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Quality evaluation of *Platycladus orientalis* (L.) Franco through simultaneous determination of four bioactive flavonoids by high-performance liquid chromatography

Yan-hua Lu [a], Zhi-yong Liu [a], Zheng-tao Wang [b,*], Dong-zhi Wei [a,*]

[a] *State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Box #311, 130 Meilong Road, Shanghai 200237, PR China*

[b] *Institute of Chinese Materia Medica, Shanghai University of TCM, Shanghai 201203, PR China*

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Abstract

Platycladus orientalis (L.) Franco (Cupressaceae), a traditional Chinese herb and food additive, has been used for treatments of gout, rheumatism, diarrhoea and chronic tracheitis. To evaluate the quality of *P. orientalis* (L.) Franco, a sensitive, simple and accurate reversed-phase high-performance liquid chromatographic (RP-HPLC) separation method with a photodiode array detector (DAD) was developed for the determination of four main bioactive flavonoids, rutin, quercitrin, quercetin and amentoflavone. Separation of the four compounds was achieved by the HPLC assay (Agilent Eclipse XDB-C 18 column with mobile phase, methanol–acetonitrile–18 mM sodium acetate buffer (pH 3.5) and recorded at UV 356 nm). This method showed good linear relation in the range of 0.8–80 μg/ml for rutin, 1.84–184 μg/ml for quercitrin, 0.72–72 μg/ml for quercetin and 0.72–72 μg/ml for amentoflavone. The correlation coefficients of the calibration curve for the analysis were all higher than 0.999. In addition, the contents of those four flavonoids in *P. orientalis* (L.) Franco growing in 12 different locations in China were compared to establish the effectiveness of the method.

Keywords: *Platycladus orientalis* (L.) Franco (Cupressaceae); Rutin; Quercitrin; Quercetin; Amentoflavone; Flavonoid; HPLC/DAD

1. Introduction

Platycladus orientalis (L.) Franco (Cupressaceae) is synonym to *Biota orientalis* Endl; *Thuja orientalis* Linnaeus; *Platycladus stricta* Spach; *Thuja chengii* Borderes & Gaussen; and *Tuja orientalis* var. argyi Lemee & H. Léveillé [1]. *P. orientalis* (L.) Franco (PO), a traditional Chinese herb and food additive, has been used for treatments of gout, rheumatism, diarrhoea and chronic tracheitis [2,3].

The flavonoid constituents of PO are responsible for major bioactivities, such as spasmolytic, antiphlogistic, antioxidatic, antiallergic and diuretic properties [4–6]. Flavonoids are a large family of over 4000 ubiquitous secondary plant metabolites, comprising five subclasses, anthocyanins, flavonols, flavones, catechins and flavonones.

Several flavonoidic constituents of the leaves of PO have been reported, such as rutin, quercitrin, quercetin, amentoflavone, aromadendrin, myricetin and hinokiflacone [4]. So far, analysis of flavonoids has been accomplished by high-performance liquid chromatography (HPLC) [7–14], but there was no report about analytical methods for determination of rutin, quercitrin, quercetin and amentoflavone simultaneously in PO. There was only previous report for determination of single flavonoid in PO [5,15]. Furthermore, amentoflavone is also the major flavonoid in PO, but there was no way to separate it from PO by HPLC in previous studies.

Thus, with the increasing applications of PO in food and the medicinal herb industry, it is necessary to establish an analytical method for quality control. The strategy we applied was to determine the four compounds, which previously [4,5] were identified as bioactive constituents to evaluate the quality of PO. This is the first report to simultaneously determine rutin, quercitrin, quercetin and amentoflavone in PO by HPLC/DAD. In addition, the contents of those four flavonoids in PO growing

* Corresponding authors. Tel.: +86 21 64252981; fax: +86 21 64250068.
E-mail addresses: luyanhua@ecust.edu.cn (Y.-h. Lu), dzhwei@ecust.edu.cn (D.-z. Wei).

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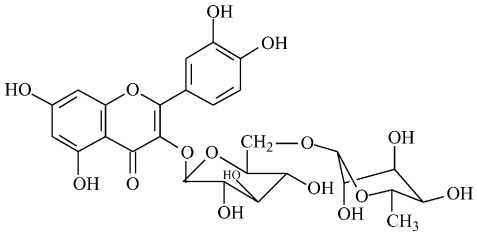
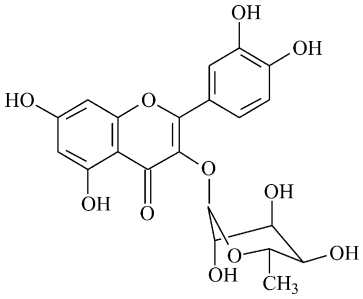
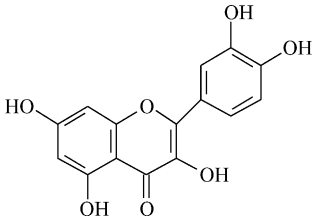
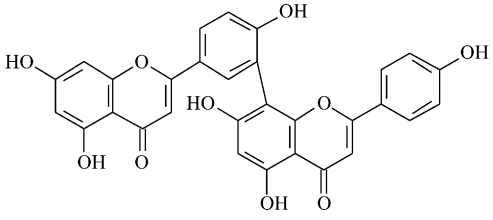
Table 1
Structure and UV_{max} for confirmation of compounds in *Platycladus orientalis* (L.) Franco

Compound	UV_{max} (nm)	Structure
Rutin	210, 260, 356	
Quercitrin	210, 260, 356	
Quercetin	210, 254, 365	
Amentoflavone	208, 270, 360	

in 12 different locations in China were compared to establish the effectiveness of the method.

2. Experimental

2.1. Plant material and chemicals

The leaves of PO samples were collected from 12 producing areas (Table 3) in China. Twelve voucher specimens, identified by Professor Zheng-tao Wang, are deposited at Institute of Biochemistry, East China University of Science and Technology, Shanghai 200237, China, in good condition.

HPLC-grade methanol and acetonitrile were purchased from Caledon Laboratories Ltd., Canada. Double distilled water was sel-prepared. Sodium acetate (≥85%) was purchased from Shanghai Chemical Co. All the solutions were filtered through 0.45 μm membranes (Schleicher & Schuell, Germany) and degassed by an ultrasonic bath before use.

The identification of four flavonoids (rutin, quercitrin, quercetin and amentoflavone; Table 1) separated in our laboratory was accomplished by direct comparison with standards from Sigma by HPLC assay and by spectroscopic methods (FAB-MS, ^{1}H NMR and ^{13}C NMR). The spectral data of the four flavonoids agree with those in literatures [16–19].

2.2. Sample preparation

Around 1 g of dried leaves was milled into powder and sonicated in 25 ml 80% (v/v) EtOH for 30 min (80% EtOH (v/v) has been tested to give the highest extraction yield for the four flavonoids). After 15-min cooling, the extract was filtered through glass wool for sample cleaning up and diluted to 50 ml with 80% EtOH. The sample solution was filtered through a 0.45 μm membrane before being injected into HPLC.

2.3. Analytical method

The main flavonoidic constituents of PO were analyzed by an Agilent 1100 series system, equipped with photodiode array detector (DAD) working in the range of 190–800 nm, quaternary pump and autosampler. The absorption was set at 356 nm

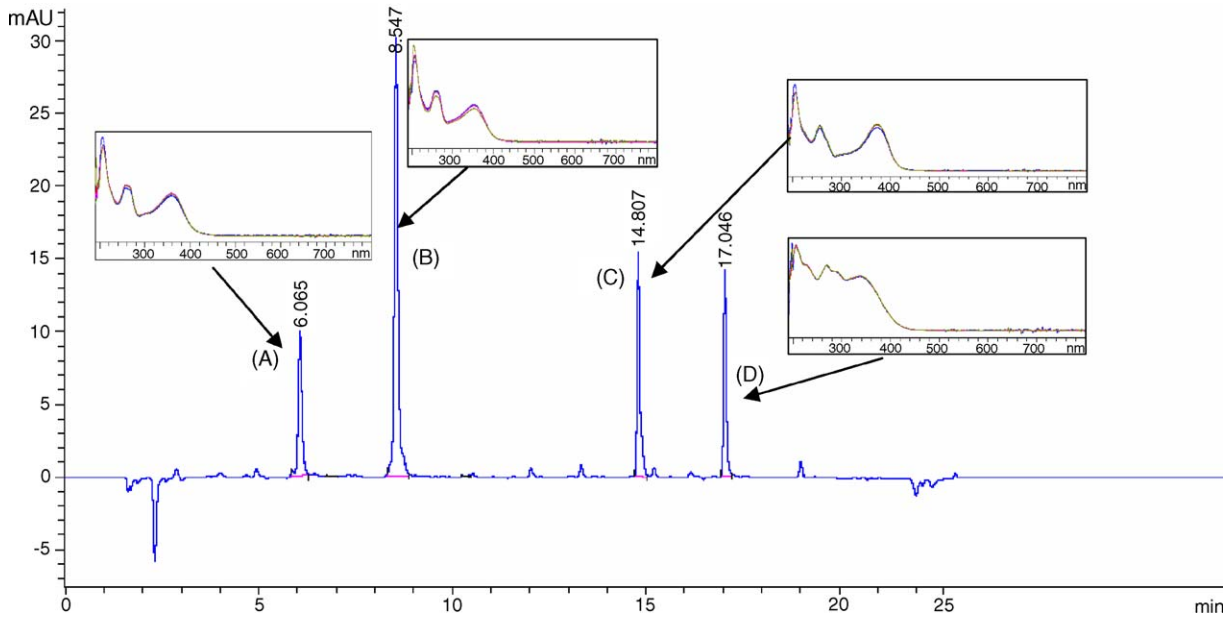


Fig. 1. HPLC chromatogram of flavonoids from *Platycladus orientalis* (L.) Franco at 356 nm wavelength. The four insets are DAD UV scan of rutin (A), quercitrin (B), quercetin (C) and amentoflavone (D) peak (190–800 nm).

for most constituents. The chromatographic data were recorded and processed with Agilent Chromatographic Work Station software. Analysis was carried out at 30 °C on an Agilent Eclipse XDB-C 18 column (3.5 μm, 150 mm × 4.6 mm, i.d.), which was protected by a guard column (3.5 μm, 12.5 mm × 4.6 mm, i.d.). The mobile phase consisted of methanol (E), acetonitrile (F) and 18 mM sodium acetate buffer adjusted to pH 3.5 with glacial acetic acid (G). The analysis was performed by use of a linear gradient program. Initial conditions were 27% E changed to 36%, 3% F changed to 4%, 70% G changed to 60% in 5 min, and in another 15 min, changed to 91% E, 9% F and 0% G. Each run was followed by an equilibration period of 10 min. The peaks were recorded using UV absorbance at 356 nm, the flow rate was kept at 0.8 ml/min and the injection volume was 10 μl. The peaks of the four main flavonoids were narrow and there were no tails; this shows that the sample in 80% EtOH has no solubility problem in the gradient phase; because the concentration of the samples is very low, they can dissolve in the gradient phase easily.

2.4. Calibration

A mixed stock solution consisting of rutin (0.2 mg/ml), quercitrin (0.46 mg/ml), quercetin (0.18 mg/ml) and amentoflavone (0.18 mg/ml) was prepared. 0.10, 0.25, 0.64, 1.6, 4.0 and 10.0 ml of the stock solution were, respectively, adjusted with 80% EtOH into six 25 ml volumetric flasks for the calibration of standard curves.

3. Results

3.1. Separation of rutin, quercitrin, quercetin and amentoflavone

Several mobile phases, including methanol–water and acetonitrile–water in combination with acetic acid or sodium acetate, were tested. Eventually, it was found that a methanol–acetonitrile system containing 18 mM sodium acetate gave the best separation of rutin, quercitrin, quercetin and amentoflavone. Fig. 1 demonstrates the separation obtained for a typical sample of rutin, quercitrin, quercetin and amentoflavone and the four insets are DAD UV (190–800 nm)) scan profiles of rutin (A), quercitrin (B), quercetin (C) and amentoflavone (D) peaks (190–800 nm). It shows that a good separation can be achieved within 20 min using the conditions described. The remainder of the gradient conditions ensures efficient column washing.

3.2. Comparison of different solvents

Ethanol, methanol, ethyl acetate and water were used to investigate the extraction effect of rutin, quercitrin, quercetin and amentoflavone comparing with water extraction. It was found that ethanol/water 80% (v/v) gave the highest extraction yield for rutin, quercitrin, quercetin and amentoflavone. The effects of extraction time on the content of rutin, quercitrin, quercetin and amentoflavone were investigated using 80% (v/v) ethanol/water as the solvent. It was found that 30 min of sonication was sufficient to extract the analytes.

3.3. Validation of the method

Typical chromatogram is shown in Fig. 1. The retention times of rutin, quercitrin, quercetin and amentoflavone were 6.1, 8.5, 14.8 and 17.0 min, respectively. Peak area (Y) of the rutin, quercitrin, quercetin and amentoflavone was measured and plotted against the concentration (X) of each compound. In the range of 0.8–80 μg/ml for rutin, 1.84–184 μg/ml for quercitrin, 0.72–72 μg/ml for quercetin and 0.72–72 μg/ml for amentoflavone, good correlation of linear-

Table 2
Recoveries of four compounds ($n = 6$)

Compound	Added (μg)	Actual* (μg)	Found* (μg)	Recovery* (%)	Average (%)	R.S.D. (%)
Rutin	93	75.9	165.2	96	98.1	2.6
	93	70.8	164.7	100.9		
	93	64.9	155.6	97.5		
Quercitrin	103	177.3	278.5	102.4	101.3	3.5
	103	171.7	277.1	104.1		
	103	111.4	270.4	97.3		
Quercetin	88	78.4	165.8	99.2	100	1.1
	88	75.2	164.1	101.2		
	88	76.3	164.1	99.7		
Amentoflavone	80	84	17	107	102	4.4
	80	85	169	102		
	80	85	16	98		

"Actual*" means the flavonoids of PO actual content. "Found*" means flavonoids of PO final content. Recovery* (%) = [(found − actual)/added] × 100%.

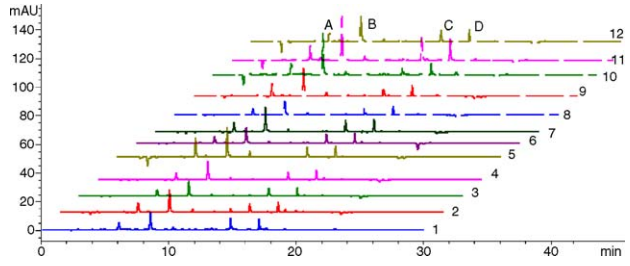


Fig. 2. HPLC chromatograms of flavonoids rutin (A), quercitrin (B), quercetin (C) and amentoflavone (D) from different sources. Sample number: (1) Zhejiang province (041224); (2) Jiangsu province (040925); (3) Anhui province (041208); (4) Guangdong province (041215); (5) Zhejiang province (041212); (6) Fujian province (041119); (7) Hebei province (041015); (8) Hebei province (031120); (9) Heilongjiang province (041223); (10) Beijing (010622); (11) Zhejiang province (050228); (12) Shanghai (041225).

ity has been achieved. The regression curves and correlation coefficients were $Y = 2.1792X + 1.1648$ ($n = 6$; $R^2 = 0.9999$) for rutin, $Y = 1165.3X - 2.3275$ ($n = 6$; $R^2 = 0.9999$) for quercitrin, $Y = 4.6216X - 0.8167$ ($n = 6$; $R^2 = 0.9998$) for quercetin and $Y = 2375.1X + 0.3363$ ($n = 6$; $R^2 = 0.9999$) for amentoflavone (X: contents of compound (μg/ml); Y: peak areas).

The limit of detection, defined as the lowest sample concentration which can be detected (signal-to-noise ratio = 3), was 0.005 μg for rutin, quercitrin, quercetin and amentoflavone, and the limit of quantification, defined as the lowest sample concentration which can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio > 10), was 0.01 μg (R.S.D. < 10%) for rutin, quercitrin, quercetin and amentoflavone.

The precision of the analytical method was determined by at least triplicate applications of each sample. One standard solution was analyzed for six times, consecutively, using the analytical method above. The relative standard deviation of peak areas was 0.8% for rutin, 0.3% for quercitrin, 0.4% for quercetin and 0.3% for amentoflavone. The study of stability was performed on 2 consecutive days ($n = 10$) indicating a relative standard deviation of 1.3% for rutin, 0.9% for quercitrin, 2.1% for quercetin and 2.6% for amentoflavone. A study of the repeatability showed relative standard deviations of 0.7% for rutin, 0.8% for quercitrin, 0.5% for quercetin and 0.5% for amentoflavone, respectively, and those for reproducibility between days ($n = 10$, 3 consecutive days) from 1.2 to 3.4%. The recovery test was used to evaluate the accuracy of the method. Known amounts of flavonoids were added to 1 g of dried leaves of PO and analyzed by the proposed methods [20]. The average recoveries of rutin, quercitrin, quercetin and amentoflavone are listed in Table 2. High recovery suggested that there was

Table 3
Contents of rutin, quercitrin, quercetin and amentoflavone in different samples

Sample number	Rutin (mg/g)	Quercitrin (mg/g)	Quercetin (mg/g)	Amentoflavone (mg/g)
Zhejiang province (041224)	0.34	1.55	0.19	0.41
Jiangsu province (040925)	0.47	1.99	0.17	0.33
Anhui province (041208)	0.30	1.37	0.14	0.29
Guangdong province (041215)	0.36	1.73	0.36	0.29
Zhejiang province (041212)	1.00	2.77	0.20	0.39
Fujian province (041119)	0.32	1.46	0.18	0.35
Hebei province (041015)	0.60	2.45	0.23	0.45
Hebei province (031120)	0.28	1.30	0.16	0.30
Heilongjiang province (041223)	0.76	3.48	0.19	0.47
Beijing (010622)	0.57	4.08	0.19	0.30
Zhejiang province (050228)	0.77	4.37	0.36	0.88
Shanghai (041225)	0.56	2.72	0.22	0.49

negligible loss of those four flavonoids during the extraction process.

3.4. Quantitative determination of PO

Twelve samples were extracted, following the procedure above, and analyzed in the HPLC system. The HPLC/DAD profiles are illustrated in Fig. 2. The content of each compound was determined by the corresponding regression equation and was summarized. Table 3 shows the content of rutin, quercitrin, quercetin and amentoflavone in 12 samples of PO. It shows that the content of rutin ranged from 0.28 to 1.00 mg/g, quercitrin from 1.3 to 4.37 mg/g, quercetin from 0.14 to 0.36 mg/g and amentoflavone from 0.29 to 0.88 mg/g.

4. Discussion

In our present study, a simple, accurate and rapid HPLC method was developed and this is the first report of a HPLC simultaneous determination of four main flavonoids in PO. The assay is reproducible, sensitive and has been fully validated. Furthermore, it was successfully applied in 12 different PO samples. The results indicate that herbals from different places show a specific and similar HPLC chromatogram and the evaluation of data might be useful in quality assurance as well as for determination of adulteration of the crude drug.

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