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Chromatographic Fingerprint Analysis and Rutin and Quercetin Compositions in the Leaf and Whole-Plant Samples of Di- and Tetraploid *Gynostemma pentaphyllum*

Zhuohong Xie,[†,‡] Yang Zhao,[†,‡,§] Pei Chen,[∥] Pu Jing,[§] Jin Yue,[§] and Liangli (Lucy) Yu[*,‡]

[‡]Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742, United States

[§]Institute of Food and Nutraceutical Science, Key Laboratory of Urban Agriculture (South), School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

[∥]Food Composition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service (ARS), United States Department of Agriculture (USDA), Beltsville, Maryland 20705, United States

ABSTRACT: *Gynostemma pentaphyllum* (Thunb.) Makino, also known as jiaogulan, has been shown to have antioxidant, antiproliferative, and anti-inflammatory activities. Flavonoid is considered a major contributor for these beneficial effects. To obtain chemical patterns of flavonoids in *G. pentaphyllum* of different genotypes (di- versus tetraploids) and different parts (leaf versus whole plant) of plants, the extraction condition was optimized and a fingerprinting approach was established by means of high-performance liquid chromatography coupled with diode array detection and mass spectrometry (HPLC−DAD−MS). Eight flavonoids were identified, among which rutin and quercetin were quantified. The highest levels of rutin and quercetin were 23.03 and 12.10 mg/g, respectively, observed in the diploid leaf sample 2L3 and 2L2, while the lowest levels of rutin and quercetin were 1.92 and 0.25 mg/g in the tetraploid whole-plant sample 4W3. The chemical patterns were further analyzed by similarity calculation and principal component analysis (PCA). Seven common characteristic peaks were found in all of the tested samples. Flavonoid patterns of tetraploids were significantly different from those of diploids, whereas different parts of plants showed less difference. The flavonoid pattern of the diploid leaf sample was most similar to that of the reference botanical *G. pentaphyllum*. The combination of chromatographic fingerprint and quantification analysis could be used for quality assessment of *G. pentaphyllum* and its derived nutraceutical products.

KEYWORDS: *Gynostemma pentaphyllum*, flavonoids, fingerprint, tetraploids

INTRODUCTION

Gynostemma pentaphyllum Makino, known as jiaogulan, is a perennial liana plant and has been traditionally used in food, tea, and folk medicines in many east and southeast Asian countries for its possible health properties, such as reducing the risk of cardiovascular diseases,[1,2] and hypoglycemic,[3] anti-inflammatory,[4,5] and anticancer[6] activities. Our recent study showed that commercial *G. pentaphyllum* Makino products contained significant levels of natural antioxidants and might suppress IL-6, Ptgs2, and TNF-α mRNA expression and inhibit HT-29 human cancer cell proliferation.[5] Flavonoids were considered one of the major components that contribute to the health beneficial properties of *G. pentaphyllum*.[7] Several flavonoids have been identified and quantified in a *G. pentaphyllum* sample.[7] These included quercetin-di(rhamno)-hexoside (548.3 μg/g), quercetin-rhamno-hexoside (1249.6 μg/g), kaempferol-rhamno-hexoside (1792.9 μg/g), kaempferol-rhamno-hexoside (2416.5 μg/g), rutin (1602.8 μg/g), kaempferol-rhamno-hexoside (170.7 μg/g), and kaempferol-3-*O*-rutinoside (429.7 μg/g).[7] In addition, a recent study from our laboratory showed that five different commercial *G. pentaphyllum* samples significantly differed in their total flavonoid, phenolic, and saponin contents and their rutin and quercetin concentrations.[5] The five *G. pentaphyllum* samples also differed in their radical-scavenging capacities, ability to suppress IL-6, Ptgs2, and TNF-α mRNA expressions, and antiproliferative effects on HT-29 human colon cancer cell lines.[5] These findings suggested the potential variation in the chemical compositions and health properties of *G. pentaphyllum* samples from different genotypes harvested in different seasons at different locations.

As a continuation of our research on *G. pentaphyllum* Makino, the present study compared di- and tetraploid *G. pentaphyllum*, their leaf and whole-plant samples for their rutin and quercetin contents, and their high-performance liquid chromatography−mass spectrometry (HPLC−MS) fingerprints of flavonoids analyzed by similarity calculation and principal component analysis (PCA). A standard *G. pentaphyllum* botanical sample was included as a reference for HPLC fingerprinting study, and four diploid leaf (2L1−2L4), four diploid whole-plant (2W1−2W4), four tetraploid leaf (4L1−4L4), and four tetraploid whole-plant (4W1−4W4) samples from the same growing location were involved to test how the di- and tetraploid samples and how leaf and the whole-botanical samples may differ from each other in their flavonoid profiles. To the best of our knowledge, this is the first time that the flavonoid fingerprint of *G. pentaphyllum* was investigated and this is also the first report of tetraploid *G. pentaphyllum* samples for their rutin and quercetin contents and their flavonoid fingerprint. The results from this

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study will be used to assess and improve the quality of *G. pentaphyllum* and to promote their use in functional foods to improve human health.

MATERIALS AND METHODS

Chemicals and Materials. Pure rutin and quercetin were purchased from Extrasynthese (Genay, Cedex, France), and their purities were above 98% by HPLC analysis. HPLC-grade acetonitrile was purchased from VWR International, Inc. (Clarksburg, MD). HPLC-grade water was prepared from distilled water using a Milli-Q system (Millipore Laboratory, Bedford, MA). HPLC-grade formic acid was purchased from Fluka Analytical (Buchs, Switzerland). All other chemicals and solvents were of the highest commercial grade and used without further purification. A total of 16 *G. pentaphyllum* Makino samples were obtained from the Asian Citrus Holdings Limited (Hong Kong), including four diploid leaf samples (2L1−2L4), four diploid whole-plant (stems and leaves) samples (2W1−2W4), four tetraploid leaf samples (4L1−4L4), and four tetraploid whole-plant (stems and leaves) samples (4W1−4W4). A *G. pentaphyllum* Makino reference botanical was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All botanical samples were grown in the Dabashan area of the Shaanxi province of China in 2009 and harvested by hand from the different locations of the same field according to a standard randomization protocol. The samples were washed using tap water, dried in a tea drier at 70−76 °C, pulverized with a conventional pulverizing machine, and kept in zip lock bags in the dark until analysis. The ploidy was determined by root tip chromosome checking (22 versus 44).

Sample and Reference Preparation. The reference chemicals, i.e., rutin and quercetin, were dissolved in methanol at 0.1432 and 0.1076 mg/mL as stock solution, respectively. The concentration range used for quantification was 0.001 432−0.1432 mg/mL for rutin and 0.001 076−0.1076 mg/mL for quercetin.

For sonication extraction, 0.5 g of pulverized samples were accurately weighed and extracted with 10 mL of methanol, ethanol, methanol/water (1:1, v/v), methanol/chloroform (1:1, v/v), and acetone, using sonication with a FS30 Ultrasonic sonicator (40 kHz, 100 W) (Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature. The mixtures were centrifuged at 5000 rpm for 5 min and filtered through a 0.45 μm membrane filter before further investigation.

For Soxhlet extraction, 5 g of pulverized samples was accurately weighed and extracted in pure methanol for 6 h using a Soxhlet apparatus. The sample solutions were filtered through a 0.45 μm membrane filter before further investigation.

Total Flavonoid Content. The total flavonoid content was determined according to an aluminum colorimetric method described previously.[8] Briefly, the sample extract/standard (150 μL) was mixed with 1 mL of 5% sodium nitrite for 6 min, followed by adding 1 mL of 10% aluminum nitrate and 4 mL of 4% sodium hydroxide. Rutin was used as the standard with a range from 0.812 to 4.06 mg/mL. Absorbance was read at 502 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 15 min of reaction. The results were reported as milligrams of rutin equivalents per gram of botanicals.

HPLC−Diode Array Detection (DAD)−MS Procedure. The fingerprint was determined by HPLC−DAD analysis according to the protocol described previously, with modification.[9] The tests were carried out by an Agilent Technologies (Palo Alto, CA) 1100 Series system comprising a binary pump with a vacuum degasser, a thermostatted column compartment, an autosampler, and a diode array detector. Compounds were separated on a reversed-phase column (Agilent Eclipse Plus C18, 150 × 2.1 mm, 1.8 μm) at a flow rate of 0.25 mL/min. The HPLC separation was accomplished using a two-solvent gradient system. The mobile phases consisted of water (containing 0.1% formic acid, A) and acetonitrile (containing 0.1% formic acid, B). The initial ratio of A/B was 90:10 (v/v); this was changed linearly to 55:45 in 20 min, to 10:90 after 30 min, and then kept at 10:90 from 30 to 40 min. The wavelength range was 190−400 nm for the detection of flavonoids and chemical fingerprint and 256 nm for quantitative analysis. The column temperature was set at 45 °C. The compounds were identified by HPLC−MS. The tests were performed by a Finnigan LCQ Classic ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) using an electrospray ionization (ESI) interface. The conditions of MS analysis were as follows: sheath gas, 80 arb; auxiliary gas, 10 arb; spray voltage, 4.5 kV; capillary temperature, 220 °C; scan mode: positive and negative; capillary voltage: 18/−12 V; MS full scan range, m/z 100−1500; collision energy for collision-induced dissociation (CID), 25−50%; source fragmentation voltage, 20%; isolation width, 3.0 Th.

Data Analysis. Data from total flavonoid, rutin, and quercetin contents were reported as the mean ± standard deviation (SD) for triplicate determinations on an "as-is" botanical weight basis. One-way analysis of variation (ANOVA) and Tukey's test were employed to identify differences in means.

The similarity was calculated on the basis of the information obtained from entire chromatographic profiles. Data analysis was performed by a software named Similarity Evaluation System (SES) for Chromatographic Fingerprint of Traditional Chinese Medicine, version 2004A (Chinese Pharmacopoeia Commission, China), which was recommended by the State Food and Drug Administration of China (SFDA). The correlation coefficients of entire chromatographic profiles of samples were calculated.

PCA was carried out using software of SIMCA-P_+ 11.5 (Umetrics AB, Sweden) based on UV (256 nm) data.

RESULTS AND DISCUSSION

Selection of the Extraction Condition. Flavonoids have been considered as one of the major health-beneficial components in *G. pentaphyllum* botanicals. Modified from recent studies, several solvent systems, including methanol, ethanol, methanol/water (1:1, v/v), methanol/chloroform (1:1, v/v), and acetone, with sonication or Soxhlet procedures were evaluated and compared for *G. pentaphyllum* flavonoid extraction.[7,10−12] As shown in Table 1, Soxhlet extraction with methanol as the solvent for 6 h was the most effective among the tested protocols and obtained the highest total flavonoid content of over 45 mg of rutin equivalents/g of botanicals, along with the greatest rutin and quercetin concentrations of 11 and 1 mg/g on a per botanical weight basis. These data suggested that Soxhlet extraction with methanol is a preferred method for estimating the *G. pentaphyllum* flavonoids in the botanicals. Also noted was that methanol with sonication obtained the second highest total flavonoids and the highest rutin content and could be a method for comparing a group of *G. pentaphyllum* botanical samples for their flavonoid contents and compositions (Table 1). MeOH−H_2O with higher polarity than MeOH did not significantly enhance the extraction of rutin nor reduce quercetin extraction (Table 1). However, replacing MeOH−H_2O with other solvents, such as dimethyl sulfoxide (DMSO), for different measurements, such as cell proliferation or antioxidant activities, will involve lyophilization and, therefore, will be much harder than using MeOH as the solvent, suggesting that MeOH−H_2O is not a preferred solvent for extraction of *G. pentaphyllum* flavonoids.

Rutin and Quercetin Contents. Flavonoids are a group of polyphenolic phytochemicals, which may be the major contributors for the health properties of vegetables, fruits (grapes and cranberries), and other botanical products.[13] Differences in the

Table 1. Effects of the Extraction Solvent and Method on Phytochemical Properties of *G. pentaphyllum*[a]

	solvent	TFC (mg of RE/g)	rutin content (mg/g)	quercetin content (mg/g)
sonication	MeOH	37.54 ± 1.86 d	9.79 ± 0.05 d	0.60 ± 0.00 c
	EtOH	20.44 ± 1.64 b	4.19 ± 0.57 c	0.30 ± 0.04 b
	MeOH/H_2O (1:1, v/v)	30.46 ± 1.00 c	10.94 ± 0.85 d	0.50 ± 0.06 c
	MeOH/$CHCl_3$ (1:1, v/v)	28.11 ± 2.39 c	2.66 ± 0.15 b	0.52 ± 0.01 c
	acetone	12.25 ± 0.51 a	1.04 ± 0.04 a	0.14 ± 0.00 a
Soxhlet	MeOH	45.63 ± 2.82 e	11.06 ± 0.02 d	1.02 ± 0.01 d

[a] Sample 4L3 was used in all tests. Different letters represent significant differences ($p < 0.05$). TFC stands for total flavonoid content by spectrometric methods. RE stands for rutin equivalents. Rutin and quercetin contents were the flavonoid profile obtained by HPLC.

Table 2. Rutin and Quercetin Contents in *G. pentaphyllum*[a]

	rutin (mg/g)	quercetin (mg/g)
2L1	18.97 ± 0.06 h	9.64 ± 0.02 i
2L2	22.98 ± 0.25 j	12.10 ± 0.07 m
2L3	23.03 ± 0.17 j	11.45 ± 0.02 l
2L4	20.97 ± 0.29 i	11.10 ± 0.06 k
2W1	14.15 ± 0.12 g	4.64 ± 0.02 h
2W2	9.15 ± 0.02 d	3.60 ± 0.02 g
2W3	8.17 ± 0.02 c	1.00 ± 0.03 e
2W4	14.13 ± 0.09 g	10.14 ± 0.05 j
4L1	11.19 ± 0.04 f	1.27 ± 0.03 f
4L2	9.74 ± 0.06 d	0.82 ± 0.04 d
4L3	11.07 ± 0.00 ef	1.02 ± 0.01 e
4L4	10.29 ± 0.03 de	0.92 ± 0.01 de
4W1	3.90 ± 0.02 b	0.52 ± 0.00 bc
4W2	6.86 ± 0.04 c	0.66 ± 0.01 c
4W3	1.92 ± 0.00 a	0.25 ± 0.00 a
4W4	3.19 ± 0.04 b	0.47 ± 0.01 b

[a] 2L1−2L4 represent diploid leaf botanicals. 2W1−2W4 represent diploid whole-plant botanicals. 4L1−4L4 represent tetraploid leaf botanicals. 4W1−4W4 represent tetraploid whole-plant botanicals. Different letters represent significant differences ($p < 0.05$).

type and amount of flavonoids exist in botanicals of different varieties and tend to follow taxonomic lines. Therefore, it is proposed that *G. pentaphyllum* of different genotypes may have different flavonoid profiles. Rutin and quercetin are two of the most abundant flavonoids found in *G. pentaphyllum*.[5,7] For the first time, the present study determined the rutin and quercetin contents in tetraploid *G. pentaphyllum* botanical samples. The leaf samples of tetraploid had a rutin content of 9.7−11.2 mg/g and a quercetin content of 0.8−1.3 mg/g, and the whole-botanical samples of tetraploid had significantly less rutin and quercetin at levels of 1.9−6.9 and 0.3−0.7 mg/g, respectively (Table 2). The concentrations are reported on a per "as-is" weight basis. The rutin contents in these tetraploid samples are similar to that in a recent study (1.6 mg/g).[7] The levels of rutin and quercetin are comparable to those of commercial *G. pentaphyllum* products (0.6−11.2 and 0.1−7.4 mg/g, respectively) in our previous study.[5] It was also observed that the diploid leaf samples had greater rutin and quercetin levels than their counterpart whole-botanical samples (Table 2). These data indicated that rutin and quercetin and possibly total flavonoids are more concentrated in the leaf samples than that in the stems and other nonleaf parts of the plant.

In addition, the rutin and quercetin contents of the di- and tetraploid *G. pentaphyllum* were compared. Diploid leaf and whole-botanical samples had significantly greater rutin and quercetin contents than their tetraploid counterparts (Table 2). Diploid leaf sample 2L3 had the highest rutin concentration of 23.03 mg/g. Rutin contents in the four tested diploid *G. pentaphyllum* samples ranged from 19 to 23 mg/g, which were 2 times higher than those in the tetraploid leaf samples, respectively. The quercetin contents in the diploid samples were about 10 times those detected in the tetraploid *G. pentaphyllum*. Rutin and quercetin contents in the diploid whole-botanical samples were also significantly higher than those in the tetraploid samples (Table 2). These results indicated that the diploid *G. pentaphyllum* may contain greater amount of rutin, quercetin, and total flavonoids than their tetraploid counterparts from the same growing locations.

Validation of Chromatographic Fingerprinting Methodology. The chromatographic fingerprinting methodology was validated for its precision, repeatability, and stability. The precision was evaluated by analysis of five injections of the same testing sample solution consecutively. The repeatability was examined by determination of five different working sample solutions prepared from the same botanical sample. The stability was examined by analysis of sample solution at different time points (0, 2, 4, 8, 16, and 24 h). The similarities of all of the tests were above 0.900, indicating that the HPLC fingerprint analysis method was valid and effective.

The fingerprinting analysis was performed using the characteristic peak approach. The detailed process has been discussed previously.[9,14] In this approach, an authentic sample of a certain botanical was selected as a reference fingerprint (RF, in this study, a *G. pentaphyllum* reference from the National Institute for the Control of Pharmaceutical and Biological Products). The most obvious peak of the RF was selected as the reference peak (RP, in this study, peak 5, rutin). The areas of all other peaks in the chromatograms were normalized against the area of the RP, and the ratios of the peaks were entered into a peak table and used for PCA analysis. As shown in Figure 1 and Table 3, a total of 24 characteristic peaks were detected in all of the tested *G. pentaphyllum* samples. Of the 24 peaks, 7 peaks, including peaks 5, 6, 8, 10, 11, 14, and 17, were detected in all samples and designated as "common peaks". Alignment of retention times of all common peaks were analyzed by SpecAlign 2.4 (Jason Wong, Oxford, U.K.). To achieve higher accuracy on the identification of flavonoids, both positive- and negative-ion modes were employed. Peaks 5 and 14 were identified to be rutin and quercetin, respectively, by comparing the UV spectra, retention time, and MS fragmentation behaviors to those of the references[15] (Figure 1 and Table 3). Peaks 1, 2, 3, 4, 8, 9, and 17 were tentatively identified as quercetin-di(rhamno)-hexoside,[7] kaempferol 3-*O*-di-*p*-coumaroylhexoside,[15] kaempferol 3-*O*-di-*p*-coumaroylhexoside,[15] quercetin-rhamno-hexoside,[7]

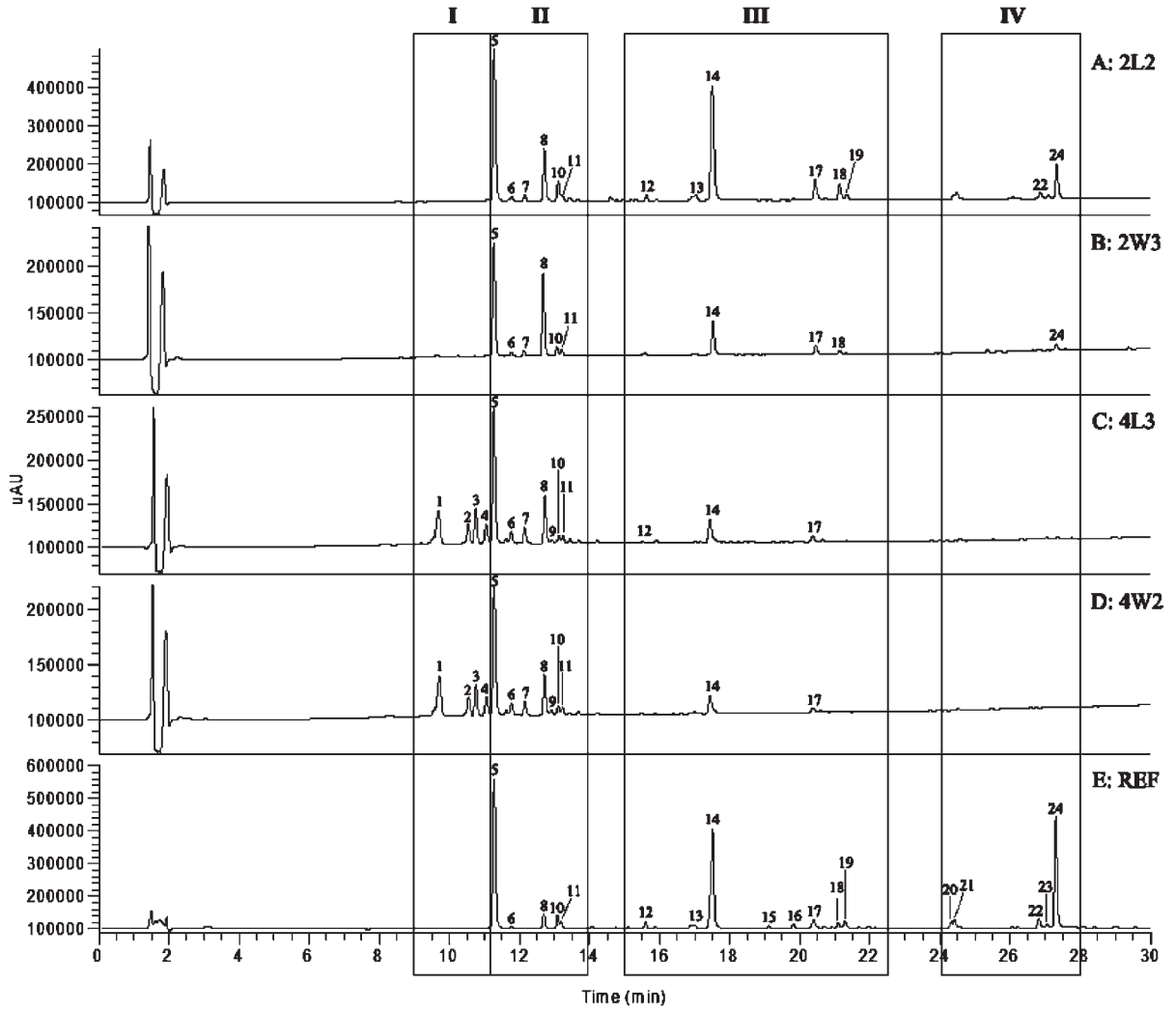

Figure 1. HPLC fingerprint of the representative *G. pentaphyllum* samples: (A) diploid leaf botanical (2L2), (B) diploid whole-plant botanical (2W3), (C) tetraploid leaf botanical (4L3), (D) tetraploid whole-plant botanical (4W2), and (E) reference botanical samples. Data was obtained at 256 nm.

kaempferol-rhamno-hexoside,[7] kaempferol-3-*O*-rutinoside,[16] and kaempferol,[15] respectively, based on the MS fragmentation behaviors under the experimental conditions and the literatures (Table 3).

The HPLC fingerprints of di- and tetraploid, leaf and whole-botanical and reference botanical samples were compared (Figure 1). REF represents the reference botanical spectrum, and 2L2, 2W3, 4L3, and 4W2 are the representative HPLC fingerprints of diploid leaf, diploid whole-botanical, tetraploid leaf, and tetraploid whole-botanical samples. The HPLC peaks could be generally grouped into regions I, II, III, and IV (Figure 1). In region I, four peaks 1−4 [quercetin-di(rhamno)-hexoside, kaempferol 3-*O*-di-*p*-coumaroylhexoside, kaempferol 3-*O*-di-*p*-coumaroylhexoside, and quercetin-rhamno-hexoside] were exclusively detected in tetraploid *G. pentaphyllum* (4L and 4W), regardless of leaf or whole-plant sample. Furthermore, peaks 5 (rutin), 6, 7, 8 (kaempferol-rhamno-hexoside), 10, and 11 were common peaks in all samples in region II, except peak 7, which was not detectable in the reference botanical sample. Peak 9 (kaempferol-3-*O*-rutinoside) was specific for tetraploid *G. pentaphyllum* (Figure 1). Diploid (2L2 and 2W3) *G. pentaphyllum* also differed from the tetraploid (4L3 and 4W2) samples in region II by having a relatively greater ratio of peak 8 and lower ratio of peaks 6 and 7 (Figure 1). No difference was observed between the leaf and whole-plant botanical samples for either di- or tetraploid *G. pentaphyllum* in their HPLC fingerprints in region II.

In region III, peaks 14 (quercetin) and 17 (kaempferol) were common for all five tested *G. pentaphyllum* samples. Peak 12 was only observed in *G. pentaphyllum* di- and tetraploid leaf and reference botanical samples but not found in any whole-botanical samples. Peaks 13 and 19 were only detected in diploid leaf and reference botanical samples under the experimental conditions. Peak 18 was found in diploid leaf and whole-botanical and reference botanical samples, whereas no peaks at the same retention time were detected in tetraploid samples. Peaks 15 and 16 were only detected in the reference botanical sample.

In region IV, peak 22 was only detected in the diploid leaf and reference botanical samples and peak 24 was observed only in the diploid leaf and whole-botanical and reference botanical samples but not in any tetraploid samples (Figure 1). Peak 23 was only seen in the reference botanical sample (Figure 1). Also noticed was that the tested *G. pentaphyllum* samples might differ in their relative levels of individual flavonoid compounds. In addition, the diploid leaf had a HPLC fingerprint most similar to that of the reference botanical sample (Figure 1).

Table 3. MS Fragmentation of the Investigated Compounds by HPLC−MS[a]

peak number	RT	UV (nm)	$[M-1]^-/[M+1]^+$	NI/PI	compound
1	9.63	256, 352	755.26/756.75		quercetin-di(rhamno)-hexoside
2	10.48	264, 346	739.23/740.66		kaempferol 3-*O*-di-*p*-coumaroylhexoside
3	10.69	266, 346	739.30/740.73		kaempferol 3-*O*-di-*p*-coumaroylhexoside
4	10.99	264, 346	609.21/610.77		quercetin-rhamno-hexoside
5	11.21	256, 354	609.19/610.91	1218.87 $[2M-1]$/633.19 $[M+Na]^+$, 464.95 $[M+H-Rham]$, 303.27 $[M+H-Rham-Glu]$	rutin
6	11.71	256, 348	609.23/610.79		unknown
7	12.09	264, 346	609.25/609.85		unknown
8	12.66	266, 348	593.18/−	285.00 $[M-H-Rham-Glu]^-$/617.17$[M+Na]^+$, 503, 287.32 $[M+H-Rham-Glu]^+$	kaempferol-rhamno-hexoside
9	12.86	256, 348	593.21/−	284.71 $[M-H-Rham-Glu]^-$/617.18 $[M+Na]^+$, 503.01, 287.26 $[M+H-Rham-Glu]^+$	kaempferol-3-*O*-rutinoside
10	13.05	254, 356	623.19/−	315.20 $[M-H-Rham-Glu]^-$/647.21 $[M+Na]^+$, 533.02, 317.25 $[M+H-Rham-Glu]^+$	unknown
11	13.17	254, 352	623.19/−	315.20 $[M-H-Rham-Glu]^-$/647.24 $[M+Na]^+$, 533.02, 317.25 $[M+H-Rham-Glu]^+$	unknown
12	15.52	266, 346	607.21/−	299.09 $[M-H-Rham-Glu]^-$/631.24 $[M+Na]^+$, 517.08, 301.30 $[M-H-Rham-Glu]^+$	unknown
13	16.84	268, 308	697.25/699.14	675, 643, 299.17/659.16, 627.16, 603.10	unknown
14	17.39	256, 360	301.07/−		quercetin
15	19.04	352	329.06/331.28	301.15/661.19$[2M+Na]^+$	unknown
16	19.74	266, 348	329.10/331.25	313.12/315.30	unknown
17	20.33	266, 368	285.23/287.27		kaempferol
18	21.04	370	315.16/317.22		unknown
19	21.16	298, 368	315.27/317.22		unknown
20	24.24	270, 364	299.15/−	−/603.08	unknown
21	24.34	270, 362	299.12/−	−/603.06	unknown
22	26.8	266, 366	299.16/−		unknown
23	26.98	272, 362	299.16/301.28		unknown
24	27.27	256, 370	299.23/−		unknown
25	30.93	266, 362	−/315.30		unknown

[a] RT, NI, and PI stand for retention time, negative-ion mode, and positive-ion mode, respectively.

Similarity Calculation of the HPLC Fingerprint Analysis. There are multiple bioactive chemical compounds in herbs, and the determination of the amount of one or several "so-called biomarkers" is not enough to evaluate the quality of herbs. Chemical quality control should also consist of chemical fingerprint, which had been introduced and accepted by the World Health Organization (WHO) in 1991[17] and State Food and Drug Administration (SFDA) of China in 2000 as an efficient approach.[18] Chromatographic fingerprint analysis emphasizes systematic characterization of chemical composition of the herbal samples. It can give an overview of the chemical pattern of different samples for quality assessment. As a suitable method for quality control, it has been widely studied and applied to different herbs in recent years.[19−21] Although it is possible to visually differentiate the chromatograms, it will result in subjective and unreliable outcomes. Therefore, the accurate similarity of the chromatograms and the chemical pattern recognition methods should be used for fingerprint analysis. The similarity evaluation of the fingerprint is one of the most important methods in quality control of traditional Chinese medicine (TCM). Generally, the similarity evaluation system (SES) is used for the calculation recommended by SFDA. The chromatograms of the samples were exported from LC as AIA or CSV formats and then imported to SES, which were recognized as data points. After the alignment and deconvolution of the original data, mean values or medians were analyzed as correlation coefficients. The correlation coefficients of chromatograms reflect the similarity.

In the present study, SES software was employed to synchronize and quantitatively compare the tested *G. pentaphyllum* samples, as well as to provide the correlation coefficients among them. The results are shown in Table 4. The closer the correlation coefficient values were to 1, the more similar the two chromatograms. The similarity values for samples within the same category were all above 0.97, except that 2W3 and 2W4 were lower than 0.93 compared to each other and to the other two diploid whole-botanical samples. These data indicated that the entire HPLC flavonoid chromatograms of the *G. pentaphyllum* samples from the leaf or whole-botanical samples of either di- or tetraploid genotype were generally consistent and stable.

The similarity values for *G. pentaphyllum* samples 2L1−2L4 to the reference botanical sample ranged from 0.914 286 to 0.936 237, and that for 2W4 was 0.933 337 (Table 4), indicating that the flavonoid profiles of 2L1−2L4 and 2W4 were more similar to the reference botanical sample. In comparison to the reference botanical sample, the similarity values were below

Table 4. Similarities of the Different *G. pentaphyllum* Samples

	2L1	2L2	2L3	2L4	2W1	2W2	2W3	2W4	4L1	4L2	4L3	4L4	4W1	4W2	4W3	4W4	reference
2L1	1	0.986124	0.98182	0.982549	0.803492	0.859403	0.656079	0.975242	0.571191	0.496461	0.566273	0.524028	0.48173	0.48772	0.450392	0.481475	0.936237
2L2		1	0.995359	0.995399	0.803781	0.856781	0.654775	0.980101	0.572706	0.492469	0.564566	0.525849	0.472899	0.480477	0.439611	0.470504	0.917554
2L3			1	0.998905	0.812297	0.862195	0.666642	0.981505	0.582484	0.503795	0.573861	0.533374	0.483229	0.490449	0.446903	0.482871	0.920604
2L4				1	0.808627	0.861312	0.663882	0.981636	0.581443	0.500737	0.573	0.533058	0.478852	0.487625	0.443626	0.476198	0.914286
2W1					1	0.982408	0.92577	0.794934	0.821243	0.826733	0.83434	0.793456	0.842474	0.829448	0.829372	0.843837	0.77219
2W2						1	0.915217	0.845239	0.790085	0.780627	0.803021	0.760531	0.788197	0.781007	0.773527	0.779442	0.807741
2W3							1	0.605709	0.892979	0.908558	0.906545	0.880039	0.894734	0.902932	0.880018	0.869591	0.595607
2W4								1	0.50946	0.441372	0.506893	0.457972	0.444409	0.436495	0.412978	0.454206	0.933337
4L1									1	0.97918	0.996025	0.991765	0.936001	0.963231	0.908074	0.908773	0.482403
4L2										1	0.985369	0.97936	0.980258	0.992039	0.964797	0.954639	0.445441
4L3											1	0.993014	0.952263	0.977174	0.929789	0.921749	0.483847
4L4												1	0.937975	0.972046	0.916668	0.898878	0.437149
4W1													1	0.989129	0.99392	0.984846	0.455602
4W2														1	0.979569	0.96028	0.439766
4W3															1	0.976477	0.433885
4W4																1	0.467272
reference																	1

0.81, 0.49, and 0.47 for *G. pentaphyllum* samples 2W1−2W3, 4L1−4L4, and 4W1−4W4 (Table 4). Taken together, the similarity analysis data suggested that the diploid leaf *G. pentaphyllum* samples had a flavonoid profile most similar to that of the reference botanical sample for *G. pentaphyllum*.

PCA. PCA transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components (PCs) and is a widely accepted mathematical approach for reducing the dimensions of multivariate problems. It can reduce the dimensionality of the original data by introducing a small number of underlying factors without losing much information.[19,22] PCA has been commonly used for summarizing chromatographic fingerprint data and may reveal more relationships of the data in a way that better show the variance of the data. Figure 2 shows the PCA scores plot prepared using the normalized areas of the 24 characteristic HPLC−UV peaks against the reference peak, peak 5 at a retention time of 11.21 min (Figure 1), while Figure 3 represents the corresponding loading plot. The scores plot reflects the original data in a rotated coordinate system, whereas the loading plot shows the weights for each original variable when calculating the PCs. The PCA scores plot indicated that the diploid (2L1−2L4) and tetraploid (4L1−4L4) leaf samples and the tetraploid whole-botanical *G. pentaphyllum* samples (4W1−4W4) can be discriminated by their positions, whereas diploid whole-plant samples (2W1−W4) were dispersed. Also noted was that the diploid leaf samples (2L1−2L4) were close to reference botanical *G. pentaphyllum* sample, suggesting close relationship and similar flavonoid profile between them, which agreed with the visual observation in Figure 1. In addition, tetraploid whole-botanical samples (4W1−4W4) were closer to their whole-botanical counterparts (4L1−4L4) than to the diploid leaf samples (2L1−2L4). Two of the tested diploid whole-botanical samples, 2W1 and 2W2, were separated from other samples including the other two diploid whole botanical samples, 2W3 and 2W4. Interestingly, 2W3 was closer to 4W1−4W4, but 2W4 was closer to 2L1−2L4, suggesting that flavonoid compositions of whole botanical samples of diploid *G. pentaphyllum* might be greatly different from each other.

Chemical Markers. Generally, the loading plot of a variable on a PC reflects both how much the variable contributed to that PC and how well that PC takes into account that variation of the variable over the data points. Loadings also describe the relationship between variables. Therefore, if the scores plot can discriminate the different classes of samples, the loading plot can partly express the influence of variables on separation between classes. These variables having the greatest influence on the scores plot are those further away from the main cluster of variables.

The loading plot of PCA (Figure 3) indicated that the peaks at retention times of 9.66 min [peak 1, quercetin-di(rhamno)-hexoside], 10.48 min (peak 2, kaempferol 3-*O*-di-*p*-coumaroyl-hexoside), 10.68 min (peak 3, kaempferol 3-*O*-di-*p*-coumaroyl-hexoside), 10.99 min (peak 4, quercetin-rhamno-hexoside), and 12.82 min (peak 9, kaempferol-3-*O*-rutinoside) may have more influence on the discrimination of the samples from different genotypes. It could be seen from Figure 1 that these "chemical markers" existed in tetraploid leaf and whole-botanical samples and not in diploid leaf or whole-plant or reference botanical *G. pentaphyllum* samples, which indicated the chemical profiles of genotypes of diploids were more similar to reference *G. pentaphyllum*.

In summary, this study showed that methanol is a preferred solvent for flavonoid extraction from *G. pentaphyllum* and Soxhlet extraction is superior to sonication for a high flavonoid yield. The

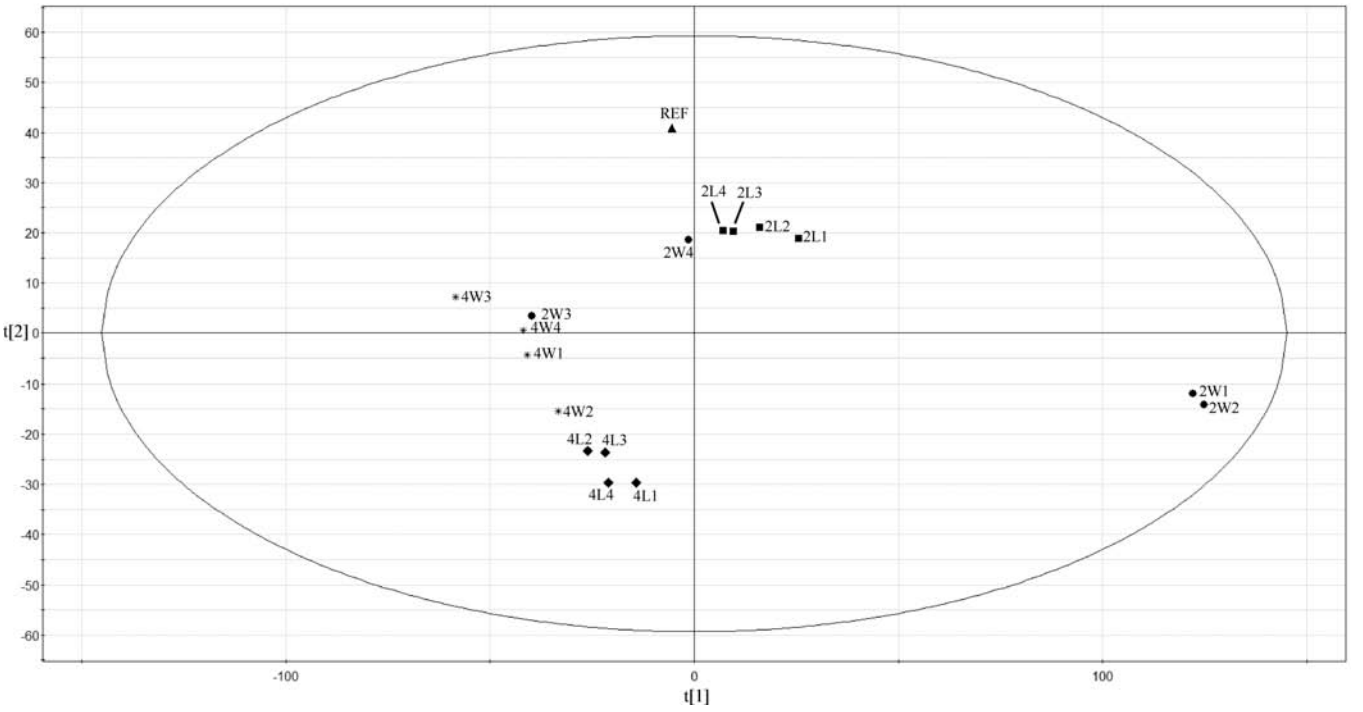

Figure 2. Scores plot of PCA of *G. pentaphyllum* samples. 2L1−2L4 represent diploid leaf botanical samples. 2W1−2W4 represent diploid whole-plant botanical samples. 4L1−4L4 represent tetraploid leaf botanical samples. 4W1−4W4 represent tetraploid whole-plant botanical samples. REF represents the reference botanical sample.

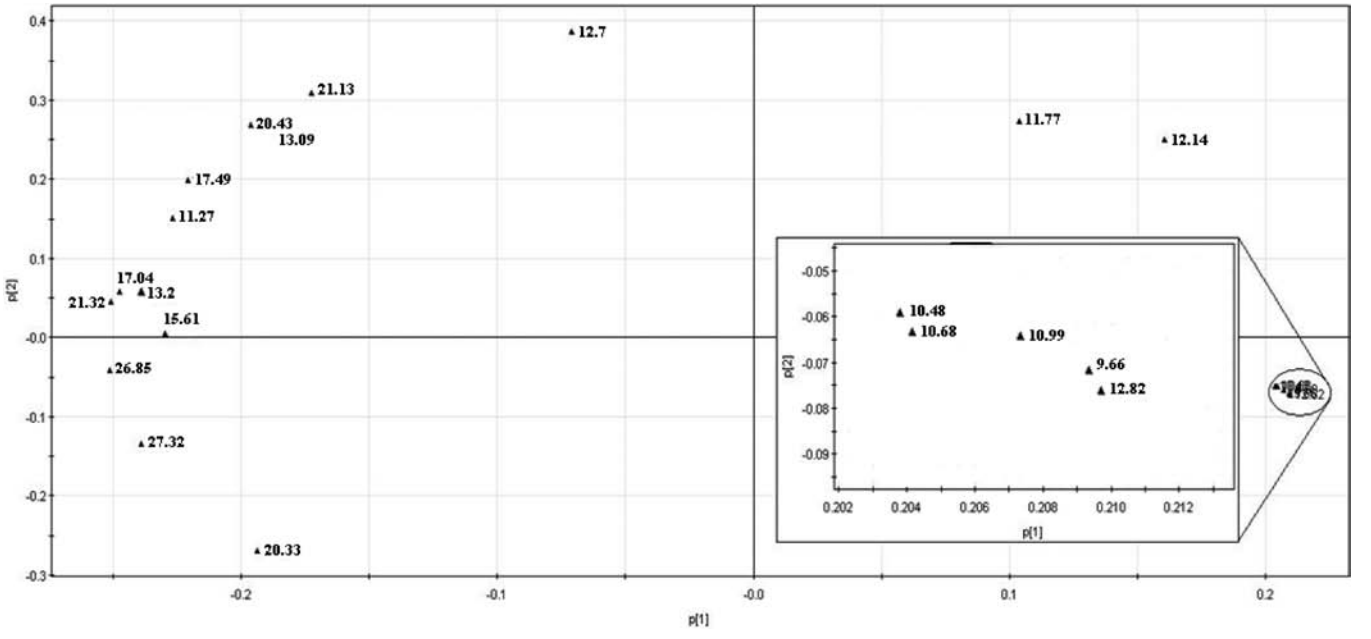

Figure 3. Loading plot of PCA. The rectangle in the bottom right corner is the enlarged graph of the circle.

present study reported the rutin and quercetin contents of leaf and whole-plant tetraploid *G. pentaphyllum* for the first time, along with a comparison to their counterpart diploid samples. PCA analysis of the HPLC−UV fingerprints showed the difference between the di- and tetraploid *G. pentaphyllum* and possibly between leaf and the whole-plant samples from the same genotype of *G. pentaphyllum*, with the diploid leaf samples most similar to the reference sample. These results may imply different physiological activities of the *G. pentaphyllum* di- and tetraploids and their potential applications in improving human health.

AUTHOR INFORMATION

Corresponding Author

*Telephone: (301) 405-0761. Fax: (301) 314-3313. E-mail: lyu5@umd.edu.

Author Contributions

[†]These authors contributed equally to this work.

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