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Simultaneous quantification of three major bioactive triterpene acids in the leaves of *Diospyros kaki* by high-performance liquid chromatography method

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Abstract

The leaf of *Diospyros kaki*, which is a traditional Chinese medicine, has been used for the treatment of various diseases. In order to improve the quality assurance of the leaves of *D. kaki*, derived extracts and phytomedicines, a simple, rapid and accurate high-performance liquid chromatography (HPLC) method was developed to simultaneously assess the three bioactive triterpene acids: barbinervic acid (BA) and its epimer, rotungenic acid (RA), along with 24-hydroxy ursolic acid (HA). This HPLC assay was performed on a reversed-phase C_{18} column with methanol and aqueous H_3PO_4 as the mobile phase and using a monitoring wavelength at 210 nm. This method was successfully applied to quantify these three bioactive triterpene acids in five different solvent extracts of the leaves of *D. kaki* and in the leaves from six different locations in China. The results demonstrated the total content and quantity of each of the main bioactive compounds were strongly dependent on the extraction solvents and locations, indicating that the quality control of the bioactive ingredients in the leaves of *D. kaki*, derived extracts and phytomedicines is critical to ensure its clinical benefits. The content of the total triterpenoids was also determined by the less selective colorimetric method, and the comparison with the HPLC method was given.

Keywords: HPLC quantification; *Diospyros kaki*; Solvent extracts; Barbinervic acid; Rotungenic acid

1. Introduction

The leaf of *Diospyros kaki* (persimmon), which is a traditional Chinese medicine (TCM), has been used for years for the treatment of stroke or syndrome of apoplexy in clinics in China to improve the outcome of ischemia stroke [1,2], and utilized as a hypotensive drug in Japanese traditional medicine [3].

The three main bioactive pentacyclic triterpene acids were characterized spectroscopically as barbinervic acid (BA, 3α, 19α, 24-trihydroxy-urs-12-en-28-oic acid), rotungenic acid (RA, 3β, 19α, 24-trihydroxy-urs-12-en-28-oic acid) and 24-hydroxy ursolic acid (HA, 3β, 24-dihydroxy-urs-12-en-28-oic acid) (see Fig. 1) isolated from the leaves of *D. kaki*, which exhibit suppressing stimulus-induced superoxide generation and tyrosyl phosphorylation [4] and are used as preventives and remedies for type-IV allergy-induced inflammation [5]. Because BA is an epimer of RA, the difference between them is only the configuration of hydroxyl group at position C3: one contains axial hydroxyl group and the other equatorial hydroxyl group; it is a bit difficult to quantify them simultaneously. So it is meaningful to assess all these three compounds in the leaves of *D. kaki* simultaneously.

As a naturally occurring medicine, the type and quantity of the bioactive ingredients in the leaves of *D. kaki* vary with the growth environments and post-harvesting process [6,7]. In addition, the solvent extraction is frequently used for isolation of bioactive compounds in pharmaceutical industry. Both extraction yields and puritis of the extracts are strongly dependent on the solvents due to the different bioactive compounds with different polarities. In other words, the extraction solvent may influence the quality of this TCM [8,9] and consequently its therapeutic outcomes if the extraction solvent alters. Therefore, a method should be established, which could be utilized to determine the bioactive components not only in this TCM growing in different locations but also in its different solvent extracts, especially to the pharmaceutical industry.

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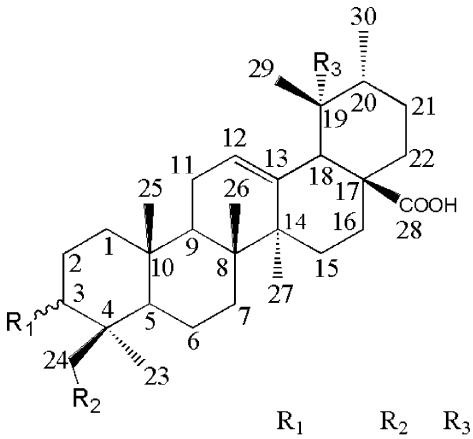

	R_1	R_2	R_3
barbinervic acid	3α-OH	OH	OH
rotungenic acid	3β-OH	OH	OH
24-hydroxy ursolic acid	3β-OH	OH	H

Fig. 1. Structure of these three triterpene acids isolated from the leaves of *Diospyros kaki*.

To date, some analytical methods have been reported on the analysis of the various bioactive ingredients in the leaves of *D. kaki*, including ursolic acid [6] and oleanolic acid [10] by TLC, fatty acids by GC–MS [11] and phenolics contents by HPLC [7]. However, no method is available for the co-quantification of these three bioactive triterpene acids by HPLC. Therefore, a reliable quality control method is needed for the qualitative and quantitative determination of these triterpene acids in the leaves of *D. kaki.*

Therefore, the present work aimed to develop an analytical method to simultaneously determine the main bioactive compounds. A simple HPLC–UV assay using an external standard method has been developed, for the first time, to simultaneously determine and quantify these three bioactive triterpene acids in the leaves of *D. kaki*. The developed method has been subsequently applied to analyze five different solvent extracts and the leaves from six different locations in China for the quantification of these three components. In order to compare with the HPLC method, the content of the total triterpenoids was also studied by the colorimetric method.

2. Experimental

2.1. Chemicals and standards

HPLC grade methanol (E. Merck, Darmstadt, Germany) was used for the HPLC analysis. Deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA).

All other organic solvents used in this study were of analytical grade from Shanghai Chemical Reagent Corporation. Barbinervic acid (BA), rotungenic acid (RA), 24-hydroxy ursolic acid (HA) and ursolic acid were isolated from the leaves of *D. kaki* growing in Zhejiang province (Hangzhou) previously by the authors' laboratory and their chemical structures were confirmed by EI-MS, ESI-MS, ^{1}H NMR and ^{13}C NMR and HPLC with their individual purities not less than 98%.

The leaves of *D. kaki* samples were collected in autumn, 2004 from Zhejiang province (Hangzhou), Jiangxi province (Fuzhou), Anhui province (Anqing), Hunan province (Changsha), Shandong province (Tai'an) and Henan Province (Luohe), respectively, in China. The dried leaves were crushed into pieces prior to extraction. The leaves of *D. kaki* sample collected from Zhejiang province (Hangzhou) were used for the assessment of the precision and recovery, limits of detection, effects of extraction solvents and effects of the leaves of *D. kaki* growing in different locations. All other samples were only used for the assessment of effects of the leaves of *D. kaki* growing in different locations.

2.2. Apparatus

HPLC was performed on an Agilent 1100 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and UV detector. The column configuration consisted of an Agilent Zorbax SB-C_{18} reserved-phase column (5 μm, 250 mm × 4.6 mm, i.d.). The colorimetric method was performed on a Lengguang® 721 spectrometer (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China). Standards were prepared from the leaves of *D. kaki* using a Model TBE-1000A HSCCC system manufactured by Tauto Biotech Co., Ltd., Shanghai, China.

2.3. Quantification of these three triterpene acids by HPLC

2.3.1. Calibration curves

To prepare the standard solutions, an accurately weighed amount of the BA, RA and HA standards (3.17, 2.46 and 0.80 mg, respectively) were dissolved in methanol (10 ml) for analysis. The standard solutions (2, 5, 10, 15, 20 μl, respectively) were injected and run for calibration curves. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with amount of analyte injected.

2.3.2. Sample preparation

An aliquot of the dried powder of the leaves of *D. kaki* (20–40 mesh, 20 g) was extracted thrice with boiling dichloromethane, ethyl acetate, acetone, methanol or 95% ethanol (aqueous solution, v/v) under reflux, respectively (200, 200 and 200 ml, each 2 h), the powder of the leaves of *D. kaki* was degreased by hot petroleum ether (bp 60–90 °C) prior to extraction. The extracts with each solvent were filtered while hot and combined. The combined solution was evaporated to dryness under reduced pressure to yield dichloromethane extract, ethyl acetate extract, acetone extract, methanol extract or 95% ethanol extract, respectively. The suitable amount of each residue was dissolved in 10 ml of methanol, respectively. The afforded solution was filtered through a 0.45 mm syringe filter prior to HPLC. All separations were performed at 30 °C. In addition, assigning peaks was based on their retention times or by spiking the extracts with standard compounds.

2.3.3. Precision and recovery studies

The measurements of intra- and inter-day variability were utilized to determine the precision of the developed assay method.

Two different concentration standard solutions containing all these three standards were prepared. Quantities for all analytes were calculated from their corresponding calibration curves. Each sample was analyzed in four times within the same day to determine the intra-day variability. The inter-day reproducibility was determined by analyzing the samples on four separate days. The relative standard deviation (R.S.D.) was taken as a measure of precision.

In addition, to further evaluate the recovery of the developed assay, into the tested plant leaves (5 g) which were degreased by hot petroleum ether (bp 60–90 °C) prior to extraction, appropriate amounts of BA, RA and HA standards were spiked. The resultant samples were then extracted with 95% ethanol and analyzed as described in Section 2.3.2. Each sample was analyzed in four times. The total concentration of each of the analytes was determined from the corresponding calibration curve, and the recovery of the measurement for each analyte was calculated by the following equation:

$$\text{recovery}(\%) = \frac{m_{\text{total}} - m_{\text{original}}}{m_{\text{spiked}}} \times 100$$

where m_{total} is the total amount of determination above, m_{original} the amount of original leaves without added standards measured in the above described experiment, and m_{spiked} is the spiked amount of each of the standards.

2.3.4. Limits of detection

The standard solutions containing all three authentic compounds were diluted with methanol to provide appropriate concentrations. The limit of detection for each analyte was determined when the ratio of the testing peak signal-to-noise was greater than 5.

2.4. Quantification of the total triterpenoids by the colorimetric method

After optimizing all experiment parameters, the content of the total triterpenoids was also determined by the colorimetric method [12] with the following procedure. The suitable amount of each extract obtained as described in Section 2.3.2 was dissolved in 25 ml of ethanol respectively, 0.2 ml of ethanol solution in graduated test tube was evaporated to dry in a boiled water bath, to the tube 0.3 ml of 5% vanillin/glacial acetic acid (w/v) and 1ml of perchloric acid solution was added successively, the sample solution was heated for 45 min at 60 °C and then cooled in an ice-water bath to the ambient temperature. The absorbance of the sample was measured at 548 nm after being added to 5 ml with glacial acetic acid, ursolic acid was used as the standard.

3. Results and discussion

3.1. HPLC separation optimization

The selection of the HPLC conditions was guided by the requirement for obtaining chromatograms with better resolution of adjacent peaks within a short time especially when large amount of samples were analyzed. Since these triterpene acids are non-chromophoric, the mixture of water and methanol was therefore the good choice for the separation. Different ratios of water and methanol were compared but no satisfied separation was reached. Hence phosphoric acid was added to optimize the separation. It was found that could improve the separation. The solvent system of A–B (A, methanol; B, 0.1% aqueous H_3PO_4) was tested by changing the volume ratio of the solvent to obtain the optimal composition. When the solvent system of A–B (80:20, v/v) was used as the mobile phase, the retention time of HA would be greater than 20 min and the peak would be extended seriously. When the solvent system of A–B (90:10, v/v) was used as the mobile phase, the retention time was too close between BA and RA to be separated. After optimizing the separation parameters, the ratio 88:12 (v/v) of the solvent system of A–B at a flow rate of 1.0 ml/min was utilized so as to ensure that each run of analysis was completed within 15 min with better resolution of adjacent peaks and low solvent consumption. 210 nm was chosen as the detection wavelength, as it is close to the maximum absorbency of these triterpene acids.

3.2. Linearity, precision and recovery of the HPLC method

Under the optimal chromatographic conditions used in this study, all these three calibration curves exhibited good linear regressions as shown in Table 1, and the limits of detection (LOD) were in the range of 0.051–0.058 μg for these three triterpene acids. The results in Table 2 demonstrated that the developed analytical method was reproducible with good accuracy and sensitivity for all analytes examined. The overall intra- and inter-day variations were less than 3.50% for all analytes. The recovery assays of the triterpene acids were carried out by adding the standards to the treated materials, and the results were shown in Table 3, from which it is clear that the recoveries for all these three triterpene acids determined were in the range of 98–107%.

3.3. Linearity, precision and recovery of the colorimetric method

Under the optimal colorimetric method conditions used in this study, calibration graphs were constructed in the range 0–0.0126 mg/ml. The regression equation of the curve was calculated as follows: $y = 0.0167x - 0.0015$ (y is the concentration (mg/ml) and x is the absorbance of the sample solution after chromogenic reaction), the correlation coefficient of the regression equation (r^2) was 0.9995. The overall intra- and inter-day variations were less than 4.00% for the total triterpenoids. The recovery assay of the total triterpenoids was carried out by adding the standard to the treated materials, and the recovery was in the range of 94–112%.

3.4. Results analysis

3.4.1. Effects of extraction solvents

In order to obtain quantitative extraction, the variables involved in the procedure such as the extraction solvent, extraction time and extraction times were optimized. The optimal

Table 1
Calibration curves and limits of the detection of these three triterpene acids in the leaves of *D. kaki*

Analyte	Retention time (min)	Standard curve	r^2	Test range (μg)	Limit of detection (μg)
BA	3.658	$Y = 433445x + 169.4$	0.9997	0.634–6.340	0.058
RA	4.490	$Y = 488935x + 132.1$	0.9998	0.492–4.920	0.051
HA	11.680	$Y = 450425x + 20.9$	0.9995	0.160–1.600	0.055

Y: peak area; x: amount of each analyte injected (mg); r^2: correlation coefficient of regression equations; limit of detection: S/N = 5.

Table 2
Intra- and inter-day precision for the determination of BA, RA and HA

Analyte	Amount injected (μg)	R.S.D. (%)	
		Intraday ($n = 4$)	Interday ($n = 4$)
BA	1.585	1.2	2.0
	3.170	0.8	1.7
RA	1.230	1.5	2.3
	2.460	1.0	1.9
HA	0.400	2.4	3.5
	0.800	2.0	3.0

experimental conditions were as follows: the powder of the leaves of *D. kaki* was extracted thrice (2 h each time) with the boiling solvent under reflux, and the ratio of the leaves of *D. kaki* to the extraction solvent was 1:10. After the extraction under the optimal experimental conditions, the residue was further extracted with the extraction solvent for additional 2 h, and almost no triterpenoids were detected by the HPLC and colorimetric method. Therefore, the later leaves of *D. kaki* were extracted under these conditions in order to assure complete extraction of the bioactive triterpene acids.

This newly developed HPLC assay method was subsequently applied to a simultaneous determination of these three triterpene acids with different extraction solvents. Representative chromatograms of the standards and the different solvent extracts from the leaves of *D. kaki* were shown in Fig. 2. The effects of the extraction solvents (dichloromethane, ethyl acetate, acetone, methanol and 95% ethanol (aqueous solution, v/v)) on the yield and purity of these three triterpene acids and the total triterpenoids in the leaves of *D. kaki* extracts were studied. The yield and puritie of these three triterpene acids were determined by HPLC and those of the total triterpenoids of the extracts by the colorimetric method (in Table 4).

Table 3
Recoveries of BA, RA and HA in the leaves of *D. kaki*

Analyte	Amount spiked (mg)	Recovery ($n = 4$)	
		Mean (%)	R.S.D. (%)
BA	0.75	102.4	2.8
	1.62	98.6	2.3
RA	0.43	103.3	2.7
	0.76	99.5	2.0
HA	0.15	106.7	3.8
	0.25	101.0	2.9

The results showed that the yield and purity of the analytes were strongly dependent on the solvents due to the different compounds with different polarities. The yield and purity of the total triterpenoids determined by the colorimetric method were much higher than the total yield and purity of these three triterpenene acids determined by HPLC in all solvent extracts analyzed. The reasons are as follows: (1) The determination of the yield and purity of the total triterpenoids by the colorimetric method is susceptible to the interference from pigments or other components of the extract, such as campesterol, stigmasterol and β-sitosterol [13]. (2) Besides the amount of these three trierpene acids, the amount determined by the colorimetric method also includes other triterpenoids [14] in the extract, such as usolic acid, oleanic acid and so on.

Ninty-five percent of ethanol was a good extraction solvent that allowed complete extraction of these three triterpene acids and the total triterpenoids with a highest yield and relatively high purity due to the co-solubility effect. Ethyl acetate and acetone were the relatively better extraction solvents due to providing relatively higher yields and purities of all these three triterpene acids and the total triterpenoids compared with the other extraction solvents. In all the five solvent extracts analyzed, BA was found to be the most abundant component and the amount of HA was rather small. Moreover, some differences in the quantity ratio of each compound in the five different solvent extracts were also observed. With a consideration of the total content of these three ingredients determined by HPLC, the ethyl acetate and methanol extracts had a similar content (1.54%), and the quantity ratio of these three major components and the content of each of the three major ingredients present in their extracts were different. For example, BA, RA and HA account for 54.54%, 31.52% and 13.64% of the total content of these three ingredients in the ethyl acetate extract, and 60.39%, 28.57% and 11.04% in the methanol extract, respectively.

Therefore, 95% ethanol was the best of these extraction solvents as it could give the highest yield and a relatively higher purity for these three triterpene acids and the total triterpenoids. In addition, the yield and purity of the analytes varied significantly with the solvents due to the different compounds with different polarities.

3.4.2. Effects of the leaves of D. kaki growing in different locations

The contents of these three constituents by HPLC and those of the total triterpenoids by the colorimetric method were also analyzed for the leaves of *D. kaki* growing in provinces of Jiangxi, Hunan, Anhui, Shandong, Henan in China. These leaves were extracted as described in Section 2.3.2. The extraction solvent

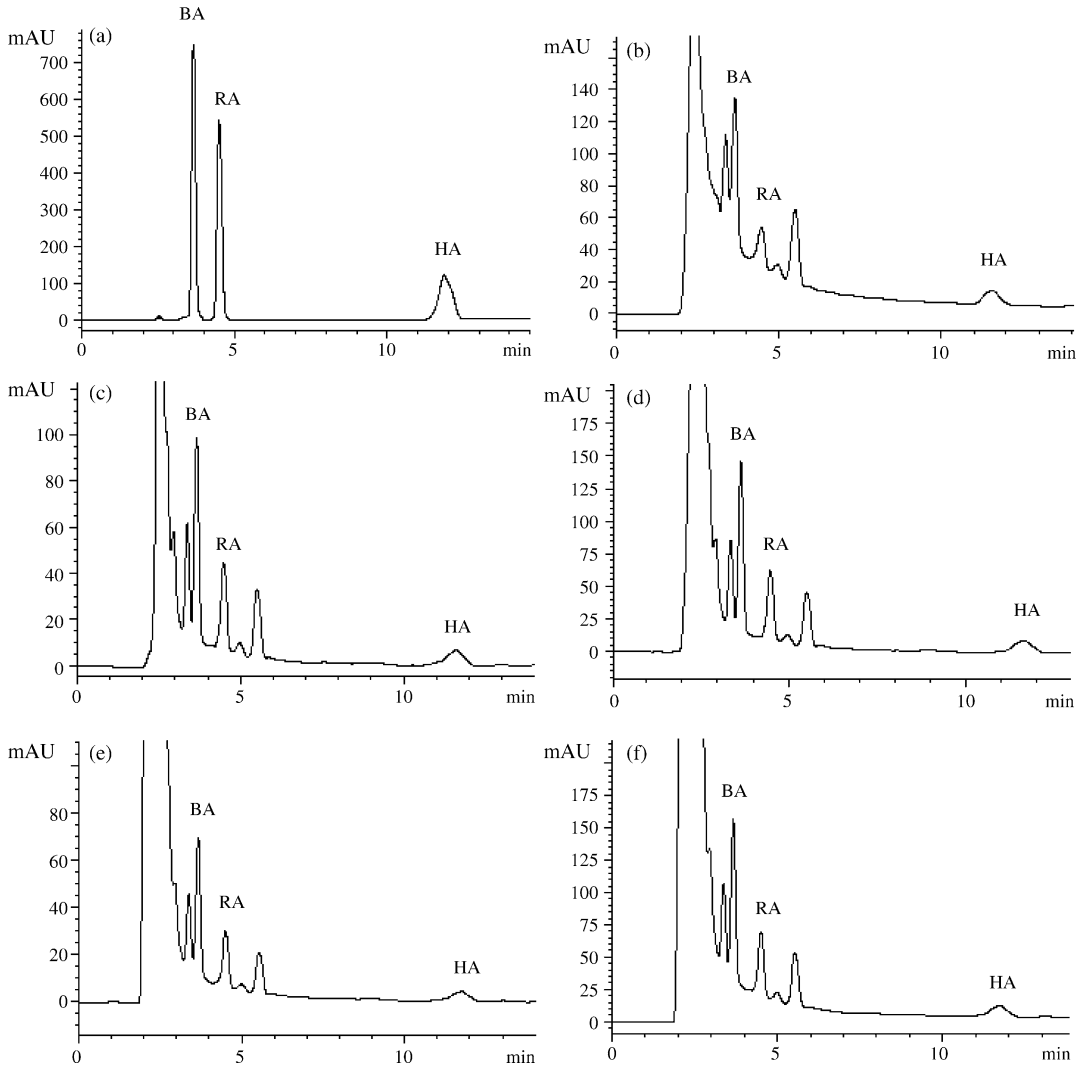

Fig. 2. Representative HPLC chromatograms of BA, RA and HA standard at a high concentration (a) and the extracts from the leaves of *Diospyros kaki* with different solvents detected at 210 nm: dichloromethane extract (b); ethyl acetate extract (c); acetone extract (d); methanol extract (e); the 95% ethanol extract (f).

was chosen as 95% ethanol because these three triterpenene acids and total triterpenods could be completely extracted from the leaves of *D. kaki* with a high yield using 95% ethanol. The results were shown in Table 5, demonstrating that all of these phytochemicals in the leaves of *D. kaki* were strikingly dependent on the location. The contents of these three major ingredients varied significantly in the leaves of *D. kaki* growing in different locations. With a consideration of the total content of these three ingredients determined by HPLC (Table 5), the sample growing in Zhjiang province had a higher content (2.32%) than all the other samples, and the three samples from Hunan, Shandong and Henan had very lower contents (Hunan: 0.99%, Shandong: 0.85%, Henan: 1.00%). The quantity ratio of these three major components and the content of each of the three major ingredients presented in the different samples were also different. For example, BA, RA and HA accounted for 58.59%, 13.13% and 28.28% of the total content of these three ingredients in the sample growing in Hunan province, and 54.12%, 31.76% and 14.12% in Shandong province, and 70.00%, 21.00% and 9.00% in Henan province, respectively.

Compared with the total yields and purities of these three triterpenene acids determined by HPLC in all samples growing in different locations, the yields and purities of the total triterpenoids determined by the colorimetric method (Table 5) were much higher, and the sort order of the yield and purity of the total triterpenoids determined by the colorimetric method was also different from the total yield and purity of these three triterpenene acids determined by HPLC in the samples growing in different locations. For example, the yield of total triterpenoids determined by the colorimetric method in the sample growing in Anhui province was the lowest, however, the total yield of these three triterpenene acids determined by HPLC was the second highest of all the samples. The reasons were similar with those given in Section 3.4.1.

In brief, the variations in the yield or purity of these three triterpenene acids determined by HPLC were inconsistent with those of the total triterpenoids by the colorimetric method in the

Table 4
Yields of the triterpenoids in the leaves of *D. kaki* and purities of the crude extracts with different extraction solvents

Extraction solvent	BA			RA			HA			Total[a]		Total[b]		
	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)
Dichloromethane	0.80	1.6	15.24	0.48	2.1	9.08	0.15	2.8	2.81	1.43	27.13	2.76	3.2	52.51
Ethyl acetate	0.84	1.4	11.62	0.49	1.9	6.70	0.21	2.4	2.89	1.54	21.21	4.37	2.4	60.33
Acetone	1.01	1.3	13.04	0.55	1.5	7.10	0.21	2.6	2.72	1.77	22.86	4.66	2.5	60.32
Methanol	0.93	1.5	7.83	0.44	2.0	3.67	0.17	2.6	1.41	1.54	12.91	3.39	3.1	28.40
95% Ethanol	1.43	0.8	11.53	0.68	1.0	5.51	0.21	2.5	1.71	2.32	18.75	6.46	2.5	52.06

Yield (%) = (amount of the analyte/amount of the leaves of *D. kaki*) × 100. Purity (%) = (amount of the analyte/amount of the crude extract of the leaves of *D. kaki*) × 100.
[a] Total is the total of these three triterpene acids determined by HPLC.
[b] Total is the total triterpenoids determined by the colorimetric method.

Table 5
Yields of the triterpenoids in the leaves of *D. kaki* growing in different locations of China and purities of the crude extracts

Location	BA			RA			HA			Total[a]		Total[b]		
	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)	Yield (mean, $n=4$, %)	Purity (%)	Yield (mean, $n=4$, %)	R.S.D. (%)	Purity (%)
Zhejiang	1.43	0.8	11.53	0.68	1.0	5.51	0.21	2.5	1.71	2.32	18.75	6.46	2.5	52.06
Jiangxi	0.63	1.2	8.35	0.34	1.3	4.50	0.25	2.0	3.38	1.22	16.23	4.68	2.0	62.46
Hunan	0.58	1.5	4.57	0.13	2.3	1.01	0.28	1.9	2.23	0.99	7.81	5.07	2.3	39.75
Anhui	0.85	1.3	9.24	0.64	0.9	6.74	0.18	2.0	2.00	1.65	17.98	3.38	2.5	36.70
Shandong	0.46	2.0	2.62	0.27	2.1	1.54	0.09	2.4	0.89	0.85	4.87	5.73	2.4	32.81
Henan	0.7	6.57	2.62	0.21	2.2	2.02	0.12	2.6	0.71	1.00	9.48	4.37	2.2	41.21

The meanings of yield, purity.
[a] Total is the total of these three triterpene acids determined by HPLC.
[b] Total is the total triterpenoids determined by the colorimetric method.

leaves of *D. kaki* using different extraction solvents or growing in different locations of China. So the study of the contents of these phytochemicals on the polarity-based solvent extraction and on the locations of the leaves of *D. kaki* was necessary. Furthermore, in order to ensure the consistency of therapeutic benefits, the determination of each of the major bioactive components was more important and meaningful than a measurement of the total content of all bioactive ingredients only prior to its use as herbal prescriptions or as the plant source for the manufacture of natural product based pharmaceutical preparations.

4. Conclusions

This is the first report on the development of a simple, sensitive and specific HPLC–UV method to simultaneously quantify these three bioactive ingredients in the leaves of *D. kaki*. The results demonstrate that the developed method is accurate and reproducible and could be readily utilized as a suitable quality control method for the quantification of the leaves of *D. kaki*, derived extracts and phytomedicines. Significant variations in the yields and purities of the major bioactive ingredients in this TCM extract with different extraction solvents or in the leaves of *D. kaki* growing in different locations of China were observed. Therefore, in order to ensure and improve the therapeutic benefits, it is necessary to quantify each of the major bioactive components in the leaves of *D. kaki*, derived extracts and phytomedicines.

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