All AI-assisted revisions were critically reviewed, edited, and verified by the authors against the original data and study procedures. The authors remain fully responsible for the accuracy, integrity, originality, and scientific interpretation of the final manuscript.

REFERENCES

Alhassan, A. M., & Ahmed, Q. U. (2016). *Averrhoa bilimbi* Linn.: A review of its ethnomedicinal uses, phytochemistry, and pharmacology. *Journal of Pharmacy and Bioallied Sciences, 8*(4), 265–271. https://doi.org/10.4103/0975-7406.199342

Alseekh, S., Aharoni, A., Brotman, Y., Contrepois, K., D'Auria, J., Ewald, J., Ewald, J. C., Fraser, P. D., Giavalisco, P., Hall, R. D., Heinemann, M., Link, H., Luo, J., Neumann, S., Nielsen, J., de Souza, L. P., Saito, K., Sauer, U., Schroeder, F. C., … Fernie, A. R. (2021). Mass spectrometry-based metabolomics: A guide for annotation, quantification and best reporting practices. *Nature Methods, 18*, 747–756. https://doi.org/10.1038/s41592-021-01197-1

Arambas, J., & Vidakovic, M. (2022). Oxidative stress-mediated beta cell death and dysfunction as a target for diabetes

management. *Frontiers in Endocrinology, 13*, 1006376. https://doi.org/10.3389/fendo.2022.1006376

Aryal, S., Baniya, M. K., Danekhu, K., Kunwar, P., Gurung, R., & Koirala, N. (2019). Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants, 8*(4), 96. https://doi.org/10.3390/plants8040096

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT - Food Science and Technology, 28*(1), 25–30. https://doi.org/10.1016/S0023-6438(95)80008-5

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis, 10*(3), 178–182.

Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products: Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry, 34*, 540–560. https://doi.org/10.1016/j.ultsonch.2016.06.035

Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences, 13*(7), 8615–8627. https://doi.org/10.3390/ijms13078615

Dirir, A. M., Daou, M., Yousef, A. F., & Yousef, L. F. (2022). A review of alpha-glucosidase inhibitors from plants as potential candidates for the treatment of type-2 diabetes. *Phytochemistry Reviews, 21*(4), 1049–1079. https://doi.org/10.1007/s11101-021-09773-1

Genitsaridi, I., Salpea, P., Salim, A., Sajjadi, S. F., Tomic, D., James, S., Thirunavukkarasu, S., Issaka, A., Chen, L., Basit, A., Luk, A. O. Y., Ma, R. C. W., Mbanya, J. C., Ramachandran, A., Wild, S. H., Duncan, B. B., Boyko, E. J., & Magliano, D. J. (2026). 11th edition of the IDF Diabetes Atlas: Global, regional, and national diabetes prevalence estimates for 2024 and projections for 2050. *The Lancet Diabetes & Endocrinology, 14*(2), 149–156. https://doi.org/10.1016/S2213-8587(25)00299-2

Gülçin, İ., & Alwasel, S. H. (2023). DPPH radical scavenging assay. *Processes, 11*(8), 2248. https://doi.org/10.3390/pr11082248

Hadidi, M., Liñán-Atero, R., Tarahi, M., Christodoulou, M. C., & Aghababaei, F. (2024). The potential health benefits of gallic acid: Therapeutic and food applications. *Antioxidants, 13*(8), 1001. https://doi.org/10.3390/antiox13081001

Harborne, J. B. (1998). *Phytochemical methods: A guide to modern techniques of plant analysis* (3rd ed.). Chapman & Hall.

Kashtoh, H., & Baek, K. H. (2022). Recent updates on phytoconstituent alpha-glucosidase inhibitors: An approach towards the treatment of type two diabetes. *Plants, 11*(20), 2722. https://doi.org/10.3390/plants11202722

Kumar, J., & Berlin, S. V. M. (2017). Phytochemical profile and free radical nitric oxide (NO) scavenging activity of *Averrhoa bilimbi* L. fruit extract. *3 Biotech, 7*(1), 85. https://doi.org/10.1007/s13205-017-0678-9

Kumar, K., Srivastav, S., & Singh, V. (2021). Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. *Ultrasonics Sonochemistry, 70*, 105325. https://doi.org/10.1016/j.ultsonch.2020.105325

Liao, J., Guo, Z., & Yu, G. (2021). Process intensification and kinetic studies of ultrasound-assisted extraction of flavonoids from peanut shells. *Ultrasonics Sonochemistry, 76*, 105661. https://doi.org/10.1016/j.ultsonch.2021.105661

Ministry of Health of the Republic of Indonesia. (2022). *Suplemen I Farmakope Herbal Indonesia Edisi II*. Ministry of Health of the Republic of Indonesia.

Pan, G., Lu, Y., Wei, Z., Li, Y., & Li, L. (2024). A review on the *in vitro* and *in vivo* screening of α-glucosidase inhibitors. *Heliyon, 10*(18), e37467. https://doi.org/10.1016/j.heliyon.2024.e37467

Proença, C., Freitas, M., Ribeiro, D., Oliveira, E. F. T., Sousa, J. L. C., Tomé, S. M., Ramos, M. J., Silva, A. M. S., Fernandes, P. A., & Fernandes, E. (2017). α-Glucosidase inhibition by flavonoids: An *in vitro* and *in silico* structure–activity relationship study. *Journal of Enzyme Inhibition and Medicinal Chemistry, 32*(1), 1216–1228. https://doi.org/10.1080/14756366.2017.1368503

Setyawan, H. Y., Sukardi, S., & Nareswari, B. F. (2021). The phytochemical potential of *Averrhoa bilimbi* - A review. *IOP Conference Series: Earth and Environmental Science, 733*(1), 012091. https://doi.org/10.1088/1755-1315/733/1/012091

Shaikh, J. R., Patil, M. K., & Udgir, A. S. (2020). Qualitative tests for preliminary phytochemical screening: An overview. *International Journal of Chemical Studies, 8*(2), 603–608. https://doi.org/10.22271/chemi.2020.v8.i2i.8834

Sulastri, L., Zulfadhli, M., Alwie, R. R., Ramani, S., Prasetiyo, A., & Simanjuntak, V. S. (2024). Aktivitas penghambatan enzim α-glukosidase senyawa β-sitosterol dari fraksi etil asetat daun salam [*Syzygium polyanthum* (Wight.) Walp]. *Jurnal Fitofarmaka Indonesia, 11*(1), 9–16. https://doi.org/10.33096/jffi.v11i1.981

Suleria, H. A. R., Barrow, C. J., & Dunshea, F. R. (2020). Screening and characterization of phenolic compounds and their antioxidant capacity in different fruit peels. *Foods, 9*(9), 1206. https://doi.org/10.3390/foods9091206

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., Fan, T. W. M., Fiehn, O., Goodacre, R., Griffin, J. L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A. N., Lindon, J. C., Marriott, P., Nicholls, A. W., … Viant, M. R. (2007). Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics, 3*(3), 211–221. https://doi.org/10.1007/s11306-007-0082-2

Widiastuti, D., Sinaga, S. E., Warnasih, S., Syahputri, Y., Saputri, N. B., Sustiprijatno, & Putra, W. E. (2024). Antidiabetic

activity of *Averrhoa bilimbi* L. fruit extracts and the identification of active compounds using LC-MS and in silico methods. *Indonesian Journal of Pharmacy, 35*(2), 282–291. https://doi.org/10.22146/ijp.7746

World Health Organization. (1998). *Quality control methods for medicinal plant materials*. World Health Organization.

Yuan, H., Xie, Q., Liang, L., Luo, J., Jiang, S., Peng, C., & Wang, W. (2024). An efficient workflow for quality control marker screening and metabolite discovery in dietary herbs by LC-Orbitrap-MS/MS and chemometric methods: A case study of *Chrysanthemum* flowers. *Foods, 13*(7), 1008. https://doi.org/10.3390/foods13071008

Zhao, H.-Y., Lan, Q., He, S., Su, B.-J., Wang, Y.-Q., Liao, H.-B., Wang, H.-S., & Liang, D. (2021). Chebulic acid derivatives from *Balakata baccata* and their antineuroinflammatory and antioxidant activities. *Bioorganic Chemistry, 116*, 105332. https://doi.org/10.1016/j.bioorg.2021.105332

Publisher's Note & Disclaimer

All statements, opinions, and data in this publication were solely the responsibility of the individual authors or contributors and did not necessarily reflect the views of the publisher or editors. The publisher and editors did not guarantee the accuracy, completeness, or reliability of the content, and were not legally responsible for any errors, omissions, or consequences arising from its use. The publisher and editors also disclaimed any liability for injury, damage, or loss to persons or property resulting from the application of ideas, methods, or products mentioned herein. Readers were advised to independently verify all information before relying on it. The publisher accepted no responsibility for any consequences arising from the use of this publication's materials.