




Article

Purification Process of Mangiferin from *Mangifera indica* L. Leaves and Evaluation of Its Bioactivities

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Abstract: Mangiferin (C₁₉H₁₈O₁₁) is a C-glucoside xanthone that is mainly found in the leaves of mango (*Mangifera indica* L.). The compound has been recognized for several pharmacological values, including antioxidant and antibacterial activities. Therefore, the present study aims to utilize the old leaves of mango as a potential source for mangiferin extraction and purification, and evaluate its antimicrobial and antioxidant properties. In the present study, mangiferin was extracted from a local variety of mango leaves using ultrasonic-assisted extraction methods and ethanol as the extraction solvent. The crude ethanolic extract of mangiferin was then purified by the liquid–liquid fractionation method with dichloromethane and ethyl acetate, then further separated by HPLC. The crude extract, ethyl acetate fractions and purified mangiferin were investigated for anti-microorganism activity against *Escherichia coli*, *Salmonella* spp. and *Aspergillus flavus* by using the paper disc diffusion assay. The results have shown that the extraction efficiency was 14.17%. The obtained mangiferin was 1.97 g of content and 94.2% of purity. The oxidation resistance of purified mangiferin was 1.77 times higher than the crude extract and 1.05 times higher than the standard mangiferin (IC₅₀ = 13.841 µg/mL). However, the purified mangiferin has shown no inhibitory action against the experimental strains of microorganisms. The findings from the present study suggest an effective scheme of extraction and purification to obtain mangiferin from the local variety of mango with high purity and antioxidant potential.

Keywords: mangiferin; *Mangifera indica* L.; ultrasonic-assisted extraction; liquid–liquid fractionation; column chromatography; antioxidant activity; antimicrobial activity



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1. Introduction

Mangifera indica L. or mango is one of the common species of the genus *Mangifera* (family Anacardiaceae) [1]. It is a medium-to-large evergreen tree with various parts (roots, barks, leaves, ripe and unripe pulps, seeds, and flowers) that have long been used in ethnomedicines [2]. More than 1000 varieties of mango have been identified and distributed mainly in the tropical areas of South and Southeast Asia [3,4]. During the last decade, the global production and export volume of mango took the leading position in the tropical fruit section, reaching approximately 47.1 and 1.69 million tons, respectively [5,6]. Nowadays, in the situation where the consumers are actively searching for health-promoting alternatives for traditional crops, mango is one of the tropical fruits that has been in high demand [7,8]. The pharmaceutical activities of mango are tremendously diverse, which is considered to derive from the bioactive compounds present in different mango plant

parts, including protein, vitamin A, vitamin C, carotenoids, phenolic compounds (e.g., mangiferin, catechin, quercetin, gallotannins, and iriflophenones), gallic acid, benzoic acid, fiber, carbohydrates, and minerals [9–11]. The phytochemical profile of mango, and consequently its biological effects, can be greatly varied according to the variety, maturity stage and cultivation areas [12].

One of the major constituents of mango leaves, barks, peel and flesh is C-2- β -d-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone or mangiferin (C₁₉H₁₈O₁₁) [13]. Numerous studies have reported a broad range of biological activities displayed by mangiferin, leading to its role as a potential therapeutic agent for pharmaceutical and cosmetic fields [14]. The abundant presence of mangiferin in mango urges for an effective and sustainable extraction process that can utilize all plant parts, especially old leaves, in order to carry the economic implications in minimizing agricultural wastes [15–17]. Previously, mangiferin was extracted by conventional methods such as Soxhlet, heat reflux and maceration extraction [18]. However, these methods consumed a significant amount of time and solvents, and the obtained compound was highly sensitive to the operation conditions [19]. With the recent development of novel extraction methods, such as microwave-assisted extraction, ultrasonic extraction, and subcritical fluid, several studies have reported the effectiveness of mangiferin extraction from various sources [20–23]. For instance, ultrasonic extraction alone and coupled with three-phase partitioning have produced a significantly high extraction efficiency of mangiferin, which were 58.46 ± 1.27 mg/g and 41 mg/g, respectively, under optimal conditions [24,25]. These studies confirmed that the use of ultrasonic extraction improved the mangiferin extraction and shortened the extraction time period, thus this method is economically feasible and highly applicable on an industrial scale. However, the extraction efficiency can be significantly interfered with by the presence of impurities in the crude extract. Furthermore, the biological activities of the desired compound, as well as the safety and production cost, would also be affected, especially in large-scale production [26]. For these reasons, it is highly demanding to develop an effective purification scheme to associate with the extraction process. In this regard, column chromatography is favorably used due to cost effectiveness and applicability of a wide range of stationary phases [27,28]. To our updated knowledge, ultrasonic extraction using the leaves of mango trees grown in Vietnam has not been performed, and a compatible process for mangiferin purification from this source has not been established.

In addition, mangiferin has been well-recognized for its antioxidant and anti-microorganism potentials [29,30]. The antioxidant activity of the compound lies on the formation of mangiferin–iron chelating complexes and the generation of reactive oxygen species, whereas the mechanism of action of its anti-microorganism activity has remained unexplored [31]. Mangiferin exhibited antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria and has also been employed in combinatory therapy to potentiate the anti-staphylococcal activity of several antibiotics [32,33]. Besides, mangiferin and its derivatives at a high concentration (30%) effectively prevented *Thermoascus aurantiacus* and *Aspergillus flavus* [34]. However, the effect of mangiferin purity on its anti-microorganism and antioxidant activities was not highlighted. Although it is known that both the antioxidant and anti-microorganism activities of a compound are closely linked to its purity, to our best knowledge, there are little to no studies on evaluating these activities of mangiferin extracted from a local variety of mango leaves.

Considering all the aforementioned gaps, the present study aims to purify mangiferin from extracts obtained from the ultrasonic extraction of local old mango leaves using liquid–liquid fractionation and the high-performance liquid chromatography (HPLC) technique, then evaluate its antimicrobial and antioxidant capabilities.

2. Materials and Methods

2.1. Plant Materials

Approximately 300 g of fresh and healthy dark-green colored old leaves were harvested from a local orchard located in Da Nang (Vietnam). The harvested leaves were washed thoroughly with tap water for three times, allowed to dry at 45–50 °C for 24 h, ground into fine powder of 1–2 mm in size and stored in zip bags at room temperature to avoid material degradation.

2.2. Chemical and Reagents

The reference mangiferin was purchased from Central Institute of Drug Quality Control of Vietnam (Hanoi, Vietnam) in the form of light yellow powder with 98.6% of purity. Ethanol (EtOH) ($\geq 96\%$), dichloromethane, ethyl acetate, chloroform, NaOH, FeCl₃, HCl, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid (vitamin C) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water used in all experiments was prepared by using a Milli-Q water purification system (EMD Millipore, Billerica, MA, USA). Luria–Bertani (LB) medium, Hansen liquid medium, potato dextrose agar (PDA) and ampicillin (50 µg/mL) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All reagents and chemicals used in the experiments were of analytical grade.

2.3. Ultrasonic Extraction

Ultrasonic extraction was performed in an ultrasonic bath (Elmasonic S100H model, 37 kHz, 600 W) as previously described by Zou et al. [24]. The mango leaves powder (3 g) was dissolved in ethanol 60% in a glass vessel, which was then immersed into water and sonication was proceeded under the following conditions: the liquid-to-solid ratios of 5/1, 10/1, 20/1, 30/1, 40/1 and 50/1 *v/w*, ethanol concentration of 20°, 40°, 60°, 80° and 96°, extraction temperatures of 15 °C, 30 °C, 45 °C, 60 °C and 75 °C and extraction time of 1, 2, 3, 4, 5, 7, 10 and 15 min. After the extraction process had completed, the extract was centrifuged at 13,000 rpm for 15 min and then subjected to an absorbance measurement at the wavelength of 318 nm. The experiment was repeated three times and results were the average values presented as mean \pm standard deviation (S.D.). The yield of crude extracts was calculated using the following formula:

$$\text{Extraction yield} = \frac{\text{total mass of crude extract (g)}}{\text{total mass of mango leaves powder (g)}} \times 100. \quad (1)$$

2.4. Fractionation and Purification of Crude Extract

The crude mango leaves extract was subjected to fractionation and purification by using liquid–liquid fractionation combined with column chromatography as previously described by Singh et al. (2012) [31]. Briefly, the crude extract was dissolved in 60% ethanol and extracted with dichloromethane (1:1 *v/v*) three times for 24 h, forming upper and bottom layers. The upper layer was then continuously extracted with ethyl acetate (1:1 *v/v*) three times for 24 h, forming upper and bottom layers. Both layers were collected for HPLC analysis and the layer that had a high mangiferin content was selected for normal-phase column chromatography, using a glass column filled with silica gel 60 (0.04–0.063 mm) as stationary phase and chloroform:ethanol with increasing polarity (90:10 to 50:50 *v/v*) as mobile phase. Then, mangiferin crystals were collected from vacuum evaporation and impurities were washed with methanol and acetone [35]. The purified mangiferin was analyzed by HPLC.

2.5. High-Performance Liquid Chromatography (HPLC) Analysis

Identification of mangiferin content in the crude extracts, as well as its dichloromethane and ethyl acetate fractions was performed by using HPLC, following the previous protocol with some modifications [33]. Briefly, 0.01 g of crude mango leaves extract, 0.03 g of upper and 0.095 g of bottom layers of ethyl acetate fraction, as well as 1 mg of standard mangiferin

were accurately weighed. Each sample was then dissolved in 60% ethanol and filtered through a 0.22- μm nylon filter (Sigma-Aldrich, St. Louis, MO, USA).

The presence of mangiferin was detected by using Agilent 1260 Infinity II HPLC coupled with a UV/Vis detector and C-18 column (4.6 \times 50 mm, 5 μm). Acetonitrile and acetic acid 0.5% (1:1 *v/v*) were used as the mobile phase. The flow rate and temperature were 0.6 mL/min and 25 $^{\circ}\text{C}$, respectively. A total of 5 μL of each prepared sample was injected onto the column. The detection wavelength was 318 nm. The chromatogram processing involved the use of Agilent ChemStation software (Agilent Technologies, Santa Clara, CA, USA). The mangiferin content and retention time (R.T.) were calculated using a standard curve obtained from reference mangiferin and results were represented as milligrams mangiferin per milliliter of extracts ($\text{mg}\cdot\text{mL}^{-1}$).

2.6. DPPH Scavenging Activity Assay

The antioxidant activity of mango leaves crude extract, ethyl acetate fraction, purified mangiferin and standard mangiferin was tested by DPPH scavenging assay following the procedure by Alam et al. (2019) [36]. In brief, the sample was firstly diluted with ethanol 60 to 1/1700, 1/1500, 1/1300, 1/1100, 1/900, 1/700, 1/300 and 1/100 of the initial concentrations. Then, 2 mg of DPPH was dissolved in 20 mL of 99.7% ethanol. Afterwards, 1 mL of sample and 1 mL of DPPH solution were mixed thoroughly with 99.7% ethanol, vortexed, incubated in the dark at room temperature for 30 min and measured for absorbance (Abs) at 517 nm by using UV-Vis spectrometer (Hitachi, Tokyo, Japan). The negative and positive controls were pure 60% ethanol and vitamin C, respectively. The DPPH scavenging activity (in %) was calculated using Formula (2) as follows. The results were presented as half maximal inhibitory concentration (IC_{50} value) [37].

$$\% \text{ inhibition of the DPPH} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100 \quad (2)$$

where *Abs control* was the Abs of the blank and DPPH mixture and *Abs sample* was the Abs of the fraction and DPPH mixture.

2.7. Antimicrobial Assay

The antimicrobial activities of purified mangiferin, reference mangiferin, crude mango leaves extract, and its ethyl acetate fraction were investigated by using disc diffusion method as previously described by Mostafa et al. (2018) [38]. *E. coli* (ATCC 25922), *Salmonella* spp. (ATCC 700623), and *A. flavus* (NBRC 33021) were kindly provided by the Institute of Biotechnology, Hue University, Vietnam. The bacterial strains and the fungal strain were maintained in LB broth and liquid Hansen medium (pH = 5.6) at -80°C , respectively. Before the experiment, a seed culture of each strain was prepared by inoculating a single colony in fresh media and incubating for 12 h at 37°C under continuous agitation (100 rpm). The suspensions were then diluted with the same types of media to yield a final cell density of approximately 10^6 colony-forming units (CFU) per mL at 600 nm wavelength.

The prepared cultures of *E. coli*, *Salmonella* spp. and *A. flavus* (10 μL) were uniformly spread onto the surface of LB agar plates (for *E. coli* and *Salmonella* spp.) and PDA plates (for *A. flavus*) using a spreader and allowed to dry for 5 min. Meanwhile, the purified mangiferin, reference mangiferin (which had been dissolved in distilled water), crude extract, and ethyl acetate fraction at concentrations of 12.5, 25, 50, 100 and 200 mg/mL were pipetted onto sterilized Whatman paper discs (6 mm in diameter) which were placed on an empty sterile Petri dish and allowed to dry for about 20 min. Ampicillin antibiotic (50 $\mu\text{g}/\text{mL}$) which acted as positive control for the assay was also applied following the same procedure. Subsequently, these sample- and control-loaded paper discs were carefully and aseptically transferred onto the inoculated agar surface. The plates were incubated at 37°C for 24 h. After 24 h of incubation, the diameter of the inhibition zone (mm)

surrounding the paper discs was measured. The experiment was performed in triplicates and the results of inhibition zone measurements were presented as mean \pm S.D.

2.8. Statistical Analysis

Statistical analysis in the present study was performed using Excel 2010 and Minitab software version 16 (Minitab, Pennsylvania State University, PA, USA). All results from the present study were subjected to one-way ANOVA and were expressed as mean \pm S.D. The difference between means was calculated by using Tukey's multiple comparison test and was considered to be significant at p -value < 0.05 .

3. Results and Discussion

3.1. Effects of Extraction Methods on the Mangiferin Content

3.1.1. Effect of Liquid-to-Solid Ratio

The effect of different liquid-to-solid ratios on the mangiferin content was examined while the other conditions were set as follows: extraction temperature of 60 °C and extraction time 10 min. The results demonstrated in Figure 1A have shown that the content of mangiferin decreased significantly from 0.814 to 0.372 Abs as the liquid-to-solid ratio decreased from 5/1 to 50/1. The mangiferin content at 10/1 of liquid-to-solid ratio was not significantly different to that of 5/1 ($p < 0.05$). Furthermore, this ratio allowed the compound to dissolve in a sufficient quantity of solvents, thus facilitating the subsequent collection of the extract. Therefore, the liquid-to-solid ratio of 10/1 was selected for the consecutive experiment.

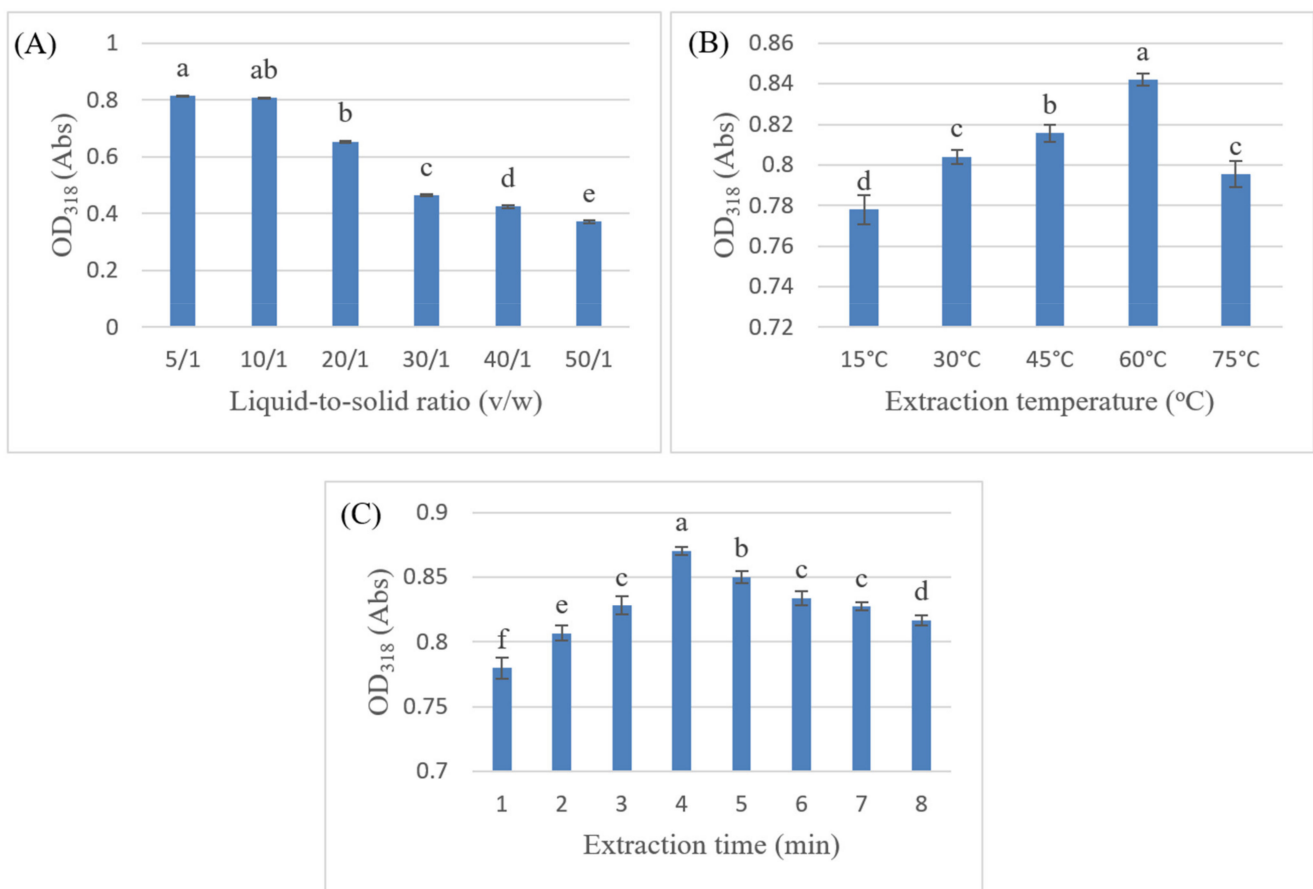


Figure 1. Effects of different parameters of extraction method on the mangiferin content, as follows: (A) effect of liquid-to-solid ratio (v/w) on the mangiferin content; (B) effect of extraction temperature (°C) on the mangiferin content; and (C) effect of extraction time (min) on the mangiferin content. Values showing different letters in the same column indicated significant difference ($p < 0.05$).

3.1.2. Effect of Extraction Temperature

The effect of different extraction temperatures on the mangiferin content was examined while the other conditions were set as follows: liquid-to-solid ratio of 10/1 and extraction time 10 min. Overall, as shown in Figure 1B, the mangiferin content increased significantly from 0.778 to 0.842 Abs when the extraction temperature increased from 15 °C to 60 °C. However, as the temperature continued to rise to 75 °C, the content started to decline to 0.79533 Abs. Previous studies have also reported that the temperature of 60 °C was suitable to promote the solubility and desorption of mangiferin [24]. In the present study, increasing the extraction temperature level to higher than 60 °C has resulted in solvent evaporation and mangiferin decomposition. For this reason, the extraction temperature of 60 °C was selected for consecutive experiments.

3.1.3. Effect of Extraction Time

The effect of different extraction times on the mangiferin content was examined while the other conditions were set as follows: liquid-to-solid ratio of 10/1, ethanol concentration 60° and extraction temperature of 60 °C. The results are demonstrated in Figure 1C. It can be observed that the mangiferin content reached the maximum level (0.87 Abs) after 4 min of extraction. However, as the extraction time prolonged from 5 to 15 min, the mangiferin content slowly declined. This observation could be explained by the fact that as mangiferin has achieved the equilibrium state of dissolution at 4 min, extending the extraction time to more than 4 min would cause evaporation of the solvent as well as exposure to the environmental degradation of the compound [39]. Therefore, 4 min was selected as a suitable extraction time for consecutive experiments.

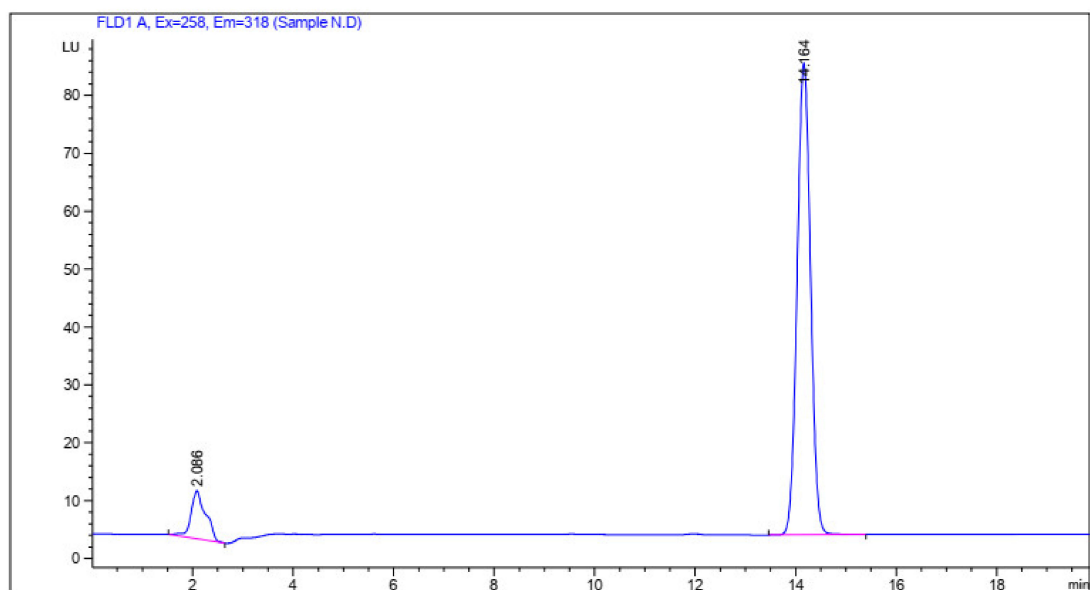
3.2. Mangiferin Quantification by HPLC Analysis

In the present study, the crude extract of local mango leaves has been produced by using the ultrasonic extraction method and ethanol as the extraction solvent. The extraction efficiency as calculated from Equation (1) was 14.17% (*w/w*), which was marginally higher than the results of a previous work by Akinpelu and Onakoya (2006) (13.93% *w/w*) [40]. However, our procedure showed superiorities in terms of the extraction time and economic efficiency. To be specific, Akinpelu and Onakoya (2006) selected methanol as the solvent and the extraction process was prolonged for 4 days, while the current process with the combination of ethanol solvent and ultrasonic extraction yielded a similar efficiency (14.17%) and only required 4 min of extraction time [40]. For this reason, the current process can be considered as an improvement in terms of the extraction efficiency and time, thereby providing an essential insight for future studies on optimization and industrial-scaled experiments.

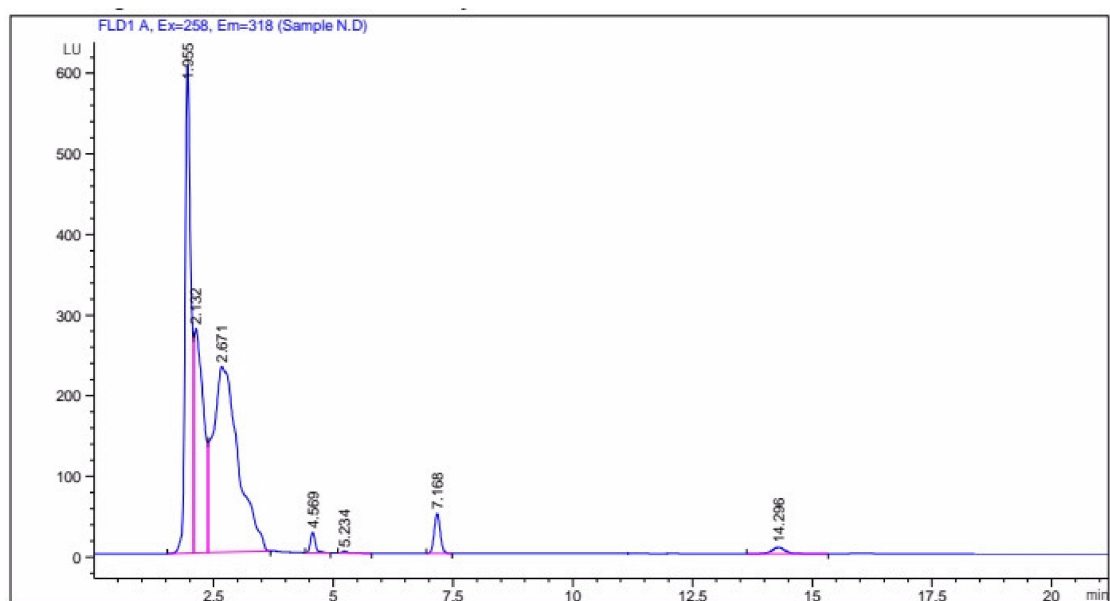
The results of the HPLC analysis of mangiferin in the crude extract of mango leaves obtained from UE are presented in Table 1 and Figure 2. Briefly, when analyzing the crude extract, there are seven peaks shown with different R.T. and peak areas (Figure 2A). Two out of these seven peaks were shown at 2.132 and 14.296 min, which were relatively close to the peaks of the standard mangiferin, shown at 2.086 and 14.164 min (Figure 2B). Similar results have been obtained by Fernández et al. (2015) [41], confirming the presence of mangiferin in the crude mango leaves extract. However, the peaks shown at 2.132 min and at 2.086 min of crude extract and standard mangiferin, respectively, were most likely corresponding to mangiferin isomers such as isomangiferin and homomangiferin, which are present in mango leaves extract in a minor quantity [2,42]. Therefore, based on the concentration and the peak area of the crude mango leaves extract shown at 14.296 min, the presence of mangiferin was identified at a content of 9.510 mg/g.

Table 1. HPLC analysis of mangiferin content (mg/g) in standard mangiferin, crude mango leaves extract, upper layer of dichloromethane fraction, bottom and upper layers of ethyl acetate fraction and purified mangiferin.

Sample	Conc. (g/mL)	Retention Time (min)	Peak Area (LU*s)	Mangiferin Content (mg/g)
Standard mangiferin	0.001	14.164	1506.542	1000
Crude leaves extract	0.01	14.296	143.269	9.51
Dichloromethane fraction (upper layer)	0.01	14.286	637.637	42.325
Ethyl acetate fraction (upper layer)	0.03	14.286	4846.101	107.223
Ethyl acetate fraction (bottom layer)	0.095	14.271	125.46	0.877
Purified mangiferin	0.8	14.103	1135.188	941.882

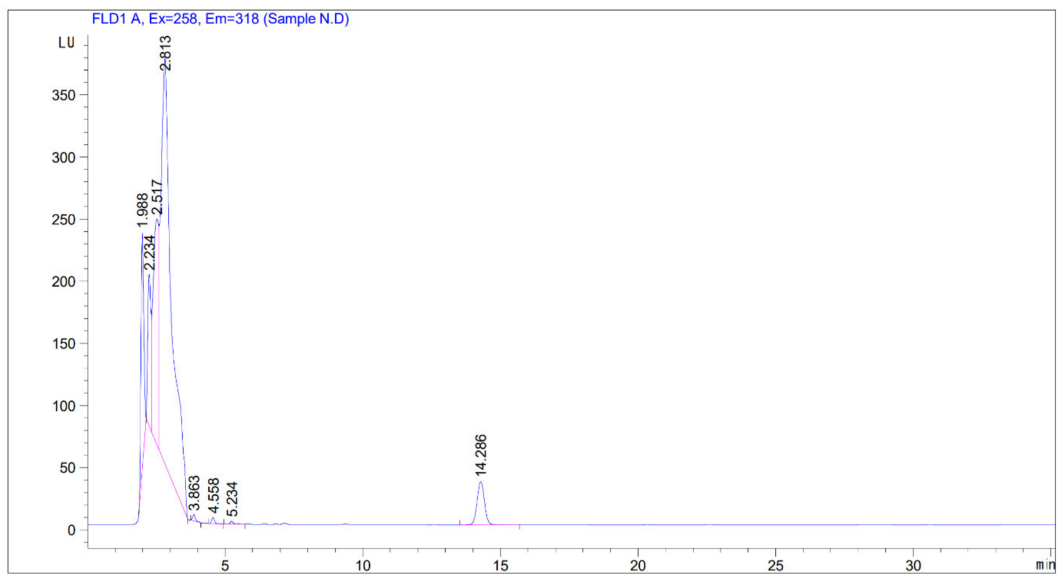


(A)

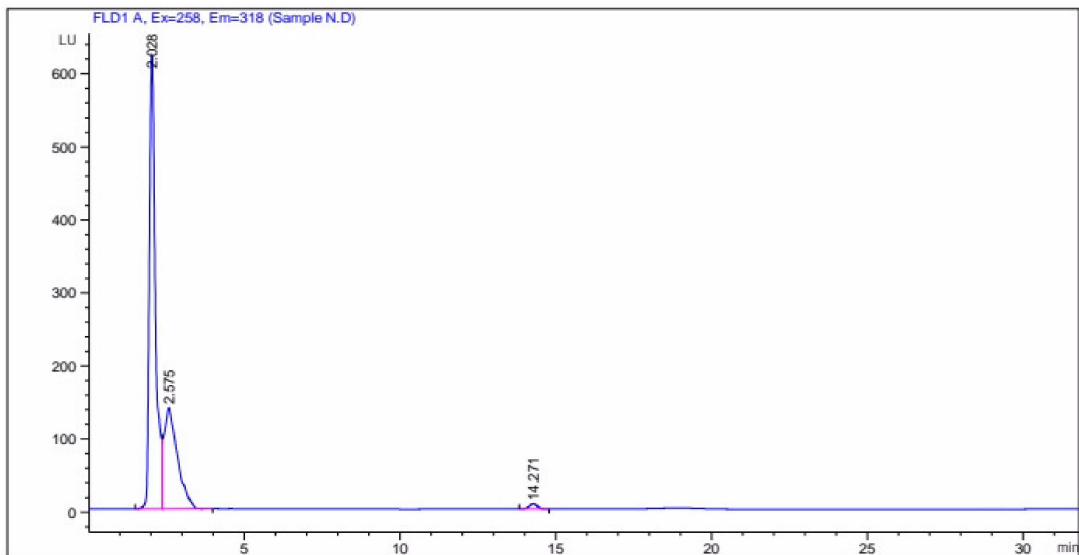


(B)

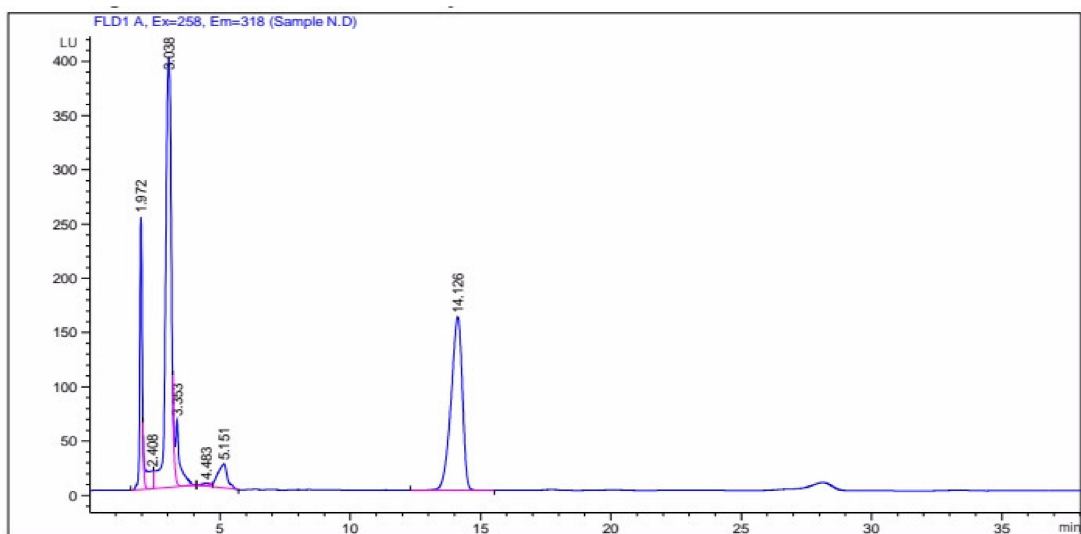
Figure 2. Cont.



(C)



(D)



(E)

Figure 2. Cont.

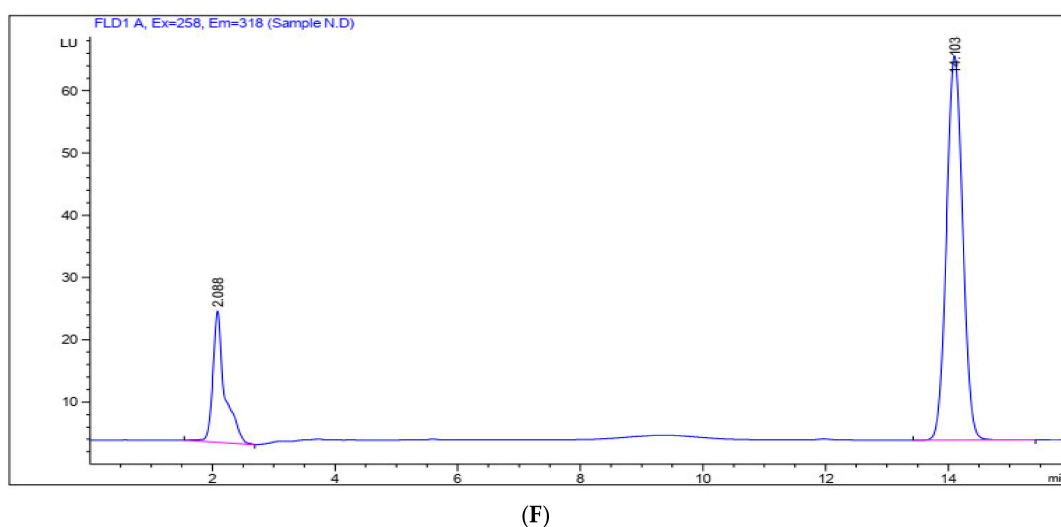


Figure 2. Results of HPLC analysis of (A) standard mangiferin, (B) crude mango leaves extract, (C) upper layer of dichloromethane fraction, (D) bottom layer of ethyl acetate fraction, (E) upper layer of ethyl acetate fraction, and (F) purified mangiferin.

In the present study, purification was performed to purify mangiferin from the crude extract of old mango leaves. This step aims to remove the impurities (e.g., colorants and compounds with weak polarity), which interfere with the efficiency of the extraction process as well as the biological activities of mangiferin [27,43]. Previously, mangiferin has been successfully purified from Chinese mango cultivars by using macroporous HPD100 resin chromatography combined with high-speed counter-current chromatography (HSCCC) [43]. However, studies on mangiferin purification from old leaves of mango trees grown in Vietnam have not been established. Therefore, in this study, mangiferin was purified from Vietnamese mango leaves extract by liquid–liquid fractionation (e.g., dichloromethane and ethyl acetate) and column chromatography.

The results from the HPLC analysis revealed the presence of mangiferin in the upper layer of the dichloromethane fraction, as well as both layers of the ethyl acetate fraction (Table 1). Firstly, for the dichloromethane fraction, the bottom layer mostly contained impurities while the upper layer showed eight peaks, and the peak shown at 14.286 min of R.T. corresponded to mangiferin presence (Figure 2C). The mangiferin content of the upper layer of the dichloromethane fraction was calculated as 42.325 mg/g and was taken for subsequent fractionation with ethyl acetate. Secondly, for the ethyl acetate fraction, mangiferin presence was detected in both the upper and bottom layers, as shown by the peaks at 14.271 and 14.286 min, respectively (Figure 2D,E). However, as the mangiferin content was more concentrated in the upper layer than the bottom layer (Table 1), the upper layer of the ethyl acetate fraction was selected for column chromatography. After the process had finished, washing with acetone and methanol helped remove the remaining solvent and impurities without dissolving the mangiferin crystals. As evidenced by the HPLC results, the mangiferin obtained after the two-step purification process showed two peaks at 2.088 and 14.103 min, which approached closely to the peaks of standard mangiferin at 2.086 and 14.164 min (Figure 2F). Therefore, similar to the standard mangiferin, the purified mangiferin may also contain a small quantity of mangiferin isomers, which corresponded to the peak shown at 2.088 min. Therefore, based on the peak shown at 14.103 min, the content of the purified mangiferin was identified at a content of 1.97 g (Table 1).

In the present study, a total amount of 1.97 g of mangiferin was obtained from the extraction and purification of a local variety of mango leaves. The compound recovery was 0.82% and the purity was 94.2%. This result was compared to previous studies, which were also performed on the leaves of mango trees grown in Vietnam yet employed macroporous D101 and solvents of a different polarity [44,45]. In these studies, it can be seen that the recovery and purity of the obtained mangiferin were ranged from 0.37–0.49% and 68–72%,

respectively, which were lower than the present study. The purity of the mangiferin in the present study can be comparable to Nian et al. (2016), in which mangiferin of 92.15% purity was extracted from *Anemarrhenae* Rhizome by using polyamide and macroporous HPD400 adsorption resins chromatography [46], as well as Luo et al. (2012), in which mangiferin (99.13% purity) was purified from Chinese mango cultivars using macroporous HPD100 resin chromatography associated with HSCCC [43]. Based on the obtained results, the present study proposed that liquid–liquid fractionation can be combined with column chromatography to form a cost-effective purification scheme for mangiferin from mango leaves extraction. Furthermore, HPLC analysis has confirmed that with well-developed extraction and purification techniques, the local mango leaves appeared to be a highly available source of mangiferin, thus adding to the value of this by-product and reducing agricultural waste.

3.3. Antioxidant Activity of Purified Mangiferin

The antioxidant activity of crude ethanol extract, ethyl acetate fractions, standard mangiferin, and purified mangiferin obtained from UE assisted with the purification, as shown in Table 2.

Table 2. Antioxidant activity of crude extract, ethyl acetate fractions, standard mangiferin and purified mangiferin from mango leaves.

Sample	IC ₅₀ (µg/mL)	Regression Equation	r ²
Crude extract	27.522	y = 2.3945 x ^{0.9167}	0.9918
Ethyl acetate fraction (bottom layer)	68.769	y = 8.0215 x ^{0.6964}	0.9915
Ethyl acetate fraction (upper layer)	15.548	y = 4.7598 x ^{0.8571}	0.997
Standard mangiferin	16.383	y = 4.1919 x ^{0.8865}	0.9925
Purified mangiferin	13.841	y = 1.7396 x ^{0.7938}	0.9978
Vitamin C	2.551	y = 33.427 x ^{0.43}	0.9915

As shown in Table 2, the purified mangiferin displayed a significantly low IC₅₀ value (IC₅₀ = 15.548 µg/mL), as compared to the standard mangiferin (IC₅₀ = 16.383 µg/mL), upper and bottom layers of the ethyl acetate fraction (IC₅₀ = 15.548 and 68.769 µg/mL) and the crude extract (IC₅₀ = 27.522 µg/mL) ($p < 0.05$). In comparison with previous studies, the free radical scavenging activity exhibited by mangiferin obtained from the present study was 2.48 and 1.43 times higher than the mangiferin purified by macroporous D101 resin (IC₅₀ = 38.5 µg/mL) and by solvents with varied polarization (IC₅₀ = 22.2 µg/mL) [45,46]. It is widely accepted that the evaluation of the biological activities of plant-based extracts such as antioxidant and anti-microorganism could provide essential insights into the efficiency of the extraction and purification methods. Therefore, from the results of the present study, it can be concluded that the combination of ultrasonic extraction, liquid–liquid fractionation and column chromatography have effectively produced mangiferin with a high antioxidant potential. Along with the benefits of lowered energy and extraction time consumption, this combinatory method is therefore highly recommended for mangiferin extraction.

3.4. Antibacterial Activity of Purified Mangiferin

The crude extracts, ethyl acetate fraction (upper layer), purified and standard mangiferin at various concentrations (12.5–200 mg/mL) were evaluated for anti-microorganism activity against *E. coli*, *Samonella* spp. and *A. flavus* by measuring the diameter of the inhibition zone which appeared after 24-h incubation at 37 °C.

As shown in Table 3, the crude extract inhibited the growth of *E. coli* in a concentration-dependent manner, with the inhibition zones ranging from 7.7 ± 0.3 to 15.6 ± 0.5 (mm). In particular, at the concentration of 25 mg/mL, this activity of mango leaves extract was lower than the methanol extract from mango stem bark, whose inhibition zone was measured as 12 mm at the concentration of 20 mg/mL [40]. In contrast, the upper layer of

the ethyl acetate fraction of the mango leaves extract showed a concentration-dependent inhibition against both *E. coli* and *Samonella* spp., indicated by the range of inhibition zones from 11.3 ± 0.4 to 21.6 ± 0.5 (mm) and from 11.5 ± 0.5 to 23.5 ± 0.5 (mm), respectively. As compared to Sija (2009), the antibacterial activity of the ethyl acetate fraction of mango leaves extract in the present study was improved against *Samonella* spp., while being comparably effective against *E. coli* [47]. The inhibitory activity by the ethyl acetate fraction of the mango leaves extract has been proposed to derive from the presence of mangiferin as well as several flavonoids and alkaloids that were previously shown to exhibit a high antibacterial effect [2]. No inhibition against *E. coli* and *Samonella* spp. was observed in the presence of both purified and standard mangiferin. All of the tested samples were unable to inhibit the growth of *A. flavus*. The inability of mangiferin to inhibit the fungal species in this study was contradictory to previous reports by Singh et al. (2012) [31], Stoilova et al. (2008) [34] and Raju et al. [48], where a high concentration of mangiferin was able to exert a killing effect on *A. flavus* and *Thermoascus aurantiacus*. Overall, these results have indicated that (1) the ethyl acetate fraction of mango leaves extract exhibited strong antibacterial activity against *E. coli* and *Samonella* spp. at 12.5–200 mg/mL, and (2) this action was not attributed to the presence of a mangiferin component.

Table 3. Anti-microorganism activity of purified mangiferin against *E. coli*, *Samonella* spp. and *A. flavus* by using disc diffusion assay.

Extract	Conc. (mg/mL)	Inhibition Zone (mm)		
		<i>E. coli</i>	<i>Samonella</i> spp.	<i>A. flavus</i>
Crude extract	200	15.6a \pm 0.5	-	-
	100	11.4b \pm 0.4	-	-
	50	10.4c \pm 0.2	-	-
	25	9.5d \pm 0.3	-	-
	12.5	7.7e \pm 0.3	-	-
Ethyl acetate fraction (upper layer)	200	21.6a \pm 0.5	23.5a \pm 0.5	-
	100	17.5b \pm 0.5	17.9b \pm 0.3	-
	50	14.6c \pm 0.5	15c \pm 0.4	-
	25	13.2d \pm 0.3	13.9d \pm 0.4	-
	12.5	11.3e \pm 0.4	11.5e \pm 0.5	-
Purified mangiferin	200	-	-	-
	100	-	-	-
	50	-	-	-
	25	-	-	-
	12.5	-	-	-
Standard mangiferin	200	-	-	-
	100	-	-	-
	50	-	-	-
	25	-	-	-
	12.5	-	-	-
Ampicilin (positive control)	200	12	19	-
	100	12	19	-
	50	12	19	-
	25	12	19	-
	12.5	12	19	-

The experiment was performed in triplicates. Values showing different letters in the same column indicated significant different ($p < 0.05$). “-” indicated no inhibition zone in case of bacteria or sparse distribution in case of fungal spores.

4. Conclusions

Mangiferin is one of the major constituents of mango that contributes to several valuable biological activities of the plant. The old leaves from a local variety which are often considered as wastes were utilized as the raw materials for the present study. By carrying out UE and developing an effective purification method that consisted of liquid–liquid fractionation and column chromatography, a total amount of 1.97 g of pure mangiferin with 0.82% of recovery and 94.2% of purity was obtained from the crude extract of the old mango leaves. The purified mangiferin showed improved scavenging activity against the DPPH free radicals, with $IC_{50} = 13.841 \pm 0.81 \mu\text{g/mL}$, which was higher than the crude extracts and its ethyl acetate fractions. However, the purified compound alone exhibited no inhibition against *E. coli*, *Salmonella* spp. and *A. flavus*. The findings from the present study provide a helpful insight into the utilization of the old leaves of mango as a promising source of mangiferin extraction to reduce agricultural wastes, and they show that ultrasound combined with fractionation and column chromatography are effective extraction and purification methods to obtain mangiferin with a high purity and antioxidant potential.

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