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Simplified isolation method of mangiferin from *Mangifera indica* L. leaves and evaluation of tyrosinase inhibitory activity

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Abstract

A method of the isolation of mangiferin from mango (*Mangifera indica* L.) leaves using a modification of Pressured Liquid Extraction (PLE), fractionation liquid-liquid partition (ethyl acetate and n-butanol), and recrystallization was applied in this investigation. Additionally, melting point, infrared (IR) spectroscopy, ultraviolet-visible spectroscopy (UV-Vis), and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy were used to validate the chemical structure of mangiferin. Tyrosinase inhibitory activity of mango leaf extracts were then compared to that of kojic acid. Mangiferin effectively inhibited tyrosinase enzyme (inhibitory concentration (IC_{50}) = 195.50±1.40 µg/mL). It was also observed that n-hexane and ethyl acetate extracts have tyrosinase inhibitory activity (IC_{50}= 210.07±1.37 µg/mL and IC_{50}= 436.79±0.89 µg/mL), respectively. These findings are important preliminary studies for further exploration of both mangiferin and the extract to develop pharmaceutical formulations of this potent tyrosinase inhibitor.

Keywords: Mangiferin, Isolation, *Mangifera indica* L., Tyrosinase inhibitory, Mango leaves

1. Introduction

Mango (*Mangifera indica* L.) is a tropical fruit found easily in Indonesia, and its use is underexploited and less diversified, as well as limited to the need for nutrients and vitamins. A previous study [1] reported that the mango plant contains valuable polyphenolic compounds, which can be developed as a pharmaceutical raw material. According to the study, the aqueous and ethanol extracts contain various phenolic compounds, which can be a potential source of natural antioxidants. Furthermore, mango peel and seed kernel extracts have also proven to be excellent tyrosinase inhibitors, antioxidants, and chelating agents [2,3].

Mangiferin is an important component of mango leaves [4], and it is a group of xanthone C-glycoside, one of the most essential tropical natural drugs [5]. It has shown several pharmacological activities, such as antioxidant [6], anti-diabetic [7], analgetic, anti-inflammatory [8], hepatoprotective [9], antiviral [10], anti-allergic [8], antiparasitic [11], antispasmodic, gastroprotective [12], antifungal [13], anti-microbial [14], and anti-cancer agent [15]. Therefore, it can be developed as a potential application in pharmaceutical and related industries.

The development of isolation methods to obtain mangiferin has interested much research around the world. Meanwhile, various conventional extraction and advanced extraction techniques have been reported. Previously, the isolation of mangiferin using the conventional method was used by Vo et al. [16]. The isolation of Mangiferin was also reported using supercritical CO_2 and low-pressure solvent extraction techniques, ultrasound-assisted extraction [17], and three-phase partitioning coupled with ultrasound from *Mangifera indica* L. leaves [18,19].

There is an increasing demand for more efficient techniques on an industrial scale. Therefore, it is necessary to optimize the extraction and isolation process for improvement and isolation efficiency and the production cost

of mangiferin [20]. In this study, a simplified method of isolating Mangiferin from *Mangifera indica* L. leaves using an environmentally friendly Pressured Liquid Extraction (PLE) was proposed.

PLE is an alternative to a classical version of solvent extraction of solids in the Soxhlet apparatus under atmospheric pressure. It is introduced to water or ethanol as the extraction solvent. Moreover, PLE has been reported to shorten the time needed for sample extraction and reduces the consumption of organic solvents. This technique has also been reported to have some benefits, such as the improvement of the antioxidant power of the obtained extracts, which allows the determination of the authentic content of compounds in the plant material and enables the study of changes in the trace levels of metabolites in plants response to stress [21]. A study revealed that extraction of *Mangifera indica* L. leaf extract enriched with potent antioxidant phenolic compounds using PLE was more efficient than Soxhlet extraction [22].

Melanins, the main pigment responsible for the color of human skin, hair, and eyes, are synthesized by melanocytes during melanogenesis [23], which was mediated by the enzyme tyrosinase and ultraviolet radiation [24]. Arbutin, kojic acid, and hydroquinone are well-known tyrosinase inhibitors used as skin-whitening agents in cosmetic industries due to their anti-pigmentation effect [25]. However, a recent study found that excessive use of kojic acid and hydroquinone has carcinogenic effects [26,27]. Non-toxic natural products utilized in the formulation of cosmetics and pharmaceuticals are of significant interest. Several natural compounds derived from plant sources have traditionally been used as whitening agents and nutritional sources. Currently, the mango leaf is a plant resource with the highest content of mangiferin [28]. Therefore, this study aimed to isolate mangiferin from mango leaves using the PLE method and evaluate the tyrosinase inhibitory activity of mangiferin and its extract.

2. Materials and methods

2.1 Chemicals and reagents

In this study, all chemicals used are analytical grade. Methanol p.a, ethanol 96%, ethyl acetate, *n*-hexane, n-butanol, aqua dest, dimethyl sulfoxide (DMSO) (Merck®), kojic acid (Sigma Chemical), mushroom tyrosinase (Sigma Chemical) and L-3, 4- dihydroxyphenyl alanine (L-DOPA) (Sigma Chemical) were used.

2.2 *Research instruments*

The structure was elucidated using high performance liquid chromatography (HPLC) (Shimadzu LC 10AD), melting point (Fisher – John Melting Point Apparatus), IR spectroscopy (Perkin Elmer), spectrophotometer ultraviolet-visible spectroscopy (UV-VIS) (Shimadzu Pharmaspec 1700) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (Bruker DMX 500®). Evaluation of tyrosinase inhibitory activity was performed on the xMark^{TM} Microplate Absorbance Spectrophotometer (Bio-Rad).

2.3 Plant sample

Leaves of *Mangifera indica* L. (1.5 kg) were collected from the botanical garden of Andalas University, Padang, Indonesia. The plants were identified and authenticated by Dr. Nurainas, M. Si, a taxonomist at Andalas University Herbarium (ANDA) (No.327/K-ID/ANDA/XII/2016).

2.4 Mangiferin isolation: simplified method

Fresh leaves of the plant *Mangifera indica* L. were collected. The isolation process was initiated by extraction and developed according to the principle of PLE [29]. In the static PLE, two valves (inlet and outlet) maintain the pressure in the vessel during extraction. Water is added manually to the extraction vessel. When the vessel is sealed and heated, the pressure increases, and the extraction occurs at the saturation pressure of the system [30]. The 1 kg of fresh mango leaves was cleaned, shredded, and steamed in pressured cooker for ten minutes. It was further transferred to the Erlenmeyer, macerated three times with 500 mL ethanol 96% for 15 minutes on the water bath, and filtered. The filtrates were combined, and the solvent was evaporated at 50°C using a vacuum rotary evaporator until it remained 500 mL. Isolation was then continued by fractionation of liquid-liquid partition using organic solvents, ethyl acetate, and *n*-butanol, sequentially. Subsequently, the solvents were evaporated under a vacuum to get dried yield extracts. The dry extract of the *n*-butanol fraction was recrystallized by redissolving in a 70% aqueous ethanolic solution and left overnight at room temperature. The process was repeated until a yellow powder was obtained (MIFB1).

Compound MIFB1: yellow powder; mp 294-296 ºC; UV (MeOH):), λ max 241, 257, 315, 367 nm, λ max (NaOMe): 233, 271, 383, 389 nm, λmax (NaOAc): 240,260, 377, 472 nm; IR: Vmax = 3364, 1648, 1619, 1250, and 1094 cm^{-1}; ^{1}H and ^{13}C NMR as shown in Table 1.

Table 1 ^{13}C and ^{1}H-NMR Spectral Data for Mangiferin.

Position	^{13}C-NMR (δ)	^{1}H-NMR (δ)
1	161.8	13.75 (br, s)
2	107.6	
3	163.9	
4	93.3	6.36, s
4a	156.2	
5	102.7	6.85, s
6	154.0	
7	143.8	
8	108.1	7.37, s
8a	111.8	
9	179.1	
9a	101.3	
10a	150.8	
Glc-1’	73.1	4.59, d, J= 10 Hz
2’	79	4.03, dd, J=7.5, 9.9 Hz
3’	79.2	3.21, m
4’	70.3	3.14, m
5’	81.6	3.12, m
6’	61.5	3.69, brd J= 10.5 Hz

2.5 Preparation of extract of plant sample

The mango leaves (*Mangifera indica* L.) were dried at 50°C for 48 h in the oven. The dried leaves were chopped mechanically using a commercial stainless-steel blender. A total of 25 g powdered mango leaves were extracted in a Soxhlet apparatus by sequential extraction using solvents of increasing polarity, non-polar (*n*-hexane, 250 mL), followed by semipolar (ethyl acetate, 250 mL) and polar (methanol, 250 mL) solvents (boiling point range 60-80°C) for 8 h on each solvent. A crude methanolic extract of *Mangiferin indica* L. was also prepared using the Soxhlet apparatus. 25 mg powdered mango leaves were extracted directly using 250 mL methanol. Furthermore, the extracts were filtered through a funnel with filter paper Whatman No 1. The extract was concentrated under low pressure at 55°C. The residues obtained were stored at a desiccator to be used later, and all residues were kept in the tightly stoppered bottle until used to evaluate tyrosinase inhibitor activity. The percentage of yield extract was determined according to the following formulation, and was obtained at 5.88%, 3.68%, 6.34%, and 16.2% for *n*-hexane, ethyl acetate, methanol, and crude methanol, respectively.

$$\text{Percentage of Yield Extact} = \frac{\text{Mass of Extract}}{\text{Mass of dry Simplicia}} \times 100\% \quad (1)$$

2.6 Evaluation of tyrosinase inhibitor activity

The method for evaluating tyrosinase inhibitor activity using L-DOPA as substrate was reported by Momtaz et al. [31]. DMSO was used as a negative control to dissolve the extracts, mangiferin, and kojic acid (as positive control). A stock solution of extracts/purified compounds was prepared at 20mg/ml in DMSO. This stock solution was then diluted to 10,000 µg/mL in phosphate buffer, pH 6.5. 50 µL of inhibitor sample solution of different concentrations (62.5-10,000 µg/mL) was mixed with 20 µL of tyrosinase enzyme solution (250 Units/mL in phosphate buffer, pH 6.5) and 30 µL of phosphate buffer in 96-well microtitre plates and incubated at room temperature for 5 minutes. Furthermore, 100 µL of the substrate (5.07 mM L-DOPA) was added to each well. The microtitre plates were incubated at room temperature for 30 minutes. The final concentrations of the extract ranged from 62.5-6000 µg/mL, and the final concentrations of pure compounds and positive controls were 18.7, 37.5, 75, 150, and 300 µg/mL. The optical densities of the wells were determined at 492 nm. The inhibitory concentration 50 (IC_{50}) was used to examine the antityrosinase activity. The evaluation was performed in triplicate and averaged. IC_{50} counting was measured by Finney Program.

2.7 Data analysis

The obtained results were expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was used to analyze the differences among means of IC_{50}, and a $p<0.05$ value was considered statistically significant.

3. Results and discussion

3.1 Mangiferin isolation and characterization

This study aimed to isolate the mangiferin from the mango leaves (*Mangifera indica* L.) using the simplified and expeditious method as well as to evaluate the tyrosinase inhibitory activity. The isolation process was initiated by extraction, which was developed by the principle of PLE. Meanwhile, static PLE is the most widely used mode. Sample and solvent are maintained for a user-specified time at constant pressure and temperature, usually, commercial devices are used [30]. Furthermore, a static PLE was performed using the commercial pressure cooker for ten minutes. The extraction time should be minimal, but sufficient for adequate mass transfer [32]. In this study, water was used as a solvent in the extraction process, which aids in the disruption of matrix-analyte interactions, leading to high recoveries of compounds from the substrates [33]. The high pressure retains solvents in a liquid condition above their boiling point, resulting in high lipid solubility and a high rate of diffusion in the solvent and penetration of the solvent into the matrix. As a result, PLE significantly improves extraction efficiency, reduces extraction time and solvent use, and has excellent reproducibility compared to other approaches [29]. In the conventional method, mangiferin was usually isolated from the polar fraction of mango leaf extract by the normal phase chromatography separation method and then purified using a Sephadex LH-20 [34]. However, these techniques use high volumes of solvents, are time-consuming, and required evaporation steps that cause the degradation of thermolabile compounds [22]. Therefore, the isolation process was simplified by the PLE extraction method and continued by fractionation of liquid-liquid partition and recrystallization. The previous study also reported that the PLE method in 50% ethanol/water of *Mangifera indica* L. leaves extract was favorable to obtaining the highest yields and proved to be more efficient than Soxhlet extraction in terms of the antioxidant activity of enriched phenolic compound extracts [22].

The isolation was then continued by fractionation of liquid-liquid partition using organic solvents, ethyl acetate, and *n*-butanol, sequentially. From the extraction, an 83% purified extract was obtained, measured by HPLC, as indicated in Figure 1. Furthermore, the purified extract was recrystallized with 70% ethanol until needle-shaped pale-yellow crystal MIFB1 (yield 0.119 %) was formed.

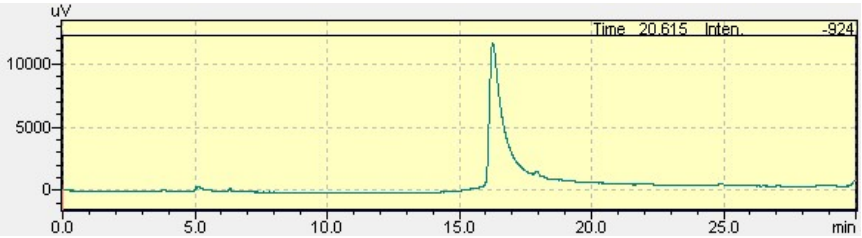

Figure 1 HPLC of purified extract.

The structure of the MIFB1 compound was characterized by melting point, and spectroscopic methods (fourier transform infrared spectrometer (FTIR), UV-Vis, ^{1}H, and ^{13} C NMR) and compared to previous literature. The melting point was determined by the temperature at which a solid becomes liquid at standard atmospheric pressure. A sharp melting point would be described as a pure crystalline compound. The melting point of the MIFB1 compound obtained was 294-296°C. The absorption bands at Vmax 3364, 1648, 1619, and 1250 cm^{-1} in the IR spectrum are characteristic of the hydroxyl, carbonyl, aromatic, and Ar-O-Ar either C-O-C stretch, respectively.

Previous studies have reported that UV spectroscopy is used to determine the structure of xanthones by detecting free hydroxyl groups on the xanthone skeleton. In general, a xanthone is characterized by a spectrum that has four maxima. Band I (225-245), Band II (245-270), Band III (300-345), and Band IV (335-410) indicated the maxima values from UV spectra of xanthones [5].

As shown in Table 1, the ^{1}H-NMR spectra of the isolated compound indicated that the presence of a hydroxyl group δ 13.75 (1H, bs) is a signal from of -OH group. Its position can form a hydrogen bond with a highly hydrophilic group (such as carbonyl). Three aromatic proton signals were at δ 7.37, 6.85, 6.36 (each 1H, s), and the six proton signals of the sugar moiety were at δ 4.59, 4.03, 3.21, 3.14, 3.12, and 3.69. It is indicated that MIFB1 is a xanthone C-glycoside. The ^{13}C-NMR spectrum reveals 19 carbons, suggesting that the structure is a xanthone containing a sugar moiety. The ^{13}C-NMR spectra of the isolated compound indicated that the presence of a carbonyl group δ 179.1 that assigned C-9 and the six-carbon signals of the sugar moiety was at δ 73.1 (C1'), 79 (C2'), 79.2 (C3'), 70.3 (C4'), 81.6 (C5'), 61.5 (C6'). Compared with the corresponding data of the known xanthones in the literature [35], the data identically to Mangiferin (Figure 2).

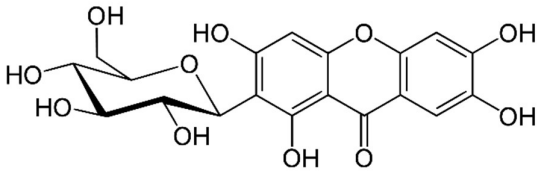

Figure 2 Mangiferin.

3.2 Evaluation of tyrosinase inhibitory activity

Melanins, the primary pigment responsible for the pigmentation of human skin, hair, and eyes, are formed by melanocytes during the process of melanogenesis. Meanwhile, melanogenesis and skin pigmentation are essential photoprotective elements in response to UV radiation damage and skin photo carcinogenesis [23]. Tyrosinase or other related melanogenic enzymes can be downregulated to pharmacologically control melanogenesis. It is the rate-limiting enzyme involved in regulating melanin synthesis [36]. The use of tyrosinase inhibitors is the most promising method for melanogenesis inhibition [37].

A previous study reported positive correlations between mangiferin with total phenolic content, total flavonoid content, and antioxidant activity ($p < 0.05$) [38]. According to Zengin et al. [39], tyrosinase inhibitory activity was also positively correlated with antioxidant activity and total phenolic content of plant extracts.

The tyrosinase inhibitory activity was achieved using L-DOPA as a substrate. In Table 2, it was shown that the mangiferin had stronger inhibition tyrosinase activity than extracts (IC_{50}= 195.50±1.40 µg/mL) but was weaker than kojic acid (IC_{50}= 24.65±0.61 µg/mL). The inhibitory activity of mangiferin may contribute due to the phenolic and carbonyl groups in the structures that play a crucial role in copper chelation. Referring to the property that tyrosinase is a metalloenzyme, copper chelators such as phenolic and polyphenolic compounds, namely mangiferin, can inhibit tyrosinase competitively at the active sites by mimicking the substrate [25]. It was also observed that *n*-hexane and ethyl acetate extracts have tyrosinase inhibitory activity with IC_{50}= 210.07 µg/mL and IC_{50}= 436.79 µg/mL, respectively.

Table 2 Tyrosinase Inhibitory Activity of Mangiferin and Extract of *M. indica* L.

Sample	IC_{50} diphenols (µg/mL)
Crude methanol extract	583.90±1.17^{d}
n-hexane extract	210.07±1.37^{b}
Ethyl acetate extract	436.79±0.89^{c}
Methanol extract	5964.79±27.51^{e}
Mangiferin	195.50±1.40^{b}
Kojic Acid (Positive control)	24.65±0.61^{a}
DMSO (Negative control)	-

Data are expressed as mean ±SD of triple replicates; Statistical significance was determined using one-way ANOVA; Data with different superscripts are significantly different (p<0.05).

Kojic acid has been found to have adverse effects such as tumor promotion, genotoxicity, and weak carcinogenicity. Experts suggest that it should only be used in 1% of cosmetic products [40]. According to the study [41], the skin-lightening effect of kojic acid was statistically significant at a concentration of 4%, indicating that it is not an effective skin-lightening agent in cosmetic products. Therefore, mangiferin may be a potent alternative because it is an abundant source in Indonesia and has no toxic effects after dermal exposure to 2,000 mg/kg [42].

This study was in line with Shi et al. [43], which demonstrated that the ethyl acetate fraction of mango leaves has favorable tyrosinase inhibitory activity with the IC_{50} value of 17.62 ± 1.26 µg/mL. Another study [44] reported interesting results that mango leaves fermented for 8 days at different degrees of ripeness showed higher tyrosinase activity than unfermented ones. The fermented light brown leaves sample (87.96%), fermented light green leaves (80.90%), and fermented green leaves (79.93%) had tyrosinase inhibition activity that was indicated as significant as anti-tyrosinase to kojic acid (86.35%)

Well-known tyrosinase inhibitors, such as arbutin, kojic acid, and hydroquinones, suffer from toxicity and lack efficacy. In contrast, safety is essential for the development of tyrosinase inhibitors as skin-whitening agents in cosmetic industries. For these reasons, it is an excellent opportunity to develop mangiferin and the *n*-hexane and ethyl acetate extracts of mango leaves as alternative tyrosinase inhibitors.

4. Conclusion

This study successfully isolated mangiferin from mango leaves using PLE modification and Fractionation liquid-liquid partition (ethyl acetate and *n*-butanol). Furthermore, mangiferin has shown promising results as a renewable bioresource for the development of tyrosinase inhibitors, although it was weaker than kojic acid. These findings pave the way for further scientific research on mangiferin and studies on extraction and purification procedures. Therefore, further research on mangiferin and its extract are needed to develop therapeutic formulations of this potent tyrosinase inhibitor.

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