HPLC methods for quality control of *Moringa oleifera* extract using isothiocyanates and astragalin as bioactive markers

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ABSTRACT: Herbal extracts are gaining widespread acceptance as ingredients in cosmetic and health products. *Moringa oleifera* Lam. is an interesting candidate as it has multiple biological properties. However, methods for quality control of the use of *M. oleifera* are still required. This study proposes the use of isothiocyanate compounds and astragalin as chemical markers to standardize *M. oleifera* extracts. Both of these compounds are known to have anti-inflammation properties, and astragalin is also reported to have antioxidant properties. The HPLC-based cyclocondensation method was further developed and validated specifically for quantitative analysis of the isothiocyanate compounds in the *M. oleifera* extracts, and the concentration of astragalin in the *M. oleifera* extracts was determined by the HPLC method. Both methods gave acceptable linearity, accuracy, and precision with the limit of detection of 0.13 µg/ml for total isothiocyanate and 0.10 µg/ml for astragalin. The uses of the methods were demonstrated in the analyses of the components in the extracts taken variously from the leaves, immature seeds, mature seeds, petioles, and pods of the *M. oleifera* tree. Our results suggest that the methods developed could be used in the quality control of *M. oleifera* extracts to develop cosmetic and natural health products.

KEYWORDS: anti-inflammation, antioxidant, chemical analysis, cyclocondensation

INTRODUCTION

Moringa oleifera Lam. (Moringaceae family) is a plant commonly found in several tropical and subtropical countries. Traditionally, *M. oleifera* has been used for its medicinal benefits, including anti-inflammation[1–3], anti-oxidation[4–6], anti-bacterial, and anti-fungal[7–9] properties, wound healing[10–12], and skin revitalization[13]. Currently, many cosmetics and natural health products use *M. oleifera* extracts as an active ingredient. This situation therefore deserves more analysis and understanding of the *M. oleifera*, as evidenced by the call of the Dietary Supplements and Herbal Medicines (DSHM) Nomenclature Subcommittee, the U.S. Pharmacopeial Convention (USP) for a Priority New Dietary Supplement Monograph on the subject (www.usp.org).

The bioactive constituents of *M. oleifera* related to cosmeceutical and medicinal actions are astragalin, a major flavonoid from the leaves, and isothiocyanate glycosides (Fig. 1). Astragalin has been shown to exhibit anti-skin inflammation action in mice[14], an effect that may relate to the inhibition of nuclear factor-kappa B (NF-κB) signalling pathway[15–18]. Astragalin is also known to have antioxidant properties[19–21]. Isothiocyanate glycosides, found in leaves, seeds, and pods, exhibit strong anti-inflammatory activity by regulating the production of nitric oxide (NO), inducible NO synthase (iNOS), and cytokines of macrophages in vitro[22–25].

In the present study, we propose to use astragalin and isothiocyanates as chemical markers for quality control and standardization of *M. oleifera* extract. A procedure for the quality control of *M. oleifera* leaves has been extensively described in the Ayurvedic Pharmacopoeia of India. However, the chemical analysis described in that monograph
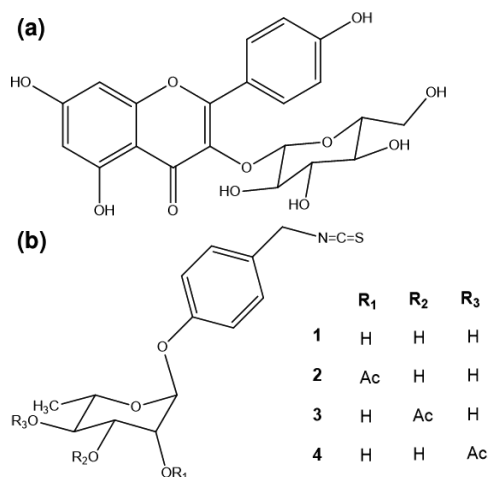
Fig. 1 Structure of (a) astragalin and (b) *Moringa oleifera* isothiocyanates (1–4): (1) 4-[(α-L-rhamnosyloxy)benzyl] isothiocyanate, (2) 4-[(2′-*O*-acetyl-α-L-rhamnosyloxy)benzyl] isothiocyanate, (3) 4-[(3′-*O*-acetyl-α-L-rhamnosyloxy)benzyl] isothiocyanate, (4) 4-[(4′-*O*-acetyl-α-L-rhamnosyloxy)benzyl] isothiocyanate.

was conducted via thin-layer chromatography system without any chemical markers[26]. Astragalin has been used as a chemical marker for quality control of *M. oleifera* using the high performance liquid chromatography (HPLC) method in the Malaysian Herbal Monograph but the run time is quite long (55 min per sample)[27]. In this study, we develop and validate the HPLC-condensation method for quantitative analysis of total isothiocyanate compounds and the HPLC method for the quantitative analysis of astragalin in *M. oleifera* extracts. The extracts of the leaves, immature seeds, mature seeds, pods, and petioles from *M. oleifera* were determined for the presence of these compounds using both proven methods.

MATERIALS AND METHODS

Chemicals and reagents

Standard compounds of astragalin and phenethyl isothiocyanate were purchased from Sigma-Aldrich (Steinheim, Germany). 1,2-benzenedithiol was acquired from Tokyo Chemical Industry (Tokyo, Japan). All solvents used were of HPLC grade and were obtained from RCI Labscan (Bangkok, Thailand).

Plant materials

Leaves, immature seeds, mature seeds, pods, and petioles of *M. oleifera* were collected from Phitsa-

nulok Province, Thailand. After cleaning, the plant samples were dried in a hot air oven at 50 °C. The dried samples were ground into coarse powder. The voucher specimen was kept at Faculty of Science, Naresuan University (Phitsanulok, Thailand). The plant was identified by Dr Pranee Nangngam.

Plant extractions

The rough powdered *M. oleifera* leaves were extracted in three different solvents: water, 50% ethanol, and 95% ethanol. For extraction by water, the powder was infused with water at 60–70 °C, and shaken for 45 min. The resulting extract was filtered through filter paper, and the water was removed by freeze drying. For the 50% ethanol extract, the powder was macerated with 50% ethanol for 48 h while shaking and then filtered through filter paper. The ethanol was then evaporated by a rotary evaporator. The residual water was then removed by freeze drying. For the 95% ethanol extract, the powder was macerated with 95% ethanol for 48 h while shaking and then filtered through filter paper. The ethanol was then evaporated using a rotary evaporator. The other plant parts (immature seeds, mature seeds, pods, and petioles) were coarsely powdered. The powder was then macerated in 50% ethanol to obtain the crude extracts by the methods described earlier. After extraction, the crude extracts were weighed and stored in a freezer (−20 °C) for further use.

Plant extract solutions

Stock solutions of *M. oleifera* extract were prepared at 10 mg/ml. The water extracts and 50% ethanol extracts were dissolved in distilled water, and the 95% ethanol extracts were dissolved in ethanol. The stock solutions of the extracts were then diluted to 1 mg/ml in the mobile phase before HPLC analyses.

HPLC-based cyclocondensation method to determine total isothiocyanates

Standard stock solutions of phenethyl isothiocyanate were prepared by dissolving the substances in methanol to a final concentration of 1 mg/ml. The standard working solutions were made by diluting the stock solutions with mobile phase.

The cyclocondensation assay method was modified from the previous report[28, 29]. Briefly, in a 5-ml glass vial, 0.25 ml of each phenethyl isothiocyanate sample was mixed with 0.25 ml of 100 mM K_3PO_4 buffer (pH 8.5), 0.5 ml of 10 mM 1,2-benzenedithiol in methanol. The reaction mixture was incubated

for 2 h at 65 °C, and then cooled to room temperature. The mixture was filtered through 0.45 μm nylon membrane. A 0.1 ml aliquot of the mixture was injected into the HPLC system for analysis of the isothiocyanates content.

The HPLC system used in the analysis consisted of Shimadzu LC-2OAT pumps, DGU-20A5 degasser SPD-M10Avp diode array detector, and Phenomenex Gemini 5 μm C18 column. The isocratic mobile phase was methanol: water (4:1 v/v), at a flow rate of 1.2 ml/min. The eluates were monitored at 365 nm. Each sample was eluted for a total of 7 min.

HPLC method for determination of astragalin

Standard stock solutions of astragalin were prepared by dissolving the substances in methanol to a final concentration of 1 mg/ml. The standard working solutions were made by diluting the stock solutions with the HPLC mobile phase.

The accuracy was evaluated by performing the recovery study. Solutions of three known concentrations of standard compound were added to 100 μg/ml of *M. oleifera* leaf extract samples. The recovery of each standard concentration from the spiked samples was calculated.

The method was modified from the previous study[30]. The HPLC system configuration as mentioned above was used together with Phenomenex Luna 5 μm C18 column. The isocratic mobile phase was 0.1% acetate buffer: acetonitrile (3:1 v/v), pH 4.5 at a flow rate of 1 ml/min. The eluates were monitored at 267 nm. The total run time was 12 min.

Method validation

Both methods were validated for range of linearity of the calibration curve, precision, accuracy, LOD, and LOQ values.

The calibration curves were plotted by peak areas and the concentrations of each standard. The stock standard solutions were diluted to 6 different concentrations. The linear regression equations were $y = ax \pm b$, where x was the concentration and y was the peak area of each standard. The linearity was verified by linear regression (R^2).

The precision was assessed by repeated injection at each of 3 concentrations in calibration curve. The intra-day precision was determined by 5 replicates for a day, and the inter-day precision was determined by 5 replicates for each of 3 days. The precision was assessed by calculating the relative standard deviation (RSD).

Limit of detection (LOD) was defined as the lowest concentration which can still be detected by the analysis method but does not have an accurate value. This was determined by the signal-to-noise ratio of 3:1. Limit of quantification (LOQ) was defined as the lowest sample concentration which can still be quantitatively detected with accuracy and precision. It was determined by the signal-to-noise ratio of 10:1.

Determination of astragalin and total isothiocyanates in *M. oleifera* leaf extracts

Total isothiocyanates and astragalin in various *M. oleifera* extract samples were determined using the validated HPLC methods.

RESULTS AND DISCUSSION

Validation of HPLC method for determination of astragalin and total isothiocyanates

The cyclocondensation assay depends on the carbon atom of the −N=C=S group of the isothiocyanate compound reacting with the thiol groups of 1,2-benzenedithiol to form 1,3-benzodithiole-2-thione and the corresponding amine (Fig. 2).

The product of the reaction was determined by HPLC. The typical chromatograms of 1,3-benzodithiole-2-thione from cyclocondensation of phenethyl isothiocyanate standard is shown in Fig. 3a. The retention time of 1,3-benzodithiole-2-thione was 4.13 min. The amount of total thiocyanates in the samples determined was reported as equivalent to phenethyl isothiocyanate. For the analysis of another active constituent, astragalin, an HPLC method was also used. A typical chromatogram of astragalin standard is shown in Fig. 4a. Astragalin was eluted at 5.41 min. Linearity was achieved in the range 0.5–16 μg/ml for both methods. The calibration curves were linear with excellent correlation coefficients ($R^2 > 0.999$). The regression equations
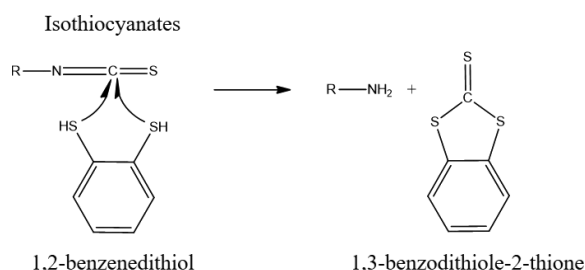

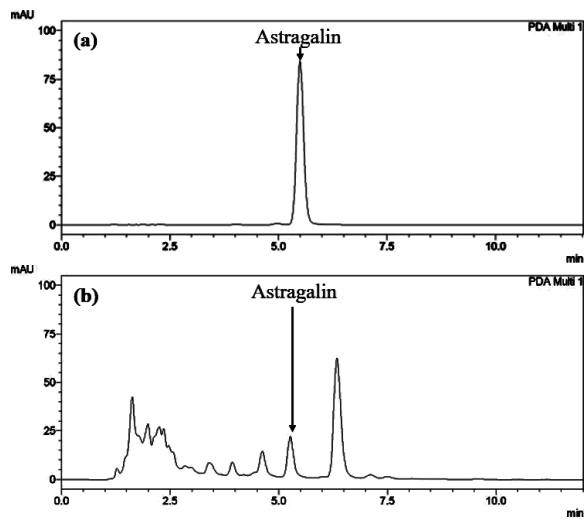**Fig. 2** Reaction of isothiocyanates with 1,2-benzenedithiol.

Fig. 3 Typical HPLC chromatograms showing reaction products from cyclocondensation of (a) phenethyl isothiocyanate standard (16 μg/ml) and (b) 50% ethanol extract of *M. oleifera* leaves (1 mg/ml).
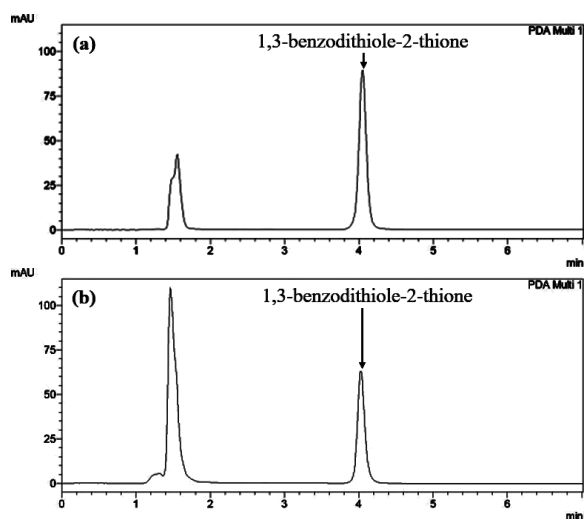

Fig. 4 Typical HPLC chromatograms showing (a) astragalin standard (16 μg/ml) and (b) 50% ethanol extract of *M. oleifera* leaves (1 mg/ml).

were $y = 43028x + 7966.5$ for phenethyl isothiocyanate and $y = 50495x - 723.02$ for astragalin. The minimum concentration levels which can be detected (LOD) and quantified (LOQ) in the analysis were found to be 0.13 μg/ml and 0.5 μg/ml for phenethyl isothiocyanate, 0.1 μg/ml and 0.5 μg/ml for astragalin, respectively. This indicated that both HPLC methods are properly sensitive for the quantification of these bioactive compounds. The RSD

Table 1 Linear range (μg/ml), linear equation, and linearity (R^2) of total isothiocyanates and astragalin obtained from HPLC methods.

Compound	Range	Linear equation	R^2
Isothiocyanates	0.5–16	$y = 43028x + 7966.5$	1.000
Astragalin	0.5–16	$y = 50495x - 723.02$	0.999

and recovery values were within acceptable ranges based on the AOAC Guidelines for Single Laboratory Validation of Chemical Methods (www.aoac.org). All method validation parameters are shown in Tables 1 and 2. These results demonstrated that both methods were reproducible with good accuracy and precision. System suitability parameters for isothiocyanates and astragalin demonstrated that the methods are suitable for determining these compounds in *M. oleifera* extracts.

Determination of total isothiocyanates and astragalin in *M. oleifera* extracts

To pinpoint *M. oleifera* extracts that had high amount of bioactive compounds, the HPLC methods were applied for determination of total isothiocyanates and astragalin contents in different extracts of leaves, immature seeds, mature seeds, pods, and petioles of *M. oleifera*. The representative chromatogram of reaction product from cyclocondensation of *M. oleifera* extract and the representative chromatogram of *M. oleifera* extract are shown in Figs. 3b and 4b, respectively. The extraction yields and the contents of total isothiocyanates and astragalin in different extracts are exhibited in Table 3. The extracts from *M. oleifera* leaves, extracted by 50% ethanol, had the highest extraction yield compared to those extracted with water and 95% ethanol.

Although a quantitative analysis of isothiocyanate contents in *M. oleifera* leaf extract using liquid chromatography mass spectrometry has previously been reported, only compounds 1 and 4 were determined[25]. Hence, our study is the first report on analyses of total isothiocyanate contents (compounds 1–4) in the extracts taken variously from the leaves, immature seeds, mature seeds, petioles, and pods of the *M. oleifera* tree. Isothiocyanates were detected in all extracts and ranged from 0.72–27.84 mg of phenethyl isothiocyanate equivalent/g extract. In comparison with the other extracts, the mature seed extract had the highest amount of total isothiocyanates. The leaf extract prepared by 50% ethanol had a higher amount of

Table 2 Precision, accuracy, LOD, and LOQ values of total isothiocyanates and astragalin obtained from HPLC methods.

Compound	RSD (%)		Recovery (%)		LOD (μg/ml)	LOQ (μg/ml)
	Intra-day ($n = 5$)	Inter-day ($n = 15$)	Intra-day ($n = 3$)	Inter-day ($n = 9$)		
Total isothiocyanates	1.80–3.49	5.22–5.67	104.1–109.0	105.5–106.6	0.13	0.5
Astragalin	0.96–2.58	0.70–3.17	98.4–107.7	104.8–108.2	0.10	0.5

Table 3 Extraction yields (% w/w), total isothiocyanate (mg of phenethyl isothiocyanate equivalent/g extract), and astragalin (mg/g extract) in various *M. oleifera* extracts.

Analysis	Water	95% Ethanol	50% Ethanol				
	Leaves	Leaves	Leaves	Immature seeds	Mature seeds	Pods	Petioles
Extraction yield	7.54	7.20	11.71	15.55	11.85	11.90	5.75
Isothiocyanates	2.46 ± 0.11	0.72 ± 0.10	7.52 ± 0.44	13.9 ± 1.0	27.84 ± 0.34	7.22 ± 0.80	17.9 ± 1.5
Astragalin	1.41 ± 0.27	6.88 ± 0.91	4.73 ± 0.41	nd	nd	< LOQ	1.90 ± 0.33

Values are means ± SD from triplicate experiments; nd: not detected.

total isothiocyanates than in the extracts by the other solvents. Nevertheless, the amount of total isothiocyanates in leaf extracts in our study was lower than in the extracts by water in the previous study. The variation may be possibly caused by the effect of different cultivated area and extraction process.

For the HPLC analyses of astragalin in *M. oleifera* extract, our method needed shorter run time than the previous methods. Astragalin was not detected in the immature seed and mature seed extracts and could not be quantified (< LOQ) in the pod extract. The 95% ethanol extract of leaves had the highest amount of astragalin followed by the 50% ethanol extract of leaves. The amount of astragalin in the leaf extracts prepared by 95% ethanol and 50% ethanol were higher than in those prepared in 70% ethanol extract in the previous study.

CONCLUSIONS

The two developed HPLC methods for the quantitative determination of anti-inflammatory and antioxidant compounds, total isothiocyanates, and astragalin in *M. oleifera* extracts are simple, rapid, precise, and accurate. Consequently, these methods can be used for quality control and standardization of *M. oleifera* raw materials, extracts, and finished products. The 50% ethanol extract of mature seeds had the highest content of total isothiocyanates without the presence of astragalin. The high content of total isothiocyanates and astragalin that was found in 50% ethanol extract of leaves indicates that this extract is a potential active-ingredient in cosmetic and health products.

REFERENCES

1. Mahajan SG, Mali RG, Mehta AA (2007) Protective effect of ethanolic extract of seeds of *Moringa oleifera* Lam. against inflammation associated with development of arthritis in rats. *J Immunotoxicol* **4**, 39–47.
2. Muangnoi C, Chingsuwanrote P, Praengamthanachoti P, Svasti S, Tuntipopipat S (2012) *Moringa oleifera* pod inhibits inflammatory mediator production by lipopolysaccharide-stimulated RAW 264.7 murine macrophage cell lines. *Inflammation* **35**, 445–55.
3. Fard MT, Arulselvan P, Karthivashan G, Adam SK, Fakurazi S (2015) Bioactive extract from *Moringa oleifera* inhibits the pro-inflammatory mediators in lipopolysaccharide stimulated macrophages. *Pharmacogn Mag* **11**, S556–63.
4. Siddhuraju P, Becker K (2003) Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. *J Agr Food Chem* **51**, 2144–55.
5. Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G, Singh HB (2009) Oxidative DNA damage protective activity, antioxidant and anti-quorum sensing potentials of *Moringa oleifera*. *Food Chem Toxicol* **47**, 1109–16.

6. Shih MC, Chang CM, Kang SM, Tsai ML (2011) Effect of different parts (leaf, stem and stalk) and seasons (summer and winter) on the chemical compositions and antioxidant activity of *Moringa oleifera*. *Int J Mol Sci* **12**, 6077–88.

7. Vieira GHF, Mourão JA, Ângelo AM, Costa RA, Vieira RHSF (2010) Antibacterial effect (in vitro) of *Moringa oleifera* and *Annona muricata* against Gram positive and Gram negative bacteria. *Rev Inst Med Trop S Paulo* **52**, 129–32.

8. Peixoto JRO, Silva GC, Costa RA, Fontenelle JLS, Vieira GHF, Fonteles Filho AA, Vieira RHSF (2011) In vitro antibacterial effect of aqueous and ethanolic *Moringa* leaf extracts. *Asian Pac J Trop Med* **4**, 201–4.

9. Galuppo M, de Nicola GR, Iori R, Dell'Utri P, Bramanti P, Mazzon E (2013) Antibacterial activity of glucomoringin bioactivated with myrosinase against two important pathogens affecting the health of long-term patients in hospitals. *Molecules* **18**, 14340–8.

10. Rathi BS, Bodhankar SL, Baheti AM (2006) Evaluation of aqueous leaves extract of *Moringa oleifera* Linn for wound healing in albino rats. *Indian J Exp Biol* **44**, 898–901.

11. Muhammad AA, Pauzi NAS, Arulselvan P, Abas F, Fakurazi S (2013) In vitro wound healing potential and identification of bioactive compounds from *Moringa oleifera* Lam. *BioMed Res Int* **2013**, 974580.

12. Gothai S, Arulselvan P, Tan WS, Fakurazi S (2016) Wound healing properties of ethyl acetate fraction of *Moringa oleifera* in normal human dermal fibroblasts. *J Intercult Ethnopharmacol* **5**, 1–6.

13. Ali A, Akhtar N, Chowdhary F (2014) Enhancement of human skin facial revitalization by moringa leaf extract cream. *Postęp Dermatol Alergol* **31**, 71–6.

14. Kotani M, Matsumoto M, Fujita A, Higa S, Wang W, Suemura M, Kishimoto T, Tanaka T (2000) Persimmon leaf extract and astragalin inhibit development of dermatitis and IgE elevation in NC/Nga mice. *J Allergy Clin Immunol* **106**, 159–66.

15. Kim MS, Kim SH (2011) Inhibitory effect of astragalin on expression of lipopolysaccharide-induced inflammatory mediators through NF-κB in macrophages. *Arch Pharm Res* **34**, 2101–7.

16. Soromou LW, Chen N, Jiang L, Huo M, Wei M, Chu X, Millimouno FM, Feng H, et al (2012) Astragalin attenuates lipopolysaccharide-induced inflammatory responses by down-regulating NF-κB signaling pathway. *Biochem Biophys Res Comm* **419**, 256–61.

17. Li F, Liang D, Yang Z, Wang T, Wang W, Song X, Guo M, Zhou E, et al (2013) Astragalin suppresses inflammatory responses via down-regulation of NF-κB signaling pathway in lipopolysaccharide-induced mastitis in a murine model. *Int Immunopharmacol* **17**, 478–82.

18. Li F, Wang W, Cao Y, Liang D, Zhang W, Zhang Z, Jiang H, Guo M, Zhang N (2014) Inhibitory effects of astragalin on lipopolysaccharide-induced inflam-matory response in mouse mammary epithelial cells. *J Surg Res* **192**, 573–81.

19. Han JT, Bang MH, Chun OK, Kim DO, Lee CY, Baek NI (2004) Flavonol glycosides from the aerial parts of *Aceriphyllum rossii* and their antioxidant activities. *Arch Pharm Res* **27**, 390–5.

20. Choi J, Kang HJ, Kim SZ, Kwon TO, Jeong SI, Jang SI (2013) Antioxidant effect of astragalin isolated from the leaves of *Morus alba* L. against free radical-induced oxidative hemolysis of human red blood cells. *Arch Pharm Res* **36**, 912–7.

21. Luo C, Wang A, Wang X, Li J, Liu H, Wang M, Wang L, Lai D, Zhou L (2015) A new proline-containing flavonol glycoside from *Caragana leucophloea* Pojark. *Nat Prod Res* **29**, 1811–9.

22. Faizi S, Siddiqui BS, Saleem R, Aftab K, Shaheen F, Gilani AH (1998) Hypotensive constituents from the pods of *Moringa oleifera*. *Planta Med* **64**, 225–8.

23. Guevara AP, Vargas C, Sakurai H, Fujiwara Y, Hashimoto K, Maoka T, Kozuka M, Ito Y, et al (1999) An antitumor promoter from *Moringa oleifera* Lam. *Mutat Res* **440**, 181–8.

24. Cheenpracha S, Park EJ, Yoshida WY, Barit C, Wall M, Pezzuto JM, Chang LC (2010) Potential anti-inflammatory phenolic glycosides from the medicinal plant *Moringa oleifera* fruits. *Bioorg Med Chem* **18**, 6598–602.

25. Waterman C, Cheng DM, Rojas-Silva P, Poulev A, Dreifus J, Lila MA, Raskin I (2014) Stable, water extractable isothiocyanates from *Moringa oleifera* leaves attenuate inflammation in vitro. *Phytochemistry* **103**, 114–22.

26. Ayurvedic Pharmacopoeia Committee (2001) *The Ayurvedic Pharmacopoeia of India*, Government of India, New Delhi, pp 163–4.

27. Malaysian Herbal Monograph Committee (2013) *Malaysian Herbal Monograph*, Forest Research Institute Malaysia, Kuala Lumpur.

28. Zhang Y, Wade KL, Prestera T, Talalay P (1996) Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzene-dithiol. *Anal Biochem* **239**, 160–7.

29. Tang L, Paonessa JD, Zhang Y, Ambrosone CB, McCann SE (2013) Total isothiocyanate yield from raw cruciferous vegetables commonly consumed in the United States. *J Funct Foods* **5**, 1996–2001.

30. Qiao C, Han Q, Song J, Wang Z, Xu L, Xu H (2007) HPLC determination of two bioactive flavone glycosides and GC-MS analysis of volatile oil constituents in *Alpinia nigra*. *Asian J Tradit Med* **2**, 85–91.