Trop J Nat Prod Res, January 2026; 10(1): 6613 - 6619

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Spectra Characterization, Antioxidant, and Antityrosinase Activities of Nonpolar to Polar Fractions from *Averrhoa bilimbi* L. Leaf Extract

Syeda Ifra Khudeja, Berna Elya*, Roshamur Cahyan Forestrania, Delly Ramadon

Faculty of Pharmacy, Universitas Indonesia, Depok 16424, Indonesia

ARTICLE INFO

Article history:
Received 01 October 2025
Revised 02 December 2025
Accepted 15 January 2026
Published online 01 February 2026

ABSTRACT

Averrhoa bilimbi L. is recognized for its rich phytochemical diversity however, the anti-tyrosinase potential of its fractions and the compounds responsible remain unexplored. This study aims to investigate the antioxidant and anti-tyrosinase activities of *A. bilimbi* non-polar to polar fractions using a bioactivity-guided approach and to characterize the major phytoconstituents of the most active fraction using liquid chromatography-mass spectrometry (LC–MS). The leaf powder was macerated with ethanol and subsequently partitioned into n-hexane, ethyl acetate, and methanol fractions. Antityrosinase activity was determined using L-dopa as the substrate, and antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays. Qualitative phytochemical screening revealed that phenolic and flavonoid compounds were present across all fractions, while alkaloids and saponins were exclusive to the more polar fractions. The ethyl acetate fraction showed significantly higher phenolic (135.9 ± 1.30 mg GAE/g extract) and flavonoid content (90.3 ± 0.14 mg QE/g extract), along with the strongest antioxidant activity (DPPH IC_{50} = 37.17 µg/mL; ABTS IC_{50} = 11.1 µg/mL; FRAP = 2410 µmol $FeSO_4$/g) and the most effective tyrosinase inhibition (IC_{50}= 1.502 ± 0.010 mg/mL), with all parameters differing significantly among the three fractions ($p < 0.05$). LC-MS profiling of the most potent fraction identified phenolic and flavonoid glycosides, including Biochanin A 7-O-rutinoside, Durantoside I and Coniferin, that have not previously been documented in this species. The results suggest that the high biological activity is linked to a synergistic action of these compounds concentrated in the ethyl acetate fraction.

Keywords: *Averrhoa bilimbi* L, Antityrosinase, Antioxidant, Total Phenolic Content, Total Flavonoid Content, Compound Profiling

Introduction

The skin, as the primary barrier and most exposed organ of the body, is highly vulnerable to external factors that generate oxidative stress and disrupt cellular function. Skin pigmentation is the major physiological defense mechanism against these detrimental effects. The pigmentation is mediated by melanin, synthesized through melanogenesis in melanocytes.[1] Although melanin offers natural defense against UV radiation and chemical exposure, its overproduction leads to dermatological disorders such as age spots, melasma, freckles, and skin cancer. Dysregulation of the melanogenesis pathway is caused by oxidative stress, resulting in abnormal skin pigmentation.[2] Tyrosinase, the enzyme which catalyzes the rate-limiting steps of melanin biosynthesis, is the primary therapeutic target for hyperpigmentation.[3] While many existing anti-tyrosinase agents, such as hydroquinone and kojic acid, are effective, they are often associated with significant drawbacks, like skin irritation and toxicity with prolonged application.[4,5]

*Corresponding author. Email: berna.elya@farmasi.ui.ac.id
Tel: +62-813-1416-1497

Citation: Khudeja SI, Elya B*, Forestrania RC, Ramadon D. Spectra Characterization, Antioxidant, and Antityrosinase Activities of Nonpolar to Polar Fractions from *Averrhoa bilimbi* L. Leaf Extract from Faculty of Pharmacy, Universitas Indonesia, Depok, Indonesia. Trop J Nat Prod Res. 2026; 10(1):6613 – 6619 https://doi.org/10.26538/tjnpr/v10i1.26

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

This has created a critical need for safer, ideal, and naturally derived alternatives. Plant-derived compounds, specifically phenolics and flavonoids, are increasingly recognized for their potent antioxidant and anti-tyrosinase properties. These phytochemicals show considerable potential as effective and non-toxic modulators of skin pigmentation.[6]

Averrhoa bilimbi L. (Oxalidaceae), commonly known as bilimbi, is native to Indonesia and widely distributed across tropical Asia, Africa, and South America. Phytochemical studies show that *A. bilimbi* contains phenolics, flavonoids, alkaloids, triterpenoids, and saponins, which account for its diverse biological activities, including antioxidant, hepatoprotective, antidiabetic, antihypertensive, antimicrobial, wound healing, and photoprotective effects.[7,8] Among these, flavonoids and phenolic compounds, with their antioxidant properties, have been reported as key contributors to tyrosinase inhibition. While previous studies reported antioxidant and antityrosinase activities in various *A. bilimbi* extracts and fractions [9–11], none have systematically evaluated non-polar, semi-polar, and polar fractions from its 70% ethanolic extract. This study addresses this gap to provide a comprehensive view of its bioactive potential.

In the current research, the extract of *A. bilimbi* was partitioned into fractions of varying polarity to separate distinct groups of compounds. This approach facilitated the assessment of the biological activity of each fraction and enabled the identification of the fraction exhibiting the highest activity. Antioxidant and tyrosinase inhibition activities were determined through standard analytical assays. LC–MS profiling was further carried out on the most active fraction to determine its major bioactive constituents. Overall, the findings provide supporting evidence for the potential of *A. bilimbi* as a natural product-based remedy for managing hyperpigmentation disorders and for cosmetic applications.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Materials and Methods

Chemicals

DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), Potassium Persulfate, 2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) (Sigma-Aldrich, United States), 70% ethanol, Ferric Chloride ($FeCl_3$), Sodium Acetate, Acetic Acid, Folin-Ciocalteu Reagent (25%), Sodium Carbonate (Na_2CO_3), Water for Injection, Ascorbic Acid, Gallic Acid, Quercetin, Aluminum Chloride ($AlCl_3$), Sodium Acetate buffer, Sodium hydroxide, Potassium Acetate, Dimethyl sulfoxide, Methanol (E.Merck, pro analysis), Tyrosinase enzyme (Sigma-Aldrich, United States; EC 1.14.18.1), 3,4-dihydroxy-L-phenylalanine (Sigma-Aldrich, United States).

Plant collection and extraction

Leaf simplicia of *Averrhoa bilimbi* L. were obtained from Bandung, West Java (−6.9175, 107.6191) in April 2024 and authenticated at the Pharmacognosy and Phytochemistry Research Laboratory, Faculty of Pharmacy, Universitas Indonesia (Voucher No.: ND-12/UN2.F15.L05/LOG.01/2024). Leaf powder was macerated in 70% ethanol for three days, with initial and subsequent ratios of 1:10 (w/v) and 1:5 (w/v), respectively. The filtrate was then concentrated using a rotary evaporator (50°C, 100 rpm).[12] This process yielded a thick, dark extract which was dried until its weight remained constant over successive measurements. The extraction yield was determined as a percentage of the initial raw material weight (equation 1).

$$\%\ Extraction\ yield = \frac{extract\ weight\ (g)}{weight\ of\ simple\ powder\ (g)} \times 100$$

(equation 1)

Fractionation by Liquid-Liquid Partitioning

The crude ethanolic extract was fractionated via sequential liquid-liquid partitioning based on solvent polarity.[13,14] First, 50 grams extract was dissolved in 500 mL water and extracted three times with equal volumes of n-hexane until the solvent layer appeared clear. The aqueous phase was then sequentially partitioned with ethyl acetate and methanol. All fractions were concentrated by rotary evaporator (50°C) and oven dried (50°C) to obtain n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar) fractions. The yield of each fraction was calculated using the following formula (equation 2):

$$\%\ Fraction\ Yield = \frac{Weight\ of\ dried\ fraction\ (g)}{Initial\ weight\ of\ crude\ extract\ (g)} \times 100$$

(equation 2)

Phytochemical Screening

Qualitative phytochemical screening of all fractions was performed following standard procedures.[15] Alkaloids were confirmed using Bouchardat's reagent after acid extraction; terpenoids by the Liebermann–Burchard reaction; phenolics by 1% $FeCl_3$; and flavonoids by the Shinoda test. Tannins were identified using the gelatin test, saponins by the foam test, and anthraquinones by acid hydrolysis followed by benzene extraction and NaOH treatment.

Determination of total phenolic content (TPC)

Total Phenolic Content (TPC) was quantified using the Folin-Ciocalteu colorimetric assay.[16] The analysis was performed in a 96-well microplate with 20 µL of sample (100 µg/mL), solvent blank, and gallic acid standards (3-8 µg/mL) in triplicate. To each well, 100 µL of 25% Folin-Ciocalteu reagent was added followed by 5-minute pre-incubation at room temperature. The chromogenic reaction was initiated by adding 80 µL sodium carbonate (100 g/L) and incubated for 2 hours at 25°C in darkness. Following incubation, absorbance was measured at 760 nm using a microplate reader (Agilent BioTek Epoch 2; United States).

Determination of total flavonoid content (TFC)

Total flavonoid content (TFC) was determined using an aluminum chloride colorimetric assay.[16] For the reaction, 20 µL of each sample (100 µg/mL) was mixed with 20 µL of 10% (w/v) aluminum chloride and 20 µL of 1 M sodium acetate. The final volume was adjusted to 200 µL with distilled water. After 30-minute incubation at room temperature, absorbance was measured at 430 nm using the microplate reader. The analyses were performed in triplicate, and TFC was expressed as mg QE/g extract using a quercetin (50–175 µg/mL) calibration curve.

DPPH antioxidant activity assay

The DPPH method was based on the principle of free radical inhibition by the antioxidant containing samples.[17–20] In a 96-well plate, 20 µL of each test fraction or ascorbic acid (positive control) was mixed with 180 µL of a 150 µM DPPH solution. The solution was incubated at room temperature in dark conditions for 30 mins. Absorbance was measured at the wavelength of 517 nm. The analyses was carried out in triplicate and absorbance data was used to calculate the % DPPH inhibition (equation 3), expressed as IC_{50} values (equation 4).

$$Percentage\ of\ Inhibition(\%) = \left(\frac{Blank\ Absorbance - Sample\ Absorbance}{Blank\ Absorbance}\right) \times 100$$

(equation 3)

Using the regression equation, IC_{50} can be calculated:

$$IC_{50} = \frac{50 - intercept}{slope}$$

(equation 4)

ABTS antioxidant activity assay

Antioxidant activity i.e. the ABTS method used ascorbic acid as a standard and was based on previous research.[21,22] The solution of ABTS radical was generated by reacting 7.0 mM ABTS with 2.73 mM potassium persulfate for 12-16 hours in dark, then diluted with methanol to give the working solution. The assay was conducted in triplicate by mixing 30 µL sample with 270 µL ABTS solution in a 96-well plate. Following a 6-minute incubation in the dark, the absorbance was measured at 737 nm. From the absorbance data, the % inhibition and IC_{50} were calculated as previously described in equations 3 and 4.

FRAP antioxidant activity assay

Herein the FRAP method employed ascorbic acid as a standard and was done with slight modification in accordance with previous research.[18,21,22] A 50 µL extract (100 µg/mL) or 20 ppm ascorbic acid control was mixed with 150 µL FRAP II reagent (300 mmol/L acetate buffer, pH 3.6, 10 mmol/L Tris Pyridyl Triazine in 40 mM HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio). The mixture was homogenized and incubated at 37 °C for 30 min in the dark, and absorbance was measured at 597 nm. The blank solution contained 50 µL methanol and 150 µL FRAP II reagent. For the calibration curve, 50 µL $FeSO_4 \cdot 7H_2O$ standard solutions (100–500 µmol/L) were mixed with 150 µL FRAP I reagent (acetate buffer, 10 mmol/L Tris Pyridyl Triazine, and water in 10:1:1). All assays were performed in triplicate. Antioxidant activity was calculated from the $FeSO_4 \cdot 7H_2O$ calibration curve and expressed as µmol Fe^{2+}/g sample using the formula (equation 5):

$$Frap\ value\ (\mu mol/g\ sample) = \frac{C \times V \times DF}{m}$$

(equation 5)

where C is the concentration of $FeSO_4 \cdot 7H_2O$ equivalent to the sample (µmol/mL), V is the sample volume (mL), DF is the dilution factor, and m is the sample mass (g).

Optimization of tyrosinase enzyme and substrate concentrations

Optimization was conducted to determine the optimum concentrations of tyrosinase enzyme and L-DOPA substrate before the inhibition assay.[23] Tyrosinase concentrations of 75-475 U/mL were tested with 4 mmol/L L-DOPA, and absorbance was measured at 490 nm. Subsequent substrate optimization was performed using 1–6 mmol/L L-DOPA with the optimized enzyme concentration (235 U/mL).

Antityrosinase enzyme inhibition activity assay

The inhibition of tyrosinase by n-hexane, ethyl acetate, and methanol fractions was tested in 96-well plates using kojic acid as control, and adapted with slight modifications from the Sigma-Aldrich protocol and

previous studies.[18,19] The reaction was conducted in a 200 µL total volume containing 50 mM phosphate buffer (pH 6.5), 40 µL of the sample solution, 235 U/mL tyrosinase, and 3 mM L-DOPA as the substrate. Appropriate controls were run in parallel, including a blank control (without enzyme and sample) and sample controls where the enzyme was replaced with buffer. The plate was homogenized for 1 minute, then incubated for 30 minutes at room temperature (25°C). Absorbance of the resulting dopachrome was measured at 490 nm using spectrophotometer (Agilent BioTek Epoch 2; United States). Each concentration was assayed in triplicate, and IC_{50} was calculated from the linear regression of the inhibition curve using the formula (equation 6):

$$\% \, Inhibition = \frac{(Blank\ absorbance - blank\ control\ absorbance) - (sample\ absorbance - sample\ control\ absorbance)}{(Blank\ absorbance - blank\ control\ absorbance)} \times 100$$

(equation 6)

Statistical Analysis

All experiments were carried out in triplicate (n=3), and the data were presented as mean ± SD. Differences among the methanol, ethyl acetate, and hexane fractions for TPC, TFC, DPPH, ABTS, FRAP, and anti-tyrosinase assays were analyzed using one-way ANOVA. When significant differences were observed, Tukey's Honestly Significant Difference (HSD) post-hoc test was applied for pairwise comparisons. Statistical analyses were performed using Python (v3.11), and a p-value < 0.05 was considered statistically significant.

LC–QTOF–MS/MS Analysis of Bioactive Constituents in the Ethyl Acetate Fraction

UHPLC–QTOF–MS/MS analysis of the active fractions were performed using an Agilent Revident 1290 Infinity II system (Agilent, Santa Clara, CA, USA) equipped with a binary pump (G7120A) and autosampler (G7129B), coupled to an Agilent QTOF mass spectrometer with an electrospray ionization (ESI) source. Samples were dissolved in methanol and 10 µL was injected into a ZORBAX Eclipse Plus C18 RRHD column (2.1 × 50 mm, 1.8 µm). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), delivered at 0.3 mL/min under a programmed gradient (2–95% B) at 30 °C. The autosampler was maintained at 10 °C. Mass spectrometry was operated in negative ESI mode with a drying gas at 325 °C (8 L/min), nebulizer pressure of 30 psi, sheath gas at 250 °C (11 L/min), capillary voltage of 3500 V, and nozzle voltage of 1000 V. Collision energies were set at 10, 20, and 40 eV. Data were acquired over m/z 100–1500 and processed using Agilent MassHunter Qualitative Analysis 12.0 for tentative compound identification based on accurate mass and MS/MS fragmentation.

Results and Discussion

Extraction and fractionation

Following maceration, the crude extract of *A. bilimbi* leaves yielded 30.2% relative to the dry plant material. The use of 70% ethanol was intentional because this solvent mixture extracts a wider spectrum of constituents than either water or absolute ethanol. [24,25] Successive solvent partitioning produced three fractions, with the ethyl acetate fraction showing the highest yield (35%) (Table 1). This indicates that the extract contains a substantial amount of semi-polar constituents, such as certain flavonoids, alkaloids and phenolic acids. These findings are consistent with previous results,[14] which reported the ethyl acetate fraction of the methanolic extract to be the largest, suggesting semi-polar compounds are key constituents.

Phytochemical screening

Qualitative phytochemical screening showed that phenolics, flavonoids, and terpenoids were present in all fractions, while alkaloids, saponins, tannins, and anthraquinones were exclusive to the more polar ethyl acetate and methanol fractions. Within these general classes, specific compounds have been isolated and identified. For instance, a review highlighted the presence of the triterpene β-amyrin and the terpene squalene,[26] while another study identified n-docosanoic acid and β-sitosterol (triterpene) from a petroleum ether fraction, as well as the glycoside flavonoid apigenin (carambolaflavone) from an ethyl acetate fraction of the ethanolic leaf extract.[27]

Table 1: Percentage yield of extraction and fractionation

Sample/Fraction	Initial weight (g)	Weight obtained (g)	Percentage yield (% w/w)
70% Ethanolic Extract	500	151	30.2%
n-Hexane Fraction	50	7.0	14.0%
Ethyl Acetate Fraction	50	17.5	35.0%
Methanol Fraction	50	8.0	16.0%

Total phenolic and flavonoid content

The biological activities of the extracts are directly linked to secondary metabolites, with total phenolic and flavonoid levels as important quantitative indicators. Based on the results (Table 2), the ethyl acetate fraction, with a polarity index of 4.4, yielded the highest concentration of both phenolics (135.9±1.30 mg GAE/g) and flavonoids (90.3±0.14 mg QE/g). This finding is in agreement with previous studies,[9] which similarly reported that the ethyl acetate fraction of *A. bilimbi* had the highest phenolic and flavonoid content when compared to the n-hexane and ethanol extracts. Phenolic and flavonoid compounds are major contributors to the plant's antioxidant potential.[28] The hydroxyl groups on the aromatic rings readily donate electrons to neutralize free radicals, thereby stabilizing these reactive species and preventing the propagation of oxidative chain reactions.[29]

DPPH, ABTS and FRAP antioxidant assays

To provide a broad antioxidant profile, three in vitro assays (DPPH, ABTS, and FRAP) were employed, using ascorbic acid as the standard (Table 2). Ascorbic acid serves as a potent non-enzymatic antioxidant agent due to its ability to donate two hydrogen atoms during oxidation to form dehydro-L-ascorbic acid.[25] A lower IC_{50} value indicates higher antioxidant strength. For the DPPH assay, IC_{50} < 50 µg/mL was classified as very strong, 50-100 µg/mL as strong, 101-150 µg/mL as moderate, and >150 µg/mL as weak.[22] Based on the DPPH IC_{50} values, the ethyl acetate fraction (37.17 ± 0.17 µg/mL) and methanol fraction (43.2 ± 0.14 µg/mL) demonstrated very strong antioxidant activity, while the n-hexane fraction showed comparatively weaker activity. The standard ascorbic acid, gave an IC_{50} value of 6.45±0.08 in the DPPH assay. This strong activity is attributed to its function as a potent reducing agent, which enables it to readily reduce two radicals from DPPH molecules at once due to its adjacent hydrogen bonds.[30] A similar trend was observed in the ABTS assay, where the ethyl acetate (11.1 ± 0.22 µg/mL) and methanol (13.5 ± 0.25 µg/mL) fractions showed stronger activity than the n-hexane fraction (15.07 ± 0.07 µg/mL). In the FRAP assay, ethyl acetate again exhibited the strongest reducing power (2410 ± 0.47 µmol $FeSO_4$/g extract), followed by methanol (2111 ± 0.81 µmol $FeSO_4$/g) and n-hexane (1515 ± 0.81 µmol $FeSO_4$/g). DPPH and ABTS assays evaluate radical-scavenging activity by determining the IC_{50} values required to quench 50% of free radicals. The DPPH method measures hydrogen atom donation to the stable purple radical, whereas the ABTS assay measures reduction of the blue-green radical cation, and is applicable to both hydrophilic and lipophilic compounds.[31,32] In contrast, the FRAP assay determines reducing power of an extract by measuring its ability to convert Fe^{3+} to Fe^{2+}, with results expressed as AAE or GAE (Ascorbic Acid/Gallic Acid Equivalent) rather than IC_{50}.[33] The findings indicate that semi-polar compounds contained in the ethyl acetate fraction are the primary contributors to the antioxidant potential of *A. bilimbi* leaves.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 2: Results of TPC, TFC, antioxidant, and tyrosinase inhibition assays of *A. bilimbi* leaf fractions

Fractions	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH IC_{50} Value (µg/ml)	ABTS IC_{50} Value (µg/ml)	FRAP (µmol $FeSO_4$/g extract)	Antityrosinase IC_{50} Value (mg/mL)
Methanol	110.9 ± 1.01^b	71.7 ± 0.15^b	43.2 ± 0.14^b	13.5 ± 0.25^b	2111 ± 0.81^b	2.05 ± 0.007^b
Ethyl Acetate	135.9 ± 1.30^a	90.3 ± 0.14^a	37.17 ± 0.17^a	11.1 ± 0.22^a	2410 ± 0.47^a	1.50 ± 0.01^a
Hexane	79.0 ± 0.13^c	48.4 ± 0.15^c	72.1 ± 1.32^c	15.07 ± 0.07^c	1515 ± 0.81^c	4.08 ± 0.02^c
Standard	-	-	6.45 ± 0.08 (AA)	4.2 ± 0.02 (AA)	2846 ± 0.00 (AA)	5.94 ± 0.02 (KA)

Values are mean ± standard deviation of three independent determinations. Different superscript letters within the same column indicate significant differences between groups ($p < 0.05$). AA, Ascorbic acid; KA, Kojic acid; ABTS, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate; DPPH, 1, 1-diphenyl-2-picrylhydrazyl; IC_{50}, half maximal inhibitory concentration; FRAP, ferric-reducing antioxidant power; µmol Fe^{2+}, micromole ferrous ions equivalent. TPC (total phenolic content) was determined using a gallic acid standard curve and expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g); TFC (total flavonoid content) was determined using a quercetin standard curve and expressed as milligrams of quercetin equivalents per gram of dried extract (mg QE/g).

Optimization of tyrosinase enzyme and substrate concentrations

Optimization results for tyrosinase and L-DOPA concentrations are presented in Figure 2. An enzyme concentration of 235 U/mL produced activity within the optimal absorbance range (0.2–0.8 AU) and was selected for subsequent assays. Tyrosinase activity increased with L-DOPA concentrations and reached a maximum rate at 3 mmol/L, after which the curve plateaued due to enzyme saturation. Thus, 3 mmol/L L-DOPA was selected as the optimal substrate concentration for all inhibition assays.

Tyrosinase inhibition assay

The antityrosinase activity of the fractions and the standard was assessed through their IC_{50} values (Table 2). The ethyl acetate fraction showed the strongest inhibition, supported by it linear regression equation ($y = 0.0191x + 21.304$; $R^2 = 0.998$), and lowest IC_{50} (1.5 ± 0.01 mg/mL), which was significantly higher than the methanol and n-hexane fractions ($p < 0.05$). These findings indicate that polar and semipolar phytochemicals are more efficacious than the nonpolar hexane-enriched nonpolar components in inhibiting tyrosinase. Kojic acid, used as a positive control, demonstrated an IC_{50} of 5.94 ± 0.02 µg/mL. Previous studies on *A. bilimbi* leaves reported considerably higher IC_{50} values for crude aqueous (83.48 ± 7.37 mg/mL) and ethanolic extracts (8.66 ± 1.61 mg/mL).[10] In contrast, the marked lower IC_{50} value of the ethyl acetate fraction in the present study demonstrates its higher inhibitory potential, which can be attributed to the enrichment of semipolar constituents through fractionation.

Mechanistically, tyrosinase activity relies on its copper-containing active site. Inhibitors act by either chelating this copper or competing with natural substrates for binding. Kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone) for example, exerts its effect through direct copper chelation.[34] Whereas, compounds such as flavonoids and phenolics bearing hydroxyl (-OH) and carboxylic (-COOH) groups share structural similarities with natural substrates like L-tyrosine and L-DOPA, and therefore can competitively inhibit the enzyme. In addition, flavonoids have been reported to scavenge free radicals or interect with the Cu cofactor, further enhancing their inhibitory activity and preventing dopachrome formation.[35]

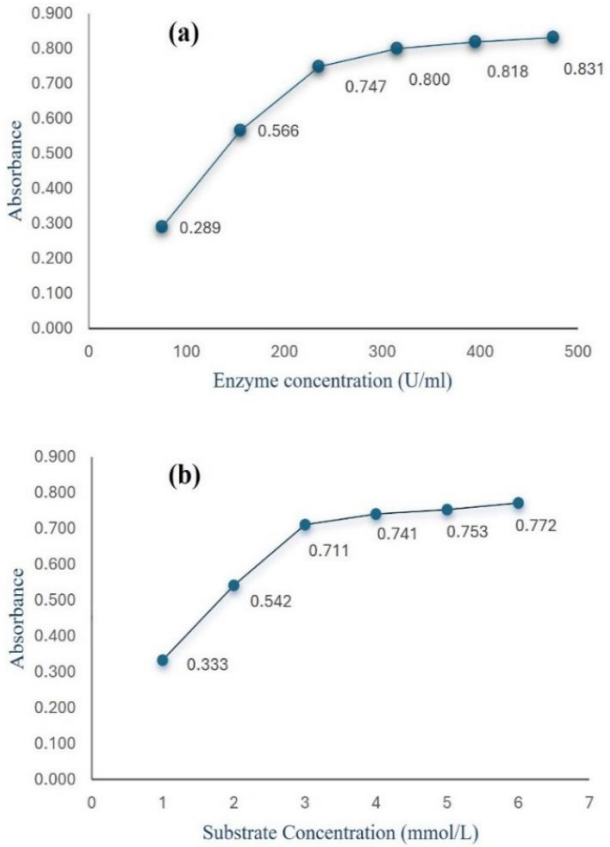

Figure 2: Enzyme and substrate optimization. (a): Optimization curve of tyrosinase enzyme, (b): Optimization curve of L-dopa substrate

Table 3: LC–MS based compound identification in the ethyl acetate fraction of *A. bilimbi* leaves

Annotated Compound	Molecular Formula	Chemical Class	Mass (Da)	RT (min)	MS m/z
D-Maltose	$C_{12}H_{22}O_{11}$	Disaccharide (Sugar)	342.12	0.56	341.11
Coniferin	$C_{16}H_{22}O_8$	Phenolic glycoside (monolignol)	342.13	3.43	401.15
Glucofrangulin	$C_{27}H_{30}O_{14}$	Anthraquinone glycoside	578.16	4.11	577.16
Arctiin	$C_{27}H_{34}O_{11}$	Lignan glycoside	534.21	4.37	579.21
Biochanin A 7-O-rutinoside	$C_{28}H_{32}O_{14}$	Isoflavonoid glycoside	592.18	4.54	591.17
Durantoside I	$C_{26}H_{32}O_{13}$	Flavonoid glycoside	552.18	4.63	551.18
Verbaspinoside	$C_{30}H_{38}O_{15}$	Phenylethanoid glycoside	638.22	4.69	697.23
Salprotoside	$C_{25}H_{30}O_{10}$	Phenolic glycoside	490.18	5.44	535.18
Acrylic acid, n-octyl ester	$C_{11}H_{20}O_2$	Fatty acid ester	184.15	6.51	183.14
Ethyl 2-acetyloctanoate	$C_{12}H_{22}O_3$	Fatty acid ester	214.16	6.60	213.15

Isocyclemone E	$C_{16}H_{26}O$	Sesquiterpene	234.20	9.39	293.21
3-Methylglutaric acid	$C_6H_{10}O_4$	Organic acid	146.06	0.61	191.06

LC-MS: liquid chromatography-mass spectrometry; RT: Retention time; MS: Mass spectra

ANOVA and Tukey's HSD post-hoc test

Significant differences were observed among fractions for all evaluated parameters (TPC, TFC, DPPH, ABTS, FRAP, and antityrosinase), and post-hoc comparisons confirmed that each fraction differed significantly from the others ($p < 0.05$). All pairwise comparisons (methanol vs. ethyl acetate, methanol vs. hexane, and ethyl acetate vs. hexane) were statistically significant, establishing the ethyl acetate fraction as the most potent bioactive fraction of *A. bilimbi* leaves. This suggest that each solvent system extracted distinct chemical profiles responsible for their differing activities.

LC-QTOF-MS profiling of the most active fraction

The LC–QTOF–MS/MS analysis of the ethyl acetate fraction identified multiple secondary metabolites, including flavonoid glycosides (e.g., Biochanin A 7-O-rutinoside, Durantoside I) and phenolic glycosides (Coniferin, Salprotoside, Verbaspinoside), and lignans (Table 3, Figure 3). Notably, Biochanin A 7-O-rutinoside, Coniferin, Glucofrangulin, Arctiin, Durantoside I, Verbaspinoside, and Salprotoside have not been previously reported in *A. bilimbi*, thereby expanding the phytochemical profile knowledge of this species. Phenolic glycosides such as glucose-, xylose-, cellobiose-, and maltose-derivatives exhibit strong tyrosinase inhibition, particularly when free of bulky substituents like methyl or benzoyl groups. Resorcinol-type glucosides are potent tyrosinase inhibitors because their two hydroxyl groups improve enzyme binding.[35] Coniferin, one of the identified metabolites, has previously been tested against mushroom tyrosinase, where it exhibited ~10.54 ± 0.81% inhibition at 25 µM and was noted to possess structural similarity to the well-known inhibitor arbutin.[36] Isoflavonoid glycosides are recognized tyrosinase inhibitors, with their inhibitory activity influenced by hydroxyl substitution patterns and the type of glycosylation present.[35] In this context, Biochanin A 7-O-rutinoside, bearing a C-7 hydroxyl linked to a rutinoside and a conjugated C-4 keto group, retains key functional groups relevant for tyrosinase interaction, thereby indicating potential inhibitory activity. Notably, several of the identified glycosides (e.g., coniferin, arctiin, biochanin A 7-O-rutinoside, durantoside I, verbaspinoside, salprotoside, and glucofrangulin) share aglycone backbones previously reported to inhibit tyrosinase.[2,37] These aglycones (Coniferyl alcohol, Arctigenin, Biochanin A, and other flavones such as apigenin, luteolin, caffeic acid, and hydroxytyrosol) have all been associated with suppression of melanogenesis through direct tyrosinase inhibition or regulation of melanogenic signaling pathways. Moreover, far-infrared–mediated deglycosylation has been shown to enhance the tyrosinase inhibitory activity of certain flavonoid glycosides.[2,34] This pattern suggests that the strong antityrosinase effect of the bioactive fractions could be attributed to the synergistic contribution of phenolic and flavonoid glycosides present in the plant.

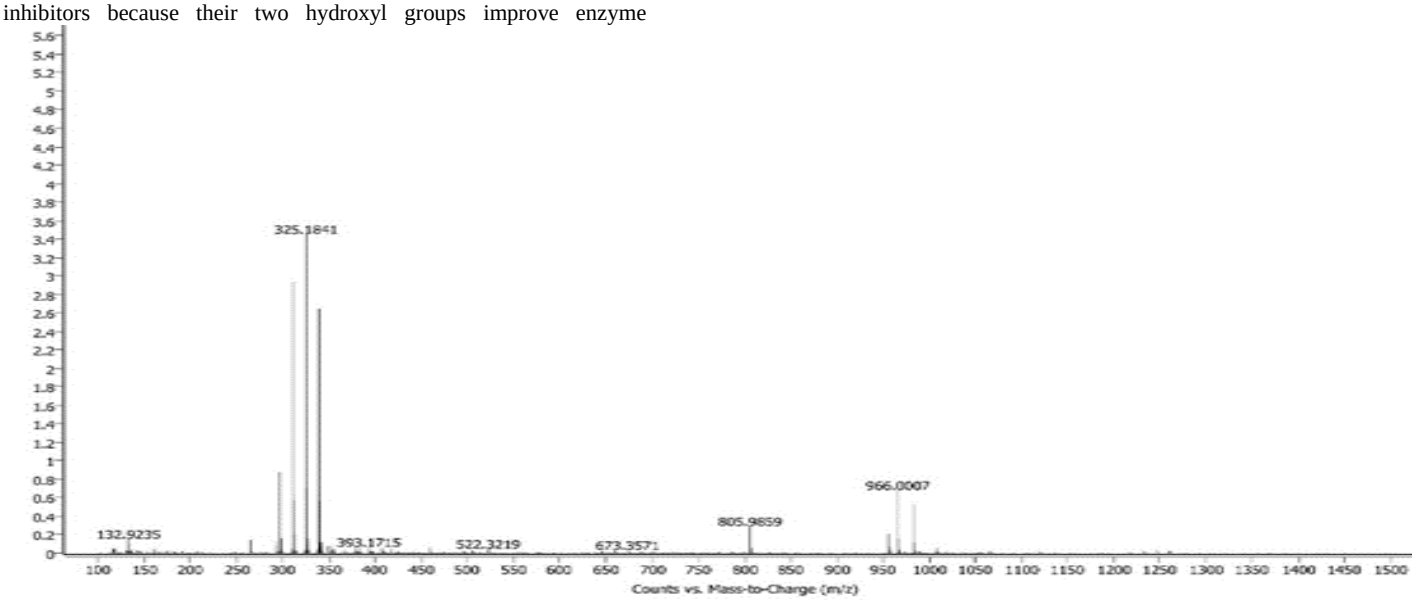

Figure 3: LC-MS chromatogram of ethyl acetate fraction from *A. bilimbi* leaf extract

Conclusion

Sequential liquid–liquid partitioning of *A. bilimbi* leaf extract yielded fractions with distinct phytochemical compositions and biological activities. In vitro and statistical analysis confirmed that the ethyl acetate fraction is the most active among the tested fractions, exhibiting significantly higher phenolic and flavonoid contents, stronger antioxidant capacity, and superior tyrosinase inhibition compared to methanol and hexane ($p < 0.05$). LC-MS profiling revealed it to be rich in phenolics and flavonoid glycosides, with tentative identification of several compounds, including biochanin A 7-O-rutinoside and coniferin, some reported for the first time in this species. These bioactive glycosides have been shown to be potent tyrosinase inhibitors. These findings suggest that the biological potential of *A. bilimbi* are linked to the synergistic contribution of phenolic and flavonoid derivatives in the ethyl acetate fraction, highlighting this plant as a valuable natural source for managing oxidative stress and hyperpigmentation. Therefore, in silico studies are warranted to elucidate the precise binding affinity and mechanism of tyrosinase inhibition of these identified compounds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

The first author gratefully acknowledges Universitas Indonesia for providing the facilities and support for this research. The authors also thank the Research Centre for Pharmaceutical Ingredients and

Traditional Medicine, BRIN, Serpong, Indonesia, for providing the LC-MS analysis facilities and technical assistance.

References

1. Chen J, Liu Y, Zhao Z, Qiu J. Oxidative stress in the skin: impact and related protection. Int J Cosmet Sci. 2021; 43:495–509. https://doi.org/10.1111/ics.12728
2. Hassan M, Shahzadi S, Kloczkowski A. Tyrosinase inhibitors naturally present in plants and synthetic modifications as anti-melanogenic agents: a review. Molecules. 2023; 28. https://doi.org/10.3390/molecules28010378
3. Thawabteh AM, Jibreen A, Karaman D, Thawabteh A, Karaman R. Skin pigmentation types, causes and treatment—a review. Molecules. 2023; 28. https://doi.org/10.3390/molecules28124839
4. Zolghadri S, Bahrami A, Khan MTH, Munoz-Munoz J, Garcia-Molina F, Garcia-Canovas F, Garcia-Molina P. A comprehensive review on tyrosinase inhibitors. J Enzyme Inhib Med Chem. 2019; 34:279–309. https://doi.org/10.1080/14756366.2018.1545767
5. Dini I, Laneri S. The new challenge of green cosmetics: natural food ingredients for cosmetic formulations. Molecules. 2021; 26. https://doi.org/10.3390/molecules26133921
6. Kim HR, Lee SH, Noh EM, Oh BJ, Kim SY, Park MH. Anti-melanogenic effect of Moju through inhibition of tyrosinase activity. Mol Cell Toxicol. 2024; 20(2):243–50. https://doi.org/10.1007/s13273-022-00329-8
7. Alhassan A, Ahmed Q. *Averrhoa bilimbi* Linn.: ethnomedicinal uses, phytochemistry, and pharmacology. J Pharm Bioallied Sci. 2016; 8:265–71. https://doi.org/10.4103/0975-7406.199342
8. Setyawan HY, Sukardi S, Nareswari BF. The phytochemical potential of *Averrhoa bilimbi*: a review. IOP Conf Ser Earth Environ Sci. 2021; 733:012091. https://doi.org/10.1088/1755-1315/733/1/012091
9. Fidrianny I, Rahmawati A, Hartati R. Comparison of different extracts of *Averrhoa bilimbi* L. on antioxidant properties and phytochemical content. Rasayan J Chem. 2018; 11(4):1628–34. https://doi.org/10.31788/RJC.2018.1143091
10. Thummajitsakul S, Silprasit K. Kinetics of tyrosinase inhibition, antioxidant activity, total flavonoid content, and FTIR analysis of *Averrhoa bilimbi* L. extracts and fruit vinegar. Trends Sci. 2023;20(2). https://doi.org/10.48048/tis.2023.3641
11. Suharsanti R, Sugihartini N, Lukitaningsih E, Rahardhian MRR. Tyrosinase inhibitory and sunscreen activity of *Averrhoa bilimbi* leaves n-hexane fraction. AIP Conf Proc. 2023. https://doi.org/10.1063/5.0120288
12. Iwansyah AC, Desnilasari D, Agustina W, Pramesti D, Indriati A, Mayasti NKI, Nurjanah S. Physicochemical properties and mineral content of *Averrhoa bilimbi* L. leaves dried extract: antioxidant and antibacterial activity. Food Sci Technol. 2021; 41(4):987–92. https://doi.org/10.1590/fst.15420
13. Abubakar AR, Haque M. Preparation of medicinal plants: basic extraction and fractionation procedures. J Pharm Bioallied Sci. 2020; 12:1–10. https://doi.org/10.4103/jpbs.JPBS_175_19
14. Duong TH, Tran TMD, To PM, Phan NHN, Nguyen TP, Le HT, Sichame J. Potential antioxidant compounds from spores of *Dicranopteris linearis* and branches of *Averrhoa bilimbi*. Antioxidants. 2024; 13(11). https://doi.org/10.3390/antiox13111319
15. Clearn AK, Elya B, Hanafi M. DPP-IV inhibition and antioxidant activity in leaf, bark, fruit, and seed of *Diospyros foxworthyi*. Int J Agric Biol. 2025; 33(6). https://doi.org/10.17957/IJAB/15.2335
16. Desmiaty Y, Saputri FC, Hanafi M, Prastiwi R, Elya B. Anti-elastase, anti-tyrosinase, and antioxidant activity of *Rubus fraxinifolius* stem extract. Pharmacogn J. 2020; 12(2):271–5. https://doi.org/10.5530/pj.2020.12.42
17. Sultana Chowdhury S, Mezbah Uddin G, Hossain M, Hasan R. In vitro antioxidant and cytotoxic potential of *Averrhoa bilimbi* L. fruits. J Pharm Sci Res. 2012. https://www.researchgate.net/publication/236635051
18. Wiliantari S, Iswandana R, Elya B. Antioxidant activity, tyrosinase inhibition, and stability of face mask cream from *Passiflora ligularis* Juss seed fraction. Res J Pharm Technol. 2023; 16(11):5255–63. https://doi.org/10.52711/0974-360X.2023.00852
19. Nur S, Hanafi M, Setiawan H, Elya B. Chemical characterization and antiaging activity of *Molineria latifolia* root extract: in silico and in vitro study. Biocatal Agric Biotechnol. 2024; 56. https://doi.org/10.1016/j.bcab.2024.103039
20. Bobo-García G, Davidov-Pardo G, Arroqui C, Vírseda P, Marín-Arroyo MR, Navarro M. Microplate methods for total phenolic content and antioxidant activity: validation and comparison. J Sci Food Agric. 2015; 95(1):204–9. https://doi.org/10.1002/jsfa.6706
21. Pereira ACH, Lenz D, Nogueira BV, Scherer R, Andrade TU, Costa HB, Silva EMA, Moreira OA. Gastroprotectiv.e activity of resin from *Virola oleifera*. Pharm Biol. 2017; 55(1):472–80. https://doi.org/10.1080/13880209.2016.1251467
22. Benkhaira N, Koraichi SI, Fikri-Benbrahim K. In vitro methods for antioxidant and biological activity of essential oils: a review. Biointerface Res Appl Chem. 2022; 12:3332–47. https://doi.org/10.33263/BRIAC123.33323347
23. Ambarwati S, Elya B. Tyrosinase inhibitory activity of extracts from *Garcinia lateriflora* Blume var. *javanica* Boerl, *Garcinia fruticosa*, and *Garcinia xanthochymus*. J Pharm Nat Sci. 2024; 2024(1):35. https://doi.org/10.70392/drty6950
24. Lee JE, Jayakody JTM, Kim JI, Jeong JW, Choi KM, Kim TS, Kim YS, Kim MJ. Influence of solvent choice on extraction of bioactives from Asteraceae. Foods. 2024; 13. https://doi.org/10.3390/foods13193151
25. Sari BP, Kustiawan PM, Madu L, Putri R, Yuliati L. Antioxidant activity of extract combination from *Averrhoa bilimbi* L. leaves and stingless bee honey. Indones J Pharm Sci. Technol. 2023; 1. https://doi.org/10.24198/ijpst.v0i0.45987
26. Leliqia NPE, Safitri I. Phytochemical properties, antibacterial activity, and toxicity of *Averrhoa bilimbi* leaves and fruit. J Pharm Sci Appl. 2021; 3(1):32. https://doi.org/10.24843/JPSA.2021.v03.i01.p04
27. Prabhu R, Fernandes R, Govinda KA. Isolation of phytoconstituents and hepatoprotective potential of *Averrhoa bilimbi* leaf extract. J Pharm Res Int. 2021; 573–81. https://doi.org/10.9734/jpri/2021/v33i58B34239
28. Widowati W, Rani AP, Hamzah RA, Arumwardana S, Afifah E, Kusuma HSW, Rihibiha DD, Nufus H, Amalia A. Antioxidant and anti-aging assays of *Hibiscus sabdariffa* extract. Nat Prod Sci. 2017; 23(3):192–200. https://doi.org/10.20307/NPS.2017.23.3.192
29. Platzer M, Kiese S, Herfellner T, Schweiggert-Weisz U, Miesbauer O, Eisner P. Trends and differences in antioxidant assays using SET-based methods. Molecules. 2021; 26(5). https://doi.org/10.3390/molecules26051244
30. Wan Amalina WM, Johari SAT, Aziz MY, Othman AS, Ali AM. DPPH scavenging, total phenolic and flavonoid content of *Catunaregam tomentosa* leaves. J Agrobiotechnol. 2021; 12(2):1–7. https://doi.org/10.37231/jab.2021.12.2.248
31. Gulcin İ, Alwasel SH. DPPH radical scavenging assay. Processes. 2023; 11. https://doi.org/10.3390/pr11082248
32. Munteanu IG, Apetrei C. Analytical methods in determining antioxidant activity. Int J Mol Sci. 2021;22. https://doi.org/10.3390/ijms22073380
33. Okolie NP, Falodun A, Davids O. Antioxidant activity of root extract of pepper fruit (*Dennetia tripetala*) and inhibition of lipid peroxidation. Afr J Tradit Complement Altern Med. 2014; 11(3):221–7. https://doi.org/10.4314/ajtcam.v11i3.31
34. Zolghadri S, Beygi M, Mohammad TF, Alijanianzadeh M, Pillaiyar T, Garcia-Molina P, et al. Targeting tyrosinase in

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

hyperpigmentation: current status and future promises. Biochem Pharmacol. 2023; 212. https://doi.org/10.1016/j.bcp.2023.115574

35. Kim HD, Choi H, Abekura F, Park JY, Yang WS, Yang SH, Kim T, Park J, Lee JE. Naturally occurring tyrosinase inhibitors classified by kinetics and copper chelation. Int J Mol Sci. 2023; 24. https://doi.org/10.3390/ijms24098226
36. Shu P, Zhu H, Liu W, Zhang L, Li J, Yu M, Wu S, Li W. Glycosidic tyrosinase inhibitors from *Typhonium giganteum* rhizomes. Rec Nat Prod. 2021;15(5):380–7. https://doi.org/10.25135/rnp.230.21.02.1965
37. Jiratchayamaethasakul C, Ding Y, Hwang O, Im ST, Jang Y, Myung SW, Kim YI, Park MS, Kwon HJ. Screening of elastase, collagenase, hyaluronidase, tyrosinase inhibitory and antioxidant activities of halophyte plant extracts. Fish Aquat Sci. 2020; 23. https://fas.biomedcentral.com/articles/10.1186/s41240-020-00149-8