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SIMULTANEOUS DETERMINATION OF FLAVONOIDS AND TRITERPENOIDS IN *CYCLOCARYA PALIURUS* LEAVES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract

Background: *Cyclocarya paliurus* is an endangered plant and endemic to China. The leaves of *C. paliurus* have been used in drug formulations and as ingredients in functional foods in China. The aim of this study was to develop an effective method to extract most of the compounds and to establish a simplified HPLC analytical method to determine the contents of major bioactive compounds simultaneously.

Materials and methods: High-performance liquid chromatography (HPLC) coupled with a photodiode array detector (PDA) was used for the simultaneous determination of the major flavonoids and triterpenoids in *C. paliurus* leaves.

Results: Ultrasonic extraction in 100% methanol for 30 min was adopted as the optimal extraction method for *C. paliurus* leaves. The separation conditions were optimized using a Phenomenex C18 ODS column (250 mm × 4.6 mm, 5 μm) with a mobile phase of acetonitrile and 0.02% formic acid and a detection wavelength of 205 nm. The validation data indicated that this new HPLC analytical method successfully quantified the provenance and seasonal variations of seven major compounds (three flavonoids and four triterpenoids) in *C. paliurus* leaves.

Conclusion: The study provided a novel and simplified approach to simultaneously determine the quantity of major flavonoids and triterpenoids in *C. paliurus* leaves. The results could promote the optimization of silvicultural systems for quality control of *C. paliurus*.

Key words: *Cyclocarya paliurus*; HPLC; flavonoids; triterpenoids

Abbreviations: High-performance liquid chromatography (HPLC); photodiode array detector (PDA); total flavonoids content (TFC); total triterpenoids content (TTC).

Introduction

Cyclocarya paliurus (Batal.) Iljinskaja is an endangered plant endemic to China and a monotypic genus of the Juglandaceae family. *C. paliurus* is commonly called “sweet tea tree” because of the sweet taste of its leaves (Fang *et al.*, 2006). Sweet tea tree is naturally distributed in many provinces of Southern China, including Anhui, Fujian, Guangxi, Henan, Hubei, Hunan, Jiangsu, Jiangxi, Sichuan, Guizhou and Zhejiang (Fang and Fu, 2007). The leaves of *C. paliurus* have been used as herbal tea for hundreds of years (Zhao *et al.*, 2013; He *et al.*, 2013), and were used for drug formulations in traditional Chinese medicine (TCM) or as ingredient of functional foods in China (Xie *et al.*, 2010). The main chemical composition of *C. paliurus* leaves include flavonoids, triterpenoids, and polysaccharides (He *et al.*, 2012). The extracts of *C. paliurus* were reported to have multiple beneficial effects, such as antihypertensive (Zhang *et al.*, 2010), antihyperlipidemic (Wang *et al.*, 2013; Kurihara *et al.*, 2003), antihyperglycemic (Wang *et al.*, 2013; Li *et al.*, 2011), antioxidant activity (Wang *et al.*, 2013), and inhibitors of PTP1B (protein tyrosine phosphatase 1B) (Zhang *et al.*, 2010).

Up to now, the raw materials of *C. paliurus* were harvested in the wild for herbal tea or nutraceutical products. As the market demand increased, the natural resources were seriously destroyed. More leaves must be produced within the plantation. Recently, a number of such plantations of *C.*

paliurus have been developed in Zhejiang and Hunan province, China. However, the chemical profile of herbal plants is readily affected by many factors such as origin, climate, harvesting time and other agricultural practices (Zhao *et al.*, 2013). The differences in chemical composition may lead to potential variations in health related effects, effective dosage, stability, shelf life, flavor, functional properties, as well as safety (Xie *et al.*, 2013). However, one or even a few active constituents in a traditional Chinese medicine do not reflect its overall efficacy (Liu *et al.*, 2010). To ensure the efficacy and safety of TCMs, qualitative and quantitative analysis of more major bioactive components is very important (Peng *et al.*, 2013). The previous studies of *C. paliurus* have focused only on the quantification of total flavonoids (Mi *et al.*, 2009; Chen *et al.*, 2009; Jin *et al.*, 2007) or a few selected flavonoids (kaempferol, quercetin and isoquercitrin) (Fang *et al.*, 2011; Yang *et al.*,2009; Xie *et al.*, 2003) because many marker compounds are not commercially available, especially some characteristic compounds. The purposes of this study were to develop an effective method to extract the overall compounds and develop a simple HPLC analytical method to quantify seven major bioactive components (isoquercitrin, kaempferol-3-*O*-*α*-L-rhamnopyranoside, kaempferol-3-*O*-*β*- glucuronide, arjunolic acid, cyclocaric acid B, pterocaryoside B and pterocaryoside A) simultaneously. Based on the established analytical method, the provenance and temporal variation of *C. paliurus* were investigated.

The results could provide useful information for quality evaluation of *C. paliurus* and for its further development in establishing a good agriculture practice (GAP) standard in China.

Experimental

Standard Chemicals and Reagents

Reference compounds isoquercitrin and kaempferol-3-*O*-*β*-glucuronide were purchased from Shanghai R & D Center for Standardization of Chinese Medicines (Shanghai, China). Arjunolic acid was purchased from BioBioPha Company limited (Yunnan Province, China). Kaempferol-3-*O*-*α*-L-rhamnopyranoside, cyclocaric acid B, pterocaryoside B and pterocaryoside A were isolated in the Laboratory of Translational Medicine, Jiangsu Province Academy of Traditional Chinese Medicine (Nanjing, China) and were identified by spectral data (UV, MS, ^{1}H NMR, ^{13}C NMR). Their structures are shown in Figure 1 and the purities of all chemicals were greater than 95%. HPLC grade acetonitrile and water were obtained from Mallinckrodt (Phillipsburg, NJ, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were all analytical grade.

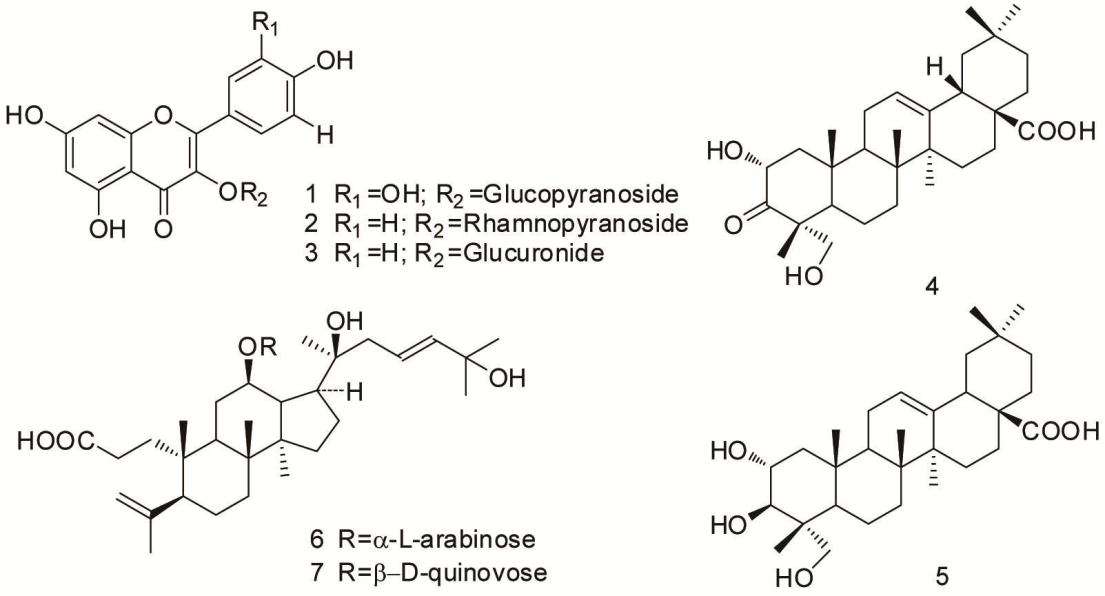

Figure 1: Chemical structures of seven bioactive compounds: 1, isoquercitrin; 2, kaempferol-3-*O*-*α*-L-rhamnopyranoside; 3, kaempferol-3-*O*-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B and 7, pterocaryoside A.

Plant Material

Seeds of *C. paliurus* from 11 provenances were collected from October to November, 2006 in China. The geographical information of the 11 provenances and seedling cultural techniques were described in detail previously (Fang *et al.*, 2011). The one-year-old seedlings were planted in Zhenjiang Nursery, Jiangsu Province at around 32°16′ N latitude and 119°32′ E longitude in Mid-March of 2008. For seasonal variation analysis, leaves of ZAJ provenance were collected in July, October, and December of 2011, respectively. For provenance variation analysis, the leaves were collected in October 2011. A voucher specimen was deposited in Silviculture Lab of Nanjing Forestry University (Voucher code: 2011ZJ).

Sample Preparation

The dried leaves were pulverized to powder and passed through a 60-mesh sieve. The accurately weighed powder (0.5 g) was suspended in 6 mL of methanol. After the weight of the whole flask was recorded, the sample was ultrasonically extracted for 30 min at 60℃ and then cooled at room temperature. The original solvent weight was restored. After centrifugation at 4000 rpm for 10 min, 2 mL supernatant was subjected to C18 column, and filtered through a 0.45 μm syringe filter (Nalgene, NY, USA) prior to HPLC. For each sample, the complete assay procedure was conducted in triplicate and the standard deviation was calculated.

HPLC Apparatus and Chromatographic Conditions

An HPLC (e2695) form Waters (Milford, MA) connected to a UV/vis Photodiode Array (PDA) 2996 detector was used for all analyses. Chromatographic separations were carried out on a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) with a C18 guard column (7.5 mm × 4.6 mm, 5 μm). A mobile phase (A: acetonitrile- D: 0.02% formic acid in water, grade elution) was delivered to the column at a flow rate of 1.0 mL/min. A pre-equilibration period of 20 min was used between individual runs. The column temperature was set at 25 ℃. All components were detected at 205 nm. An aliquot of 10 μL of each sample solution was injected and eluted by the following profile: 0-10 min, isocratic 10% A; 10-30 min, linear gradient of 10-15% A; 30-90 min, linear gradient of 15-30% A; 90-105 min, linear gradient of 30-40% A; 105-115min, isocratic 40% A; 115-145 min, linear gradient of 40-60% A; 145-155 min, linear gradient of 60-75% A; 155-165 min, linear gradient of 75-95% A; 165-170 min, linear gradient of 95-98% A; 170-175 min, linear gradient of 98-100% A; and 175-180min, isocratic 100% A.

Preparation of Standard solution

Seven stock solutions of standards (1000 μg/mL for three flavonoids and 2000 μg/mL for four triterpenoids) were prepared by dissolving them in methanol. Calibration curves were made by diluting the stock solutions with methanol. All solutions were filtered through a 0.45 μm syringe filter and stored in the refrigerator at 4 ℃ before analysis.

Method validation

Linearity, Limits of Detection, and Quantification

Calibration curves were fit to the equation $y = ax + b$ using a linear regression in which y and x were HPLC analyte peak areas of each compounds and each compound concentration. The lowest concentration of standard solution was diluted with methanol to yield a series of appropriate concentrations, and the LOD and LOQ under the chromatographic conditions were separately determined at signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Precision and Accuracy

The measurement of intra- and inter-day variability was utilized to determine the precision of this newly developed method. For intra-day precision test, the standard solution of assayed components was injected for five times within a day. The inter-day precision was determined with the same standard solution on five consecutive days. The precision and accuracy of the method were assessed by analyzing control samples at three concentrations. Precision was expressed as the coefficient of variation (CV, %), calculated as the ratio of the standard deviation to the measure mean standard concentration. Accuracy was calculated by dividing the measured mean standard concentration by the theoretical mean

standard concentration.

Recovery

A recovery test was used to evaluate the effectiveness of this method. Accurate standards were spiked with 80, 100, 120% of the amounts found in the JXS extract into the sample, then extracted and analyzed as described in the Sample Preparation section. The average percentage recoveries were evaluated by calculating the ratio of detected amount versus the added amount.

Stability

The stability of standard solutions was tested at 0, 1, 2, 3, 4, 5, 6 and 7 days. All solutions were kept at 4 ℃ before analysis.

Results and Discussion

Optimization of Extraction Methods

To achieve a complete extraction of the components, four extraction solvents (55%, 70%, 85% and 100% methanol) and four extraction times (15, 30, 45 and 60min) were investigated. Four solvents were compared with regard to marker compound extraction efficiency using ultrasonication for 30 min. The results indicated that 55% and 70% methanol could not extract any triterpenoids, and the concentrations of three flavonoids were very low (Figure 2). 100% Methanol could effectively extract the highest level of triterpenoids (compounds 4-7) and higher level of flavonoids (compounds 1-3). When samples were extracted with 100% methanol for 15 min, the extraction efficiency was low. However, the extraction efficiencies of 45 min and 60 min were similar to that of 30 min. Therefore, ultrasonically extracting in 100% methanol for 30 min was adapted as the optimal extraction method for *C. paliurus* in the present study.

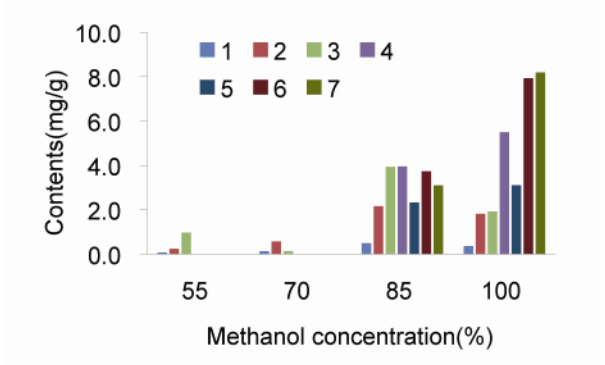

Figure 2. Effect of methanol concentration on the yields (mg/g) of marker compounds 1-7. Compounds 1, isoquercitrin; 2, kaempferol-3-*O*-*α*-L-rhamnopyranoside; 3, kaempferol-3-*O*-*β*- glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B and 7, pterocaryoside A.

Optimization of Chromatographic Conditions

To develop an accurate, valid, and optimal chromatographic fingerprint, different HPLC parameters including mobile phases (methanol-water, methanol-water-formic acid, acetonitrile-water or acetonitrile-water-formic acid) and types of column (Alltech Prevail C18 5 μm 4.6 × 250 mm, Waters Symmetry C18 5 μm 4.6 × 250 mm and Phenomenex C18 5 μm 4.6 × 250 mm) were examined and compared. The results showed that 0.02% v/v formic acid in the solvent system could give more symmetrical peaks and better separation than non-acid systems. A Phenomenex C18 ODS column provided a better separation of components in the plant than other brands of C18 columns. Finally, an optimized HPLC condition was developed by comparing the resolution, baseline, elution time and number of characteristic peaks in each chromatogram of different parameters comprehensively. Figure 3(a) showed the HPLC chromatogram of seven compounds obtained by optimized gradient elution with a mobile phase consisting of acetonitrile-water containing 0.02% formic acid. In the optimal HPLC condition, seven marker compounds showed retention times of 49.13 min, 67.32 min, 71.91 min, 127.03 min, 134.16 min, 137.08 min and 143.08 min, respectively, with good resolution and satisfactory peak shape. As shown in Figure 3 (b), the peaks of seven compounds were also well separated from other peaks found in the extracts of *C. paliurus*.

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Method Validation

The HPLC method in the present study was validated by assessing the linearity, limit of determination (LOD) and quantification (LOQ), intra- and inter-day accuracy and precision, recovery and stability. As shown in Table 1, all of the analytes showed good linearity. The correlation coefficients (R^2) of the calibration curves for the seven marker compounds were greater than 0.999 in relatively wide concentration ranging from 10 to 2060 μg/mL for all the compounds. The LOD (the limit of determination, S/N = 3) and LOQ (the limit of quantifiation, S/N = 10) were less than 92.34 and 307.86 ng/mL, respectively. The intra- and inter-day accuracy and intra- and inter-day precision were assessed at three different concentrations of the seven standards. The accuracy and precision data are presented in Table 2. The intra-day accuracy was 95.95 to 104.95% and the inter-day accuracy was 96.75 to 105.44 %. The intra-day precision was 0.83 to3.92% and the inter-day precision was 0.91 to 3.69%. As shown in Table 3, the mean recoveries found to be in the range of 96.38 and 102.20%, and their RSD values were less than 1%. As shown in Figure 4, the analytes remained very stable and remained at more than 96.30 % in the methanol solution at 4 ℃ over the tested period and the RSD values of all the analytes were less than 2.84 %. Hence, this verified HPLC method was precise, accurate and sensitive for the quantitative evaluation of seven active compounds in the extracts of *C. paliurus*.

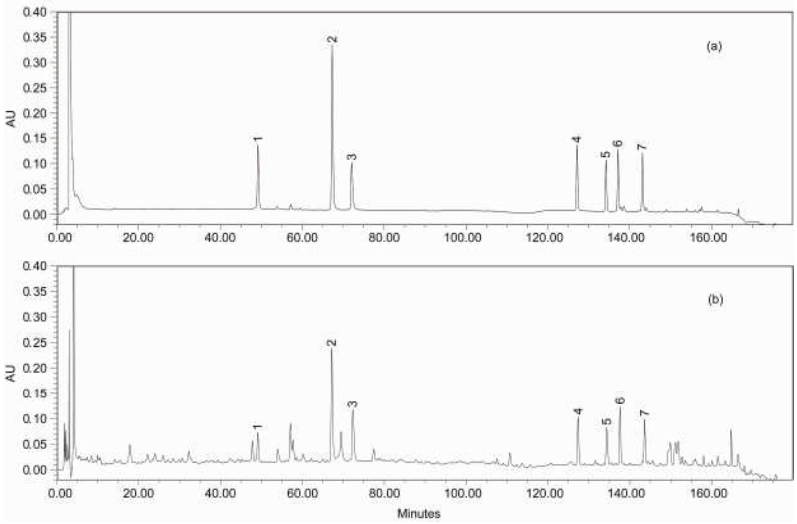

Figure 3: HPLC chromatograms of a standard mixture (a) and a *Cyclocarya paliurus* sample (b). Peak 1, isoquercitrin; 2, kaempferol-3-O-*α*-L-rhamnopyranoside; 3, kaempferol-3-O-*β*- glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B and 7, pterocaryoside A.

Table 1. Regression data, LODs, and LOQs for seven marker compounds.

Compounds[a]	Calibration curve	R^2	Linear Range (μg/mL)	LOD[b] (ng/mL)	LOQ[c] (ng/mL)
1	y = 38174x + 20081	0.9995	10-990	47.49	158.16
2	y = 40415x - 697919	0.9997	10-980	23.17	77.25
3	y = 19057x - 145577	0.9994	10-1010	58.62	159.52
4	y = 4654.1x - 292.96	0.9992	20-1060	88.31	294.53
5	y = 6938.3x - 21806	0.9992	20-1960	92.34	307.84
6	y = 3472.4x - 23720	0.9993	20-2040	78.25	260.92
7	y = 2545.1x - 50967	0.9994	20-2060	90.43	298.67

[a] Compounds 1, isoquercitrin; 2, kaempferol-3-*O*-*α*-L-rhamnopyranoside; 3, kaempferol-3-*O*-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A. [b] Limit of detection (ng/mL). [c] Limit of quantification (ng/mL).

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Analysis and evaluation of *C. paliurus* samples

Seven marker compounds in *C. paliurus* were positively identified by comparing the retention time, UV spectra and MS fragmentation with the corresponding data obtained from the analysis of reference compounds (Figure 3 (b)). In the previous studies, only isoquercitrin, kaempferol and quercetin have been used as marker compounds of *C. paliurus* (Fang *et al.*, 2011; Yang *et al.*, 2009; Xie *et al.*, 2003). Although kaempferol-3-*O*-*α*-L-rhamnopyranoside (He *et al.*, 2012), kaempferol-3-*O*-*β*-glucuronide (Zhang *et al.*, 2005), arjunolic acid (He *et al.*, 2012), cyclocaric acid B (Zhang *et al.*, 2005) and pterocaryoside B (Cui and Li, 2012) have been identified as some compounds in *C. paliurus*, there are no reports on these compounds as markers in this plant. Pterocaryoside A was identified and used as a marker compound in *C. paliurus* for the first time.

Table 2: Intra- and inter-day precision and accuracy in the assay of the seven compounds.

Compounds	Nominal conc. (μg/mL)	Intra-day (n=5)			Inter-day (n=5)		
		Observed (μg/mL)	Accuracy (%)	Precision (%)	Observed (μg/mL)	Accuracy (%)	Precision (%)
1	20.00	19.26	96.30	3.28	19.39	96.95	3.69
	250.00	252.03	100.81	2.01	258.12	103.25	2.52
	500.00	521.50	104.30	1.48	527.2	105.44	2.31
2	20.00	20.99	104.95	2.31	20.75	103.75	1.22
	250.00	244.81	97.92	3.03	246.67	98.67	1.39
	500.00	503.69	100.74	2.53	511.94	102.39	2.54
3	20.00	19.19	95.95	1.96	19.35	96.75	2.61
	250.00	248.32	99.33	1.02	245.89	98.36	2.84
	500.00	508.12	101.62	2.33	511.31	102.26	0.91
4	40.00	39.53	98.83	0.97	39.68	99.20	3.02
	500.00	509.65	101.93	1.38	506.83	101.37	1.74
	1000.00	1023.49	102.35	2.71	1030.64	103.06	3.49
5	40.00	40.67	101.68	1.56	40.32	100.80	0.92
	500.00	498.07	99.61	2.81	486.63	97.33	1.88
	1000.00	989.45	98.95	3.92	983.74	98.37	3.72
6	40.00	40.35	100.88	3.22	40.33	100.83	1.63
	500.00	510.89	102.18	1.35	512.05	102.41	2.87
	1000.00	1048.28	104.83	2.54	1044.72	104.47	2.53
7	40.00	39.68	99.20	0.83	39.53	98.83	1.47
	500.00	493.56	98.71	1.90	489.48	97.90	2.12
	1000.00	984.17	98.42	3.57	982.92	98.29	2.64

Compounds 1, isoquercitrin; 2, kaempferol-3-O-*α*-L-rhamnopyranoside; 3, kaempferol-3-O-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A. [b] Limit of detection (ng/mL). [c] Limit of quantification (ng/mL).

The data revealed that there were great provenance differences in the chemical constituents and their contents among eleven samples (Table 4). In general, levels of four triterpenoids in most of samples were higher than that of the three flavonoids. The contents of three flavonoids ranged from 0.20 to 2.29 mg/g, while the contents of four triterpenoids ranged from 0.92 to 14.12 mg/g. In samples from FZP, YKM, GZY, GLP, GJH and JXS provenances, all seven marker compounds were observed, but with a high degree of content variability. The higher content of each flavonoid and triterpenoid were found in JXS provenances, and its total contents of three flavonoids (isoquercitrin,

kaempferol-3-*O*-*α*-L-rhamnopyranoside and kaempferol-3-*O*-*β*-glucuronide) and four triterpenoids (arjunolic acid, cyclocaric acid B, pterocaryoside B and pterocaryoside A) reached its highest level, 4.00 mg/g and 25.31 mg/g, respectively. Though AQLF contained higher contents of total flavonoids, reached to 3.60 mg/g, its total triterpenoids was the lowest among eleven provenances, only 4.66 mg/g. In this study, the content of isoquercitrin was 0.23 mg/g in HHF provenance, and it was much lower than the report from Fang *et al*. (2011), likely caused by seedling ages and extraction methods. Therefore, JXS is selected to be the optimal provenance based on the quantification of seven bioactive compounds.

Seasonal variations in natural compounds are fairly common and can contribute to variations of the finished products (Chou *et al*., 2009). Black *et al*. (2011) reported that there were significant seasonal variations in fifteen constituents of Northern Labrador Tea (*Rhododendron tomentosum* ssp. *subarcticum*). The temporal variations of the three selected compounds (quercetin, kaempferol, isoquercitrin) in the leaves of *C. paliurus* were determined by Fang *et al*. (2011), who reported that the mean concentrations in most of the provenances reached the maxima in July and September. In this study, the leaves of *C. paliurus* collected in July and October also showed higher contents in three major flavonoids (Table 5). The total content of four triterpenoids was the lowest in July; however, the total triterpenoids in October was similar to that in December. In summary, July and October are the optimal harvesting seasons for flavonoids while October and December are the best harvesting time for triterpenoids.

Table 3: Recovery of the marker compounds through the addition of standards.

Comp.	Original (μg/mL)	Added (μg/mL)	Calconc. (μg/mL)	Recovery (%)	RSD (%)
1	28.56	25.0	52.72	96.64	0.11
		30.0	58.23	98.90	0.07
		35.0	64.18	101.77	0.14
2	148.60	120.0	265.91	97.76	0.13
		150.0	298.07	99.65	0.12
		180.0	326.79	98.99	0.08
3	155.85	120.0	273.16	97.76	0.22
		150.0	301.28	96.95	0.19
		180.0	330.43	96.99	0.24
4	483.47	385.0	869.22	100.19	0.15
		480.0	957.74	98.81	0.12
		580.0	1071.38	101.36	0.17
5	269.20	215.0	476.41	96.38	0.27
		270.0	551.02	104.38	0.31
		325.0	601.35	102.20	0.25
6	660.38	530.0	1182.44	98.50	0.63
		660.0	1302.53	97.30	0.37
		800.0	1447.86	98.44	0.49
7	695.06	555.0	1235.89	97.45	0.37
		695.00	1376.92	98.11	0.26
		835.00	1504.55	96.94	0.52

Compounds 1, isoquercitrin; 2, kaempferol-3-O-*α*-L-rhamnopyranoside; 3, kaempferol-3-O-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A.

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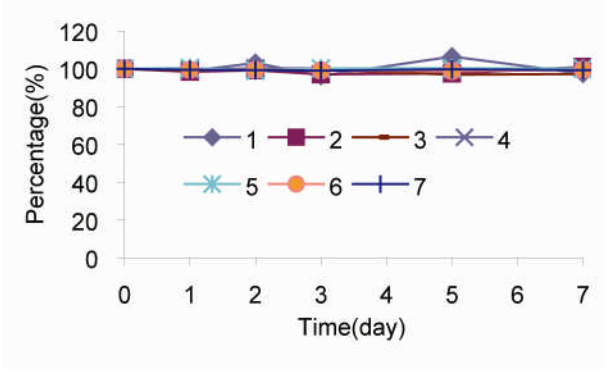

Figure 4: Stability of marker compounds 1-7. Compounds 1, isoquercitrin; 2, kaempferol-3-O-*α*-L-rhamnopyranoside; 3, kaempferol-3-O-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A.

Table 4: Quantification of seven compounds in the samples of *C. paliurus* collected from different provenances.

Provenances	Compounds							Total	
	1	2	3	4	5	6	7	TFC	TTC
FZP	0.30±0.00	1.62±0.00	1.13±0.00	10.16±0.01	7.222±0.01	1.52±0.00	0.92±0.00	3.05	19.82
HJH	0.38±0.01	0.66±0.01	1.74±0.03	4.54±0.06	ND	6.71±0.09	14.17±0.25	2.78	25.41
YKM	0.21±0.01	1.16±0.01	1.34±0.00	7.96±0.04	5.700±0.03	3.27±0.01	7.69±0.07	2.71	24.62
GZY	0.20±0.00	0.94±0.01	0.72±0.01	8.26±0.14	3.441±0.02	2.49±0.01	1.95±0.01	1.86	16.14
GLP	0.37±0.01	1.33±0.02	0.86±0.01	8.77±0.10	2.720±0.02	3.67±0.04	3.21±0.03	2.55	18.37
GJH	0.20±0.00	1.36±0.01	0.83±0.01	9.88±0.07	3.533±0.03	1.88±0.01	1.63±0.01	2.40	16.92
JXS	0.34±0.00	1.78±0.01	1.87±0.01	5.80±0.04	3.232±0.03	7.93±0.05	8.34±0.03	4.00	25.31
JLS	ND	0.94±0.00	0.56±0.00	9.27±0.03	3.596±0.02	4.38±0.02	5.01±0.01	1.50	22.25
HHF	0.23±0.01	0.98±0.02	ND	5.68 ±0.07	ND	ND	ND	1.20	5.68
AQLF	1.31±0.01	2.29±0.03	ND	3.71±0.02	ND	0.95±0.01	ND	3.60	4.66
ZAJ	ND	1.50±0.01	0.91±0.01	11.85±0.08	6.98±0.03	2.26±0.01	3.82±0.01	2.41	24.91

Compounds 1, isoquercitrin; 2, kaempferol-3-*O*-*α*-L-rhamnopyranoside; 3, kaempferol-3-*O*-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A. Unit: mg/g; TFC: contents of total three flavonoids; TTC: contents of total four triterpenoids; Mean ± standard deviation of triplicate experiments; ND: Not detectable.

Table 5: Contents of seven compounds in the ZAJ provenance collected in different seasons.

Harvesting time	Compounds							Total	
	1	2	3	4	5	6	7	TFC	TTC
July	ND	1.15±0.01	1.34±0.01	6.62±0.04	1.03±0.009	2.81±0.01	3.64±0.027	2.48	14.10
October	ND	1.50±0.01	0.91±0.01	11.85±0.08	6.98±0.03	2.26±0.01	3.82±0.01	2.41	24.91
December	ND	0.22±0.00	ND	9.31±0.299	3.67±0.093	3.59±0.08	8.07±0.29	0.22	24.65

Compounds 1, isoquercitrin; 2, kaempferol-3-O-*α*-L-rhamnopyranoside; 3, kaempferol-3-O-*β*-glucuronide; 4, arjunolic acid; 5, cyclocaric acid B; 6, pterocaryoside B; and 7, pterocaryoside A. Unit: mg/g; TFC: contents of total three flavonoids; TTC: contents of total four triterpenoids; Mean ± standard deviation of triplicate experiments; ND: Not detectable.

Conclusions

A novel and simplified HPLC analytical method was developed for the simultaneous quantification of seven bioactive components in *C.*

paliurus leaves. This HPLC method was applied successfully to determine the provenance and temporal variation of isoquercitrin, kaempferol-3-*O*-*α*-L-rhamnopyranoside, kaempferol-3-*O*-*β*- glucuronide, arjunolic acid, cyclocaric acid B, pterocaryoside B and pterocaryoside A. Obviously, the developed quantitative analytical method of these bioactive components could be helpful for the quality assessment of *C. paliurus* leaves and provide useful information toward establishing good agriculture practice standard in China.

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