RP-HPLC Method for the Quantitative Analysis of Naturally Occurring Flavonoids in Leaves of *Blumea balsamifera* DC

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Abstract

A selective and sensitive reversed-phase (RP) high-performance liquid chromatographic method is developed for the quantitative analysis of five naturally occurring flavonoids of *Blumea balsamifera* DC, namely dihydroquercetin-7,4'-dimethyl ether (DQDE), blumeatin (BL), quercetin (QN), 5,7,3',5'-tetrahydroxyflavanone (THFE), and dihydroquercetin-4'-methyl ether (DQME). These compounds have been isolated using various chromatographic methods. The five compounds are completely separated within 35 min using an RP C_{18}, Nucleosil column and with an isocratic methanol–0.5% phosphoric acid (50:50, v/v) mobile phase at the flow rate of 0.9 mL/min. The separation of the compounds is monitored at 285 nm using UV detection. Identifications of specific flavonoids are made by comparing their retention times with those of the standards. Reproducibility of the method is good, with coefficients of variation of 1.48% for DQME, 2.25% for THFE, 2.31% for QN, 2.23% for DQDE, and 1.51% for BL. The average recoveries of pure flavonoids upon addition to lyophilized powder and subsequent extraction are 99.8% for DQME, 99.9% for THFE, 100.0% for BL, 100.6% for DQDE, and 97.4% for QN.

Introduction

Flavonoids are a ubiquitous group of polyphenolic substances that are present in most medicinal plants. *Blumea balsamifera* DC (*Compositae*) is a medicinal plant, widely distributed in Malaysia. It is locally known as "Capa". The leaves of this plant are used in folk medicine as stomachic, expectorant, antispasmodic, and diaphoretic and are also believe to be useful for fever, lumbago, increasing appetite, skin diseases, wounds, liver cirrhosis, and kidney stone diseases (1,2). Phytochemical investigation of the leaves of *B. balsamifera* revealed that it contained a number of flavonoids, namely velutin, dihydroquercetin-7,4'-dimethyl ether, blumeatin, ombuine, tamarixetin, rhamnetin, luteolin-7-methyl ether, luteolin, quercetin, 5,7,3',5'-tetrahydroxyflavanone, and dihydroquercetin-4'-methyl ether (3–6). It has been found that flavonoids possess antioxidant and free radical scavenging activity (6–8), and epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer (9–13). The reputed medicinal properties of flavonoids and pythochemical findings of this plant prompt the investigation, validation, and standardization of the local plant as an herbal medicine.

High-performance liquid chromatography (HPLC) has been the most widely employed chromatographic technique in flavonoid analysis during the past 20 years (14–18). It has added a new dimension to the investigation of flavonoids in food and plant extracts. The separations are far more rapid than classical methods and provide high resolution and sensitivity (18,19). HPLC of flavonoids is a widely used methodology and easily adapted to the quantitation of individual compounds. It has the advantage of generating a chemical fingerprint, which can be used in defining the identity and quality of a given sample. Therefore, the aim of the present work was to develop a simple, routine, reproducible, and accurate HPLC method for the determination of five major flavonoids of *B. balsamifera*: dihydroquercetin-7,4'-dimethyl ether (DQDE), blumeatin (BL), quercetin (QN), 5,7,3',5'-tetrahydroxyflavanone (THFE), and dihydroquercetin-4'-methyl ether (DQME) (Figure 1). To date, this is the first report on HPLC assay of flavonoids of *B. balsamifera*.

Experimental

HPLC conditions

The HPLC system consisted of a Gilson Model-302 pump (Paris, France) coupled to a variable UV absorbance detector (Model SPD-10A, Shimadzu, Tokyo, Japan) operated at 285 nm. The chromatogram was recorded using an electronic integrator (Chromato-Integrator, Model Hitachi D-2500, Hitachi, Tokyo, Japan). Chromatographic separation was carried out using a C_{18} reversed-phase (RP) analytical HPLC column (Phenomenex

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100A, Nucleosil, 250- × 4.6-mm i.d., 5-µm particle size) (Phenomenex, Torrance, CA). The mobile phase consisted of methanol–0.5% phosphoric acid in water (50:50, v/v). The flow rate was held constant at 0.9 mL/min.

Chemicals and reagents

Methanol (HPLC and analytical grade) was obtained from Merck (Darmstadt, Germany). DQDE, BL, QN, THFE, and DQME were isolated from the leaves of *B. balsamifera* in our laboratory and characterized by UV, IR, NMR (1D and 2D) mass spectrometry (MS) (electron impact/electrospray ionization), and elemental analyses (5,6) were used as flavonoid standards for preparation of standard curves for quantitative analysis.

Plant materials

The leaves of *B. balsamifera* were collected from different regions of Malaysia, and a herbarium voucher specimen (FRI 57083) has been deposited in the Botany unit of The Forest Research Institute of Malaysia (Kuala Lumpur, Malaysia). Five sources of authenticated plant materials were used for quantitative analysis as follows: source A (HPA, Perlis); source B (Kedah); source C (Kepala Batas, Penang); source D (Botanical garden, Penang); and source E (Perak).

Sample preparation

After lyophilization, the leaves of the plant samples were ground using a Centrifugal Mill. A portion of the powdered samples (100 mg) was extracted for 6 h with 10 mL of methanol in glass-stopped vessels on a hot plate with magnetic stirring at 40°C. After centrifugation at 3000 *g* for 10 min, the extracted sample was decanted and the remaining solid residue was extracted three times with 5 mL of methanol. The extract was evaporated in vacuum to dryness at 40°C. The solid residue was reconstituted with 5 mL of methanol and 20 µL was injected into the HPLC system.

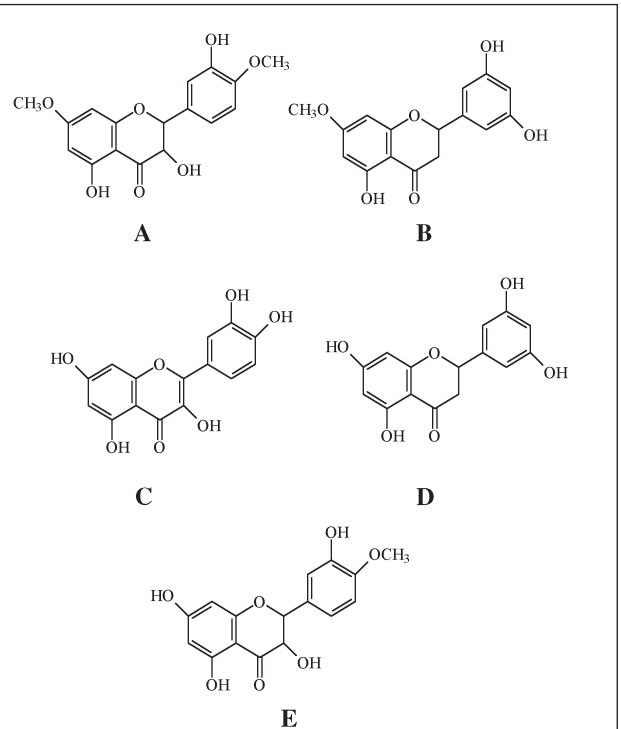

Figure 1. Chemical structures of isolated flavonoids of *B. balsamifera*, namely DQDE (A), BL (B), QN (C), THFE (D), and DQME (E).

Standard preparation

Stock solutions of standard DQDE, BL, QN, THFE, and DQME (1 mg/mL) were prepared in methanol. Stock solutions were further diluted with methanol to prepare different concentrations of flavonoid standard solutions. All standard solutions were stored at 4°C before use.

Detector linearity

The detector linearity was assessed by injecting aliquots of the standard solutions in methanol: 0.025–100 µg/mL for DQDE and BL, 0.05–100 µg/mL for QN, and 0.01–100 µg/mL for THFE and DQME, respectively. The peak area versus the amount of the flavonoids injected was plotted to determine the linearity.

Calibration

The calibration curve was established on six data points covering a concentration range of 1 to 100 µg/mL for all of the analyzed compounds (DQDE, BL, QN, THFE, and DQME). Aliquots (20 µL) were used for the HPLC injections. The peak area of the flavonoids was plotted against the corresponding concentration of the flavonoids. Linear regression of the peak area of the compounds of interests versus the compound concentration was performed in order to estimate the slope, intercept, and correlation coefficient of each calibration curve.

Recovery studies

The recovery efficiency was determined by adding measured amounts of DQME, DQDE, BL, THFE, and QN to the extraction solvent for leaves to final concentrations of 10, 50, and 100 µg/mL. The sample was prepared as described previously and 20 µL of the filtrate was injected into the HPLC. The control was prepared from the same sample. The recoveries were determined by subtracting the values obtained for the control sample preparation from those of the samples prepared with the added standards. The recovery experiment was performed with three replicates, and mean values with standard deviations (SDs) are reported.

Statistical analysis

The results of quantitative analysis of flavonoids from different sources of *B. balsamifera* leaves were expressed as mean ± SD. The data were subjected to a one-way analysis of variance. A Tukey's test ($p < 0.05$) was performed to determine the significance of the difference between means.

Results and Discussion

Development of the HPLC method

Figure 2 illustrates the separation of five flavonoids of *B. balsamifera* leaves on Nucleosil 100A C_{18} using the methanol–0.5% phosphoric acid isocratic elution system. As a means of qualitative identification, retention data, including mean values of retention times (t_R) with standard deviations of five replicates and UV absorption of maxima of each standard, are given in Table I.

The UV maxima in absolute methanol for the flavonoid compounds studied in this work have been reported previously (3–6,20). To determine the authenticity of the standards, each sample was dissolved in absolute methanol and their UV maxima were determined. The results showed excellent agreement with the reported values. The response of DQDE, BL, THFE, and DQME was stronger at 285 nm. In view of that, the detection of the compounds was monitored at 285 nm.

Calibration

The relationship between the peak area and concentration is given by a, b, and *r* values (as shown in Table II), in which a and b are the coefficients of the regression equation:

$$y = ax + b \qquad \text{Eq. 1}$$

where *x* is the concentration of flavonoid (µg/mL), *y* is the peak area, a is the slope, and r is the correlation coefficient of the equation. All of the flavonoids exhibited good linearity (r = 0.9998 – 1.000) and obeyed Beer's law in the investigated concentration range of 1–100 µg/mL. In addition, the curves remained consistent during the working life of the column, making it unnecessary to construct a new calibration curve each day. The calibration curve is normally checked by running a triplicate standard injection prior to injection of the unknown sample. Recalibration is only necessary if the mean difference between the known standard and the calculated values exceeds 5%.

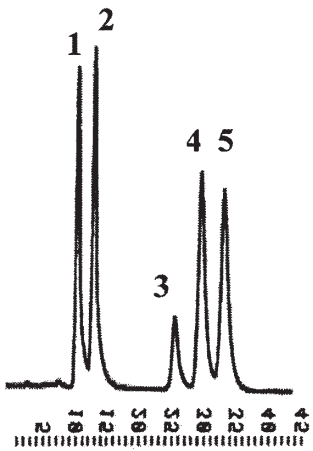

Figure 2. Isocratic RP-HPLC analysis of flavonoid standards isolated from the leaves of *B. balsamifera*. Peak numbers: DQME (1), THFE (2), QN (3), DQDE (4), and BL (5) (see Table I).

Table I. Retention Times and Absorption Maxima (λ max) of the Flavonoids of *B. balsamifera* Investigated Using Isocratic RP-HPLC

Peak no.	Common name	t_R (isocratic)* ± SD	λ max
1	DQME	10.83 ± 0.229	289
2	THFE	14.01 ± 0.315	288
3	QN	26.26 ± 0.828	371, 294, 255
4	DQDE	30.25 ± 0.785	288
5	BL	34.11 ± 0.934	287

* Quintuplicate analyses.

Recoveries of flavonoids from *B. balsamifera* leaves powder

The recoveries of the flavonoids are given in Table III. The average recoveries were 99.8% for DQME, 99.9% for THFE, 100.0% for BL, 100.6% for DQDE, and 97.4 % for QN.

Detection limit and precision

The limit of detection (LOD) was defined as the amount of flavonoid that resulted in a peak height three times the standard of the base line noise (15). According to this rule, the LODs for DQDE, BL, QN, THFE, and DQME were 10, 10, 50, 5, and 5 ng/mL, respectively. Precision was evaluated by performing five replicate analyses of the *B. balsamifera* leaves extract within the same working day. The coefficients of variation (CV) for the five

Table II. Relationships between Flavonoid Levels and Peak Areas of Flavonoids Investigated Using Isocratic RP-HPLC

Peak no.	Flavonoids	*a* (× 10^5)*	*b* (× 10^5)†	*r*
1	DQME	0.9045	–0.1212	0.9999
2	THFE	1.03973	–0.0239	0.9999
3	QN	0.3006	–0.0389	0.9998
4	DQDE	0.6705	–0.1126	0.9999
5	BL	0.9195	0.0512	0.9999

* *a* = Coefficients of the regression equation y = *a*x + *b*, where x is the flavonoid concentration (µg/mL) and y is peak area, for concentrations ranging from 1 to 100 µg/mL.
† *b* = correlation coefficients of the regression equation.

Table III. Recoveries of the Five Major Flavonoids from the Leaves of *B. balsamifera

Flavonoids	Added (µg/mL)	Detected (µg/mL)	Recovery (%)	CV (%)
DQDE	10	10.10 ± 0.36	99.60	3.63
	50	50.48 ± 0.93	100.96	1.86
	100	101.26 ± 1.57	101.26	1.55
BL	10	9.96 ± 0.36	98.80	3.64
	50	50.14 ± 1.49	100.28	2.98
	100	100.78 ± 1.65	100.78	1.64
QN	10	9.58 ± 0.42	97.00	4.51
	50	48.62 ± 1.67	97.24	3.46
	100	98.02 ± 1.59	98.02	1.63
THFE	10	9.94 ± 0.46	98.00	4.65
	50	50.78 ± 0.78	101.56	1.55
	100	100.0 ± 0.84	100.08	0.84
DQME	10	9.94 ± 0.40	99.40	4.11
	50	49.92 ± 0.58	99.84	1.17
	100	100.04 ± 1.42	100.04	1.42

* Triplicate analyses.

compounds were less than 3%. The CV was 1.48% for DQME, 2.25% for THFE, 2.31% for QN, 2.23% for DQDE, and 1.51% for BL, suggesting that the proposed HPLC method is sufficiently sensitive for the determination of flavonoids in *B. balsamifera* samples.

Table IV. Contents of Five Major Flavonoids* in Different Sources of *B. balsamifera* Leaves

Peak no.	Flavonoids	Sources† A	B	C	D	E
1	DQME	1.914 ± 0.028	2.302 ± 0.029	1.685 ± 0.021	4.937 ± 0.052	0.407 ± 0.023
2	THFE	1.384 ± 0.031	1.828 ± 0.091	0.455 ± 0.006	3.165 ± .012	0.229 ± 0.011
3	QN	0.406 ± .010	0.336 ± 0.031	0.258 ± 0.023	0.958 ± 0.053	0.021 ± 0.001
4	DQDE	0.889 ± 0.019	0.796 ± 0.012	1.147 ± 0.025	2.220 ± 0.029	0.191 ± 0.002
5	BL	1.694 ± 0.025	1.263 ± 0.022	1.129 ± 0.021	2.602 ± 0.011	0.184 ± 0.001

* In mg/g of dried leaves.
† Each value is expressed as mean (mg flavonoids/g of dried leaves powder) ± SD (n = 3). Means within a row for each compound are significantly different ($p < 0.05$). Source A, HPA (Perlis); source B, Kedah; source C, Kepala Batas (Penang); source D, Botanical garden (Penang); and source E, Perak.

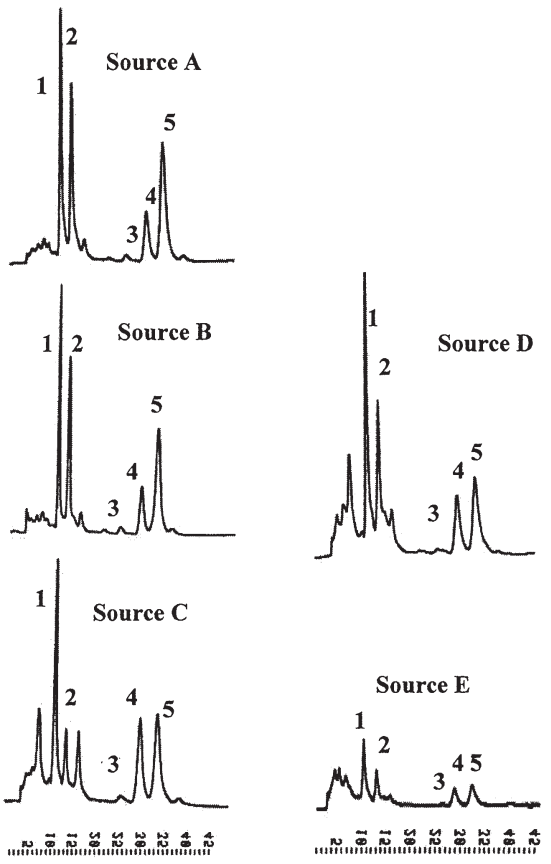

Figure 3. Isocratic RP-HPLC analysis of *B. balsamifera* leaves extract collected from different regions of Malaysia. Peak numbers: DQME (1), THFE (2), QN (3), DQDE (4), and BL (5) (see Table IV).

Quantitation of flavonoids of *B. balsamifera* collected from different regions of Malaysia

An isocratic RP-HPLC method was used for the identification and quantitation of five major flavonoids of leaves of *B. balsamifera* (Figure 3). In this study, five different sources of samples [source 1, HPA (Perlis); source 2, Kedah; source 3, Kepala Batas (Penang); source 4, Botanical garden (Penang); and source 5, Perak] were analyzed and their flavonoid contents were determined as shown in Table IV. The results were expressed as mg/g dried powders. The results indicated that there were significant differences ($p > 0.05$) of flavonoids content in different sources of leaves.

Conclusion

A simple, routine, reproducible, and accurate HPLC method for the simultaneous determination of five major flavonoids in extracts prepared from *Blumea balsamifera* leaves was established. The flavonoids of five different sources were determined to demonstrate the validity of this method. The isocratic method developed herein could be useful for routine analysis of crude drug and also applicable to determine the plasma level of major flavonoids of *B. balsamifera*. Furthermore, the use of this method would allow the identification and quantitation of the flavonoids from different sources of *Blumea balsamifera* leaves, and the finished product contained this medicinal herb.

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