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Article

Simultaneous HPLC Quantitative Analysis of Active Compounds in Leaves of *Moringa oleifera* Lam.

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***Moringa oleifera* Lam. has been used as a traditional medicine for the treatment of numerous diseases. A simultaneous high-performance liquid chromatography (HPLC) analysis was developed and validated for the determination of the contents of crypto-chlorogenic acid, isoquercetin and astragalin, the primary antioxidative compounds, in *M. oleifera* leaves. HPLC analysis was successfully conducted by using a Hypersil BDS C18 column, eluted with a gradient of methanol–1% acetic acid with a flow rate of 1 mL/min, and detected at 334 nm. Parameters for the validation included linearity, precision, accuracy and limits of detection and quantitation. The developed HPLC method was precise, with relative standard deviation < 2%. The recovery values of crypto-chlorogenic acid, isoquercetin and astragalin in *M. oleifera* leaf extracts were 98.50, 98.47 and 98.59%, respectively. The average contents of these compounds in the dried ethanolic extracts of the leaves of *M. oleifera* collected from different regions of Thailand were 0.081, 0.120 and 0.153% (w/w), respectively. The developed HPLC method was appropriate and practical for the simultaneous analysis of crypto-chlorogenic acid, isoquercetin and astragalin in the leaf extract of *M. oleifera*. This work is valuable as guidance for the standardization of the leaf extracts and pharmaceutical products of *M. oleifera*.**

Introduction

Moringa oleifera Lam. (Moringaceae) is an evergreen tree, native to India or sub-Himalayan areas and widespread throughout the tropical and subtropical areas. Different parts of this plant have been traditionally used for medicinal purposes as an antioxidant, anti-inflammatory and anti-cancer agent (1, 2). The leaves are used as antibacterial, anti-diabetic and cardiotonic agents, and also for the treatments of stomachaches, sprains and fever (1, 3). Previous phytochemical studies demonstrated that the leaves of *M. oleifera* contain glucosinolate glycosides and complex flavonoid compounds, which are responsible for anti-atherosclerotic, antioxidative and anti-diabetic activities (4–6). In addition, the leaves are extensively used as food supplements in Thailand as an adjuvant for treatment of diabetes, cardiovascular disorders, and inflammation (7).

To standardize the leaf extracts of *M. oleifera*, analytical methods have been developed for the quality assessment of these leaves. Liquid chromatography–mass spectrometry (LC–MS) was used for the quantitative analysis of several phytochemicals in the leaf extract of *M. oleifera* (8). However, this instrument requires high managerial costs. High-performance liquid chromatography (HPLC) was used to determine the nitrile glycosides, niaziridin and niazirin contents in leaves and pods of *M. oleifera* (9), and the α and γ-tocopherol contents in the leaves, flowers and fresh beans of *M. oleifera* (10). Nevertheless, there has been no report on the contents of bioactive phenolics and flavonoids related to cardiovascular disorders and antioxidation of *M. oleifera* leaf extracts (4–6). Therefore, this study was conducted to develop and validate HPLC conditions for the quantitative analysis of three major active compounds: crypto-chlorogenic acid, quercetin 3-O-glucoside (isoquercetin) and kaempferol 3-O-glucoside (astragalin) in the ethanolic extracts of *M. oleifera* leaves collected from four different locations in Thailand.

Experimental

Materials and reagents

HPLC grade methanol was purchased from Fisher Scientific (Leicester, UK). Deionized water was purified by Water Pro PS (Labconco, Kansas City, MO). Glacial acetic acid was purchased from Sigma-Aldrich (Singapore). All other reagents were of analytical grade. *M. oleifera* leaves were collected from four locations in the northern, northeastern, southern and central regions of Thailand from January to March 2011. The samples were identified by Dr. W. Gritsanapan and the voucher specimens (BVMO11001–BVMO11004) were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Thailand. The leaves were dried in a hot air oven at 60°C for 24 h. The dried samples were ground and passed through a sieve (20 mesh) and stored at room temperature (28–30°C) in a dark place until use.

Separation of crypto-chlorogenic acid, isoquercetin and astragalin

Dry powdered leaves of *M. oleifera* (300 g) were macerated with frequent agitation for 48 h with 70% ethanol (5 L × 3). The ethanol filtrates were combined and dried under reduced pressure by using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland). A portion of dried extract was suspended in distilled water (1:10, w/v) and the solution was extracted with *n*-hexane (200 mL × 3) and dichloromethane (200 mL × 3), respectively. The remaining aqueous fraction was partitioned with *n*-butanol (200 mL × 9). The *n*-butanol fractions were combined and evaporated to dryness by a rotary evaporator. This fraction was suspended in distilled water (1:10, w/v) and partitioned with ethyl acetate (250 mL × 3). The dried ethyl acetate fraction

(1 g) was subjected to column chromatography (3 × 30 cm, Sephadex LH-20) with methanol–water (9:1, v/v, 300 mL) as a mobile phase. The collected fractions were monitored by using thin-layer chromatography (TLC) to obtain 60 fractions (5 mL each) and combined to produce 12 primary ethyl acetate fractions. Fractions 5–7 were pooled together as fraction AC1, which after concentration using a rotary evaporator, yielded approximately 200 mg of dried extract. AC1 was re-subjected to another round of Sephadex LH-20 column chromatography using acetone–water (85:15, v/v, 200 mL) as a mobile phase. Ten fractions (20 mL each) were obtained and Fractions 7–8 were combined to produce Fraction C1 (approximately 25 mg), which was further subjected to low-pressure column chromatography (Lobar) using a reversed-phase C18-column (Merck, Darmstadt, Germany) and methanol–water (3:7, v/v, 30 mL) as a mobile phase. The fractions were collected according to the peaks on the chromatograph. The pure compound (approximately 10 mg) was collected and recrystallized to yield crypto-chlorogenic acid (8 mg). Meanwhile, Fraction AC2 (approximately 80 mg), combined from Fractions 8–10 of ethyl acetate, was also subjected to Lobar by using a reversed-phase C18 column and methanol–water (4:6, v/v, 50 mL) as a mobile phase to yield isoquercetin (24 mg) and astragalin (20 mg). TLC chromatograms of Fractions AC1 and AC2 from preparative columns are shown in Figure 1. The isolated pure compounds were identified by comparing their melting points, proton nuclear magnetic resonance (^{1}H-NMR) and MS with reported data. (11, 12).

Preparation of standard solutions

Stock standard solutions of crypto-chlorogenic acid, isoquercetin and astragalin were prepared in 50% methanol in water. Working standard solutions were obtained by appropriately diluting the stock solution with 50% methanol to achieve the final concentrations of 5.0, 2.5, 1.0, 0.5, 0.25, 0.125 and 0.0625 μg/mL.

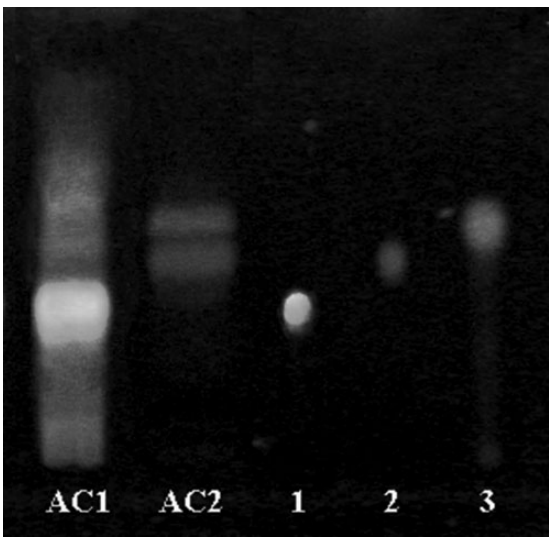

Figure 1. TLC chromatograms of Fractions AC1 and AC2 and standard compounds; crypto-chlorogenic acid (1), isoquercetin (2), astragalin (3). Absorbent: silica gel 60 GF254; solvent system: ethyl acetate–formic acid–acetic acid–water (18:3:3:3, v/v/v/v); detection: sprayed with natural product/polyethylene glycol (NP/PEG) solvent and observed under UV 366 nm.

Sample preparation

Four samples of *M. oleifera* leaf powder were accurately weighed (5.0 g) and exhaustively extracted by maceration with 70% ethanol (1:20, w/v) for 72 h at room temperature with occasional shaking. Each sample was extracted in triplicate. The extract was dried under reduced pressure at 50°C by using a rotary vacuum evaporator. An accurately weighed aliquot (100 mg) of the extract was dissolved in 50% methanol (10 mL) and analyzed by using the validated HPLC method. Before injection, each solution was filtered through a 0.2 μm nylon membrane filter and analyzed in triplicate.

HPLC apparatus and chromatographic conditions

HPLC was performed on a Shimadzu SPD-10A (Kyoto, Japan) equipped with an LC-10AD pump, a DGU-10A degasser, a SPD-10AV ultraviolet visible (UV-vis) detector and an SIL-10AD auto-injector. The separation was conducted on a Hypersil BDS C18 column (4.6 × 150 mm i.d., 5 μm) with a C18 guard column

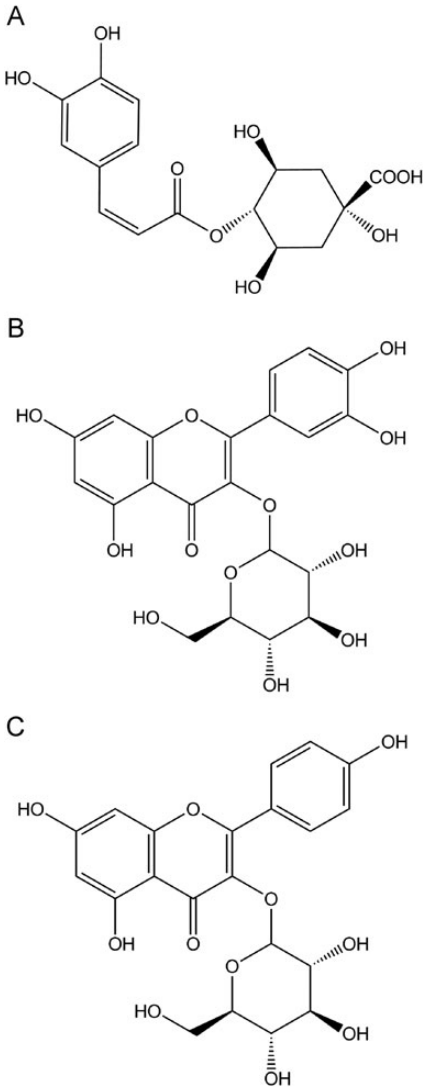

Figure 2. Chemical structures: crypto-chlorogenic acid (A); isoquercetin (B); astragalin (C).

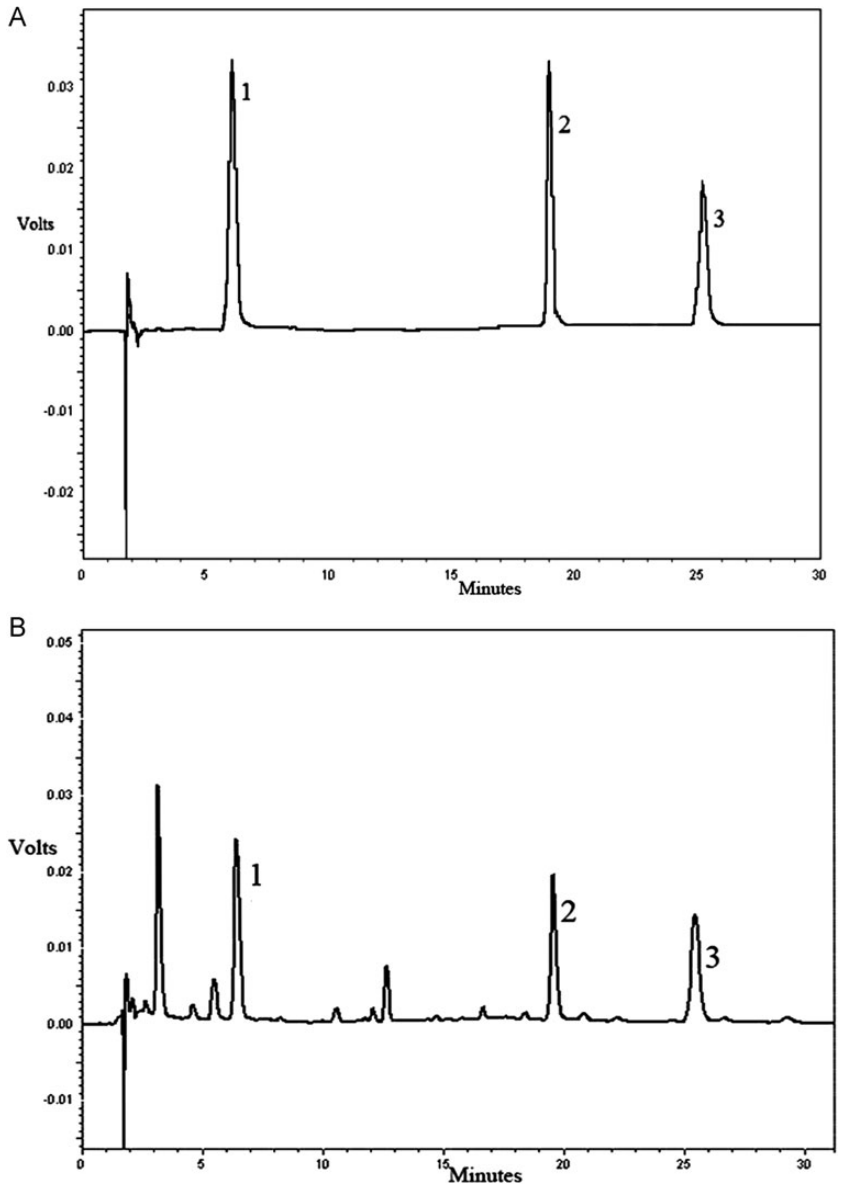

Figure 3. HPLC chromatograms: standard compounds (A); 70% ethanol extract of *M. oleifera* leaves from Lampang Province (B); crypto-chlorogenic acid (peak 1), isoquercetin (peak 2), astragalin (peak 3).

Table I
Validation Parameters of the Method for the Quantitative Analysis of Crypto-Chlorogenic Acid, Isoquercetin and Astragalin in Leaf Extracts of *M. oleifera*

Compounds	Linear range (μg/mL)	Regression equation*	r^2	Recovery (%)	LOD (μg/mL)	LOQ (μg/mL)
Crypto-chlorogenic acid	0.06–4.98	$y = 244642.89x + 14172.08$	0.9999	98.50 ± 0.93	0.008	0.024
Isoquercetin	0.04–4.98	$y = 186544.77x + 21785.67$	0.9999	98.47 ± 1.18	0.015	0.044
Astragalin	0.06–4.99	$y = 152250.67x + 7258.08$	0.9999	98.59 ± 1.21	0.011	0.032

*x represents the concentrations of crypto-chlorogenic acid, isoquercetin and astragalin in μg/mL; y represents the peak area, detected at UV 334 nm.

(Cheshire, UK). The elution was performed on a gradient solvent system using 1% glacial acetic acid (solvent A) and methanol (solvent B) as mobile phases. The ratios were as follows: 80:20 (A/B) for 3 min, 80:20 to 65:35 (A/B) in 11 min, held for 14 min, 65:35 to 0:100 (A/B) in 25 min and held for 5 min. The flow rate was 1.0 mL/min at room temperature. The UV-vis detector was monitored at 334 nm and the injection volume for all samples and standards was 20 μL. The quantitative HPLC analysis of each compound was calculated according to its peak area.

Validation of the method

The analytical method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ) according to International Conference on Harmonization (ICH) guidelines (13).

Linearity

Linearity was determined by using crypto-chlorogenic acid, isoquercetin and astragalin standard solutions in 50% methanol at concentrations of 5.0, 2.5, 1.0, 0.5, 0.25, 0.125 and 0.0625 μg/mL. Each concentration was analyzed in triplicate. The calibration curves were obtained by plotting the peak area versus the concentration of the standards.

Accuracy

Recovery was used to assess the accuracy of the method. Standard addition was conducted with pre-analyzed standard solutions. Three different concentrations (3, 2 and 1 μg/mL) of standard mixtures containing crypto-chlorogenic acid, isoquercetin and astragalin were added to the sample of *M. oleifera* extract. Spiked samples were prepared in triplicate and three determinations were performed. The percentage of recovery was calculated as follows: $recovery\ (\%) = 100 \times (amount\ found - original\ amount)/amount\ spiked$.

Precision

Using the 1 μg/mL solutions of crypto-chlorogenic acid, isoquercetin and astragalin, the intra-day precision was determined by six analyses within one day, whereas the inter-day precision was examined for three consecutive days by using the proposed method. The results were expressed as percent relative standard deviation (RSD).

LOD and LOQ

The LOD was indicated as $3.3\sigma/S$ and the LOQ was $10\sigma/S$, where σ is the standard deviation (SD) of the response and S is the slope of the calibration curve.

Results

Crypto-chlorogenic acid, isoquercetin and astragalin, the major active antioxidant constituents in *M. oleifera* leaves, were separated and identified by chromatographic and spectroscopic techniques and yielded 0.0027, 0.0080 and 0.0067% (w/w) of dried powder, respectively. The chemical structures of isolated compounds are shown in Figure 2. An HPLC method was developed to analyze the contents of three major active constituents—crypto-chlorogenic acid, isoquercetin and astragalin—in four extracts of *M. oleifera* leaves collected from different locations in Thailand. HPLC chromatograms of the extract from Lampang Province and standard compounds are shown in Figure 3. The method was validated for linearity, accuracy, precision, LOD and LOQ according to ICH guidelines (13).

The method was found to be linear within the ranges of 0.06–4.98, 0.04–4.98 and 0.06–4.99 μg/mL, for crypto-chlorogenic acid, isoquercetin and astragalin, respectively. The correlation coefficients (r^2) of all standards were 0.9999 (Table I). Recovery was used to assess the accuracy of the method. Standard addition was conducted with pre-analyzed standard solutions. The recovery values of crypto-chlorogenic acid, isoquercetin and astragalin ranged between 97.43–99.18% (average 98.50%), 97.12–99.25% (average 98.47%) and 97.24–99.60% (average 98.59%), respectively (Table I). The method also demonstrated acceptable precision, with RSD values lower than 2% (Table II). LODs, at a signal-to-noise ratio (S/N) of 3:1, were found to be 0.0078, 0.0146 and 0.0105 μg/mL for crypto-chlorogenic acid, isoquercetin and astragalin, respectively. LOQs, at S/N of 10:1, were found to be 0.0236, 0.0442 and 0.0319 μg/mL for these three compounds, respectively (Table I).

The contents of crypto-chlorogenic acid, isoquercetin and astragalin in the ethanolic extracts of *M. oleifera* leaves ranged from 0.015 to 0.150% (average 0.081%), 0.067 to 0.145% (average 0.120%) and 0.056 to 0.290% (average 0.153%), respectively. In addition, the contents of these components in the dry powdered leaves ranged from 0.006 to 0.057% (average 0.032%), 0.028 to 0.057% (average 0.047%) and 0.023 to 0.110% (average 0.060%), respectively (Table III).

Table II
Intra-Day and Inter-Day Precision of Crypto-Chlorogenic Acid, Isoquercetin and Astragalin

Compounds	Intra-day (RSD, %)			Inter-day (RSD, %)
	Day 1	Day 2	Day 3	
Crypto-chlorogenic acid	1.06	0.40	0.88	1.32
Isoquercetin	1.58	0.72	0.69	1.43
Astragalin	0.85	0.97	0.47	0.83

Discussion

Phenolics and flavonoids are active antioxidant components in the leaves of *M. oleifera* (5, 6). The major compounds were isolated and identified as crypto-chlorogenic acid, isoquercetin and astragalin (Figure 2), which expressed exceptionally strong antioxidant activity in many studies (11, 14, 15). The method also provided good separation of the compounds and the system

Table III
Percentages of Crypto-Chlorogenic Acid, Isoquercetin and Astragalin in 70% Ethanolic Extracts and Dried Powder of *M. oleifera* Leaves Collected from Four Regions in Thailand

Location (region)	Percentage of major compounds (w/w)					
	In 70% ethanol extract			In dried powder		
	Crypto-chlorogenic acid	Isoquercetin	Astragalin	Crypto-chlorogenic acid	Isoquercetin	Astragalin
Lampang (northern)	0.150 ± 0.002	0.145 ± 0.004	0.290 ± 0.003	0.057 ± 0.001	0.055 ± 0.002	0.110 ± 0.001
Surin (northeastern)	0.062 ± 0.002	0.140 ± 0.004	0.093 ± 0.003	0.026 ± 0.001	0.057 ± 0.002	0.038 ± 0.001
Ang Thong (central)	0.100 ± 0.006	0.127 ± 0.004	0.174 ± 0.009	0.038 ± 0.002	0.049 ± 0.002	0.067 ± 0.004
Phuket (southern)	0.015 ± 0.007	0.067 ± 0.008	0.056 ± 0.003	0.006 ± 0.003	0.028 ± 0.003	0.023 ± 0.001
Average	0.081 ± 0.057	0.120 ± 0.035	0.153 ± 0.104	0.032 ± 0.021	0.047 ± 0.013	0.060 ± .0038

minimized running time and costs in routine analysis. According to the HPLC chromatogram, crypto-chlorogenic acid, isoquercetin and astragalin are the principal compounds (Figure 3), contrary to studies in India and Ghana, which reported the primary compounds as chlorogenic acid and rutin (6, 16). The variation may possibly be the consequence of different plantation regions or genetic diversity of *M. oleifera*.

Conclusion

According to the validated parameters of linearity, accuracy, precision, LOD and LOQ, the proposed HPLC method was successful in simultaneously and quantitatively analyzing three major components (crypto-chlorogenic acid, isoquercetin and astragalin) in leaf extracts of *M. oleifera*. This method may be beneficial for the standardization and quality control of leaf extracts and herbal preparations of *M. oleifera*.

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