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ORIGINAL ARTICLE

Quantitative Analysis and Comparison of Four Major Flavonol Glycosides in the Leaves of *Toona sinensis* (A. Juss.) Roemer (Chinese Toon) from Various Origins by High-Performance Liquid Chromatography-Diode Array Detector and Hierarchical Clustering Analysis

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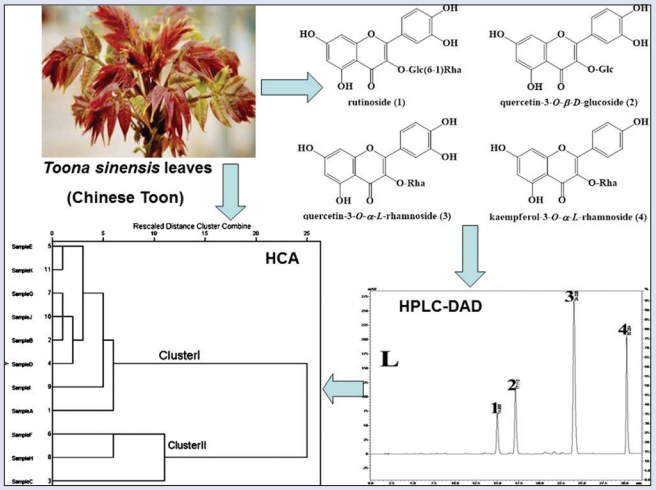
ABSTRACT

Background: *Toona sinensis* (A. Juss.) Roemer is an endemic species of *Toona* genus native to Asian area. Its dried leaves are applied in the treatment of many diseases; however, few investigations have been reported for the quantitative analysis and comparison of major bioactive flavonol glycosides in the leaves harvested from various origins. **Objective:** To quantitatively analyze four major flavonol glycosides including rutinoside, quercetin-3-O-β-D-glucoside, quercetin-3-O-α-L-rhamnoside, and kaempferol-3-O-α-L-rhamnoside in the leaves from different production sites and classify them according to the content of these glycosides. **Materials and Methods:** A high-performance liquid chromatography-diode array detector (HPLC-DAD) method for their simultaneous determination was developed and validated for linearity, precision, accuracy, stability, and repeatability. Moreover, the method established was then employed to explore the difference in the content of these four glycosides in raw materials. Finally, a hierarchical clustering analysis was performed to classify 11 voucher specimens. **Results:** The separation was performed on a Waters XBridge Shield RP18 column (150 mm × 4.6 mm, 3.5 μm) kept at 35°C, and acetonitrile and H_2O containing 0.30% trifluoroacetic acid as mobile phase was driven at 1.0 mL/min during the analysis. Ten microliters of solution were injected and 254 nm was selected to monitor the separation. A strong linear relationship between the peak area and concentration of four analytes was observed. And, the method was also validated to be repeatable, stable, precise, and accurate. **Conclusion:** An efficient and reliable HPLC-DAD method was established and applied in the assays for the samples from 11 origins successfully. Moreover, the content of those flavonol glycosides varied much among different batches, and the flavonoids could be considered as biomarkers to control the quality of Chinese Toon.

Key words: Chinese Toon, flavanoids, flavonol glycosides, *Toona sinensis*

SUMMARY

- Four major flavonol glycosides in the leaves of Toona sinensis were determined by HPLC-DAD and their contents were compared among various origins by HCA.



Abbreviations used: HPLC-DAD: High-performance liquid chromatography-diode array detector, HCA: Hierarchical clustering analysis, MS: Mass spectrometry, RSD: Relative standard deviation.

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INTRODUCTION

Toona sinensis (A. Juss.) Roemer (*T. sinensis*), originated from China, is an endemic species of *Toona* genus (*Meliaceae* family) and naturally distributed over Asian region, such as China, India, Indonesia, Korea, Malaysia, Myanmar, and Thailand. This deciduous arbor, well-known as Chinese Toon or Red Toon, is widely cultivated over China and Indian area now, as fresh young leaves and shoots of this plant have a unique and favorable texture and flavor and which is one of the most healthy and dietary foods. It is popularly consumed as a seasonal vegetable by local people in the spring and has been exported largely to Hong Kong, Taiwan, Japan, Korea, and Russia as well.[1,2] Moreover, its dried leaves, being a readily available folk

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herb, have been also extensively employed to treat many diseases such as halitosis, vomiting, dysentery, lack of appetite, enteritis, and itchiness because of its significant pharmacological effects on detoxification and anti-inflammation, without showing disgusting irreversible side effects.[3,4]

Previously, *in vitro* study has demonstrated that its crude extract has a significant anti-proliferative effect on nonsmall cell lung cancer by regulating the expression of Bcl2, Bax, cyclin D1, and CDK4.[5-9] Moreover, *T. sinensis* extract or its fraction has an antinociceptive effect comparable with nonsteroidal anti-inflammatory agents in mouse writhing test, can inhibit lipid accumulation through up-regulation of genes involved in lipolysis and fatty acid oxidation in adipocytes, and also can ameliorate insulin resistance via AMPK and PPARγ pathways and display activity against sepsis in both *in vitro* and *in vivo* models, etc.[10-17] In addition, it has also exceptionally shown an efficient anti-oxidant capacity against several chemical oxidative models such as 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay and lipid peroxidation assay in a comparative assessment of anti-oxidant capacity among 127 common and underutilized foods.[18,19]

To reveal intrinsic constituents responsible for these major activities of the extract, many efforts have been put on phytochemical investigations and instrumental analyses in this decade.[20-24] And, as a consequence, some compounds such as rutinoside, quercetin, methyl gallate, ethyl gallate, gallic acid, kaempferol, astragalin, 1,2,3,4,6-penta-O-galloyl-β-D -glucopyranose, 1,2,3,6-tetra-O-galloyl-β-D-glucopyranose, (S, S)-γ-glu tamyl-(cis-S-1-propenyl) thioglycine, kaempferol-3-O-α-L-rhamnoside, 3-hydroxy-5,6-epoxy-7-megastigmen-9-one, quercetrin, quercetin-3-O-α-L-rhamnoside, and scopoletin were isolated and identified from the leaves' extract by column chromatography or liquid chromatography in tandem with mass spectrometry (MS).[25-30] On the other hand, the anti-oxidant capacities of five flavonol glycosides and three derivatives of gallic acid were evaluated comprehensively by using various chemical methods such as DPPH-free radical scavenging assay, PMS-NADH-NBT superoxide anion scavenging assay, $FeCl_3$-$K_3Fe(CN)_6$ reducing power assay, and $FeCl_2$-ferrozine metal chelation assay.

However, until now, few investigations have been reported for the quantitative analysis and comparison of major bioactive flavonol glycosides in the leaves of *T. sinensis* harvested from different places. In this paper, for the first time, a simple and convenient method was optimized for the extraction of the glycosides from plant material, and then a fast and a reliable high-performance liquid chromatography-diode array detector (HPLC-DAD) method for the simultaneous determination of rutinoside (1), quercetin-3-O-β-D-glucoside (2), quercetin-3-O-α-L-rhamnoside (3) and kaempferol-3-O-α-L-rhamnoside (4) was developed and validated for linearity, precision, accuracy, stability, and repeatability. Their chemical structures are shown in Figure 1. And, this HPLC-DAD method established was eventually utilized in further study to explore the difference in the content of these four glycosides in the raw materials from various origins. Finally, a hierarchical clustering analysis (HCA) was carried out to classify 11 voucher specimens.[31-34]

MATERIALS AND METHODS

Chemicals

Acetonitrile (ACN) of HPLC grade was a product from Fisher Scientific (New Jersey, USA), and methanol (MeOH) for analysis was provided by Merck KGaA (Darmstadt, Germany). Trifluoroacetic acid (TFA) of reagent grade was purchased from Sigma-Aldrich Inc. (Missouri, USA). Ultrapure water (>18.2 MΩ·cm) was prepared by using an Ultra Clear compact RO system (Siemens, Barsbüttel, Germany). Rutinoside (98.0% by HPLC) was purchased from Nanjing ZeLang Medical Technology Co. Ltd., (Nanjing, China). As reported in our previous study, the other three flavonol glycosides including quercetin-3-O-β-D-glucoside (97.5%), quercetin-3-O-α-L-rhamnoside (98.3%), and kaempferol-3-O-α-L-rhamnoside (97.1%) were isolated from the leaves harvested on the campus of Jiangsu University (Zhenjiang, China) followed by spectral analyses including ^{1}H-NMR, ^{13}C-NMR, and MS. And, their purities were tested and calculated based on the normalization of peak area by the use of HPLC-electrospray ionization-MS.

Plant materials

Fresh leaves of *T. sinensis* were harvested from the following 11 origins in China:

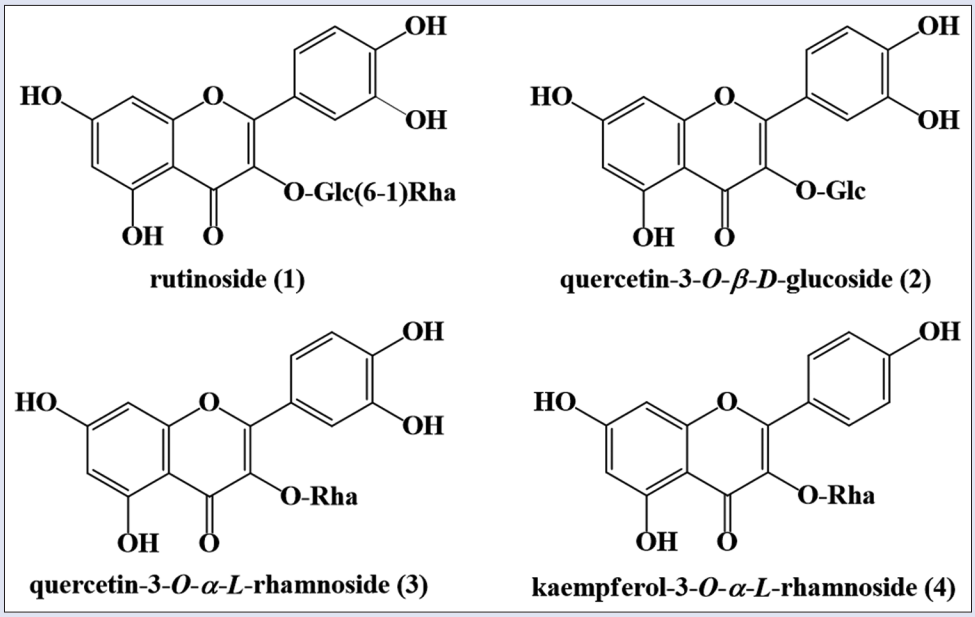

Figure 1: Chemical structures of four flavonol glycosides

A: Xiping (Henan province) in July 2013; B: Mianyang (Sichuang province) in July 2013; C: Yantai (Shandong province) in August 2013; D: Yuncheng (Shanxi province) in September 2013; E: Zhoukou (Henan province) in August 2013; F: Ximou (Shandong province) in July 2013; G: Zhenjiang (Jiangsu province) in August 2013; H: Danyang (Jiangsu province) in August 2013; I: Jiangyin (Jiangsu province) in August 2013; J: Wenxian (Henan province) in August 2013; K: Yuncheng (Shandong province) in August 2013.

All the voucher specimens had been authenticated by the Associate Professor Hongxia Chen (Department of Chinese Materia Medica and Pharmacy, School of Pharmacy, Jiangsu University, The City of Zhenjiang, Jiangsu province, China) and were deposited at the Pharmacognosy Research Facility.

After harvest, the leaves from each origin were immediately dried in a well-ventilated condition under shade to remove most of water prior to a complete lyophilization for 24 h by Freezemobile 25EL (SP Scientific, Pennsylvania, USA). The plant material was then grounded into powder form and passed through a 40-mesh sieve. The fine powder collected was stored in a dry cabinet (RH ≤30%) at room temperature before subsequent analysis.

High-performance liquid chromatography instrumentations and conditions

A prominence HPLC instrument (Shimadzu, Japan) was equipped with a DGU-20A5 degasser, a LC-20AD pump, a SIL-20AC autoinjector, a CTO-20AC column oven, a SPD-M20A DAD, and a CBM-20A communications bus module. The data were acquired and then processed using the LC Solution 1.11 SP1 software for chromatographic analysis (Shimadzu, Japan).

The chromatographic analyses were carried out on an XBridge Shield RP18 column (150 mm L. × 4.6 mm I.D., 3.5 μm) purchased from Waters Corporation (Massachusetts, USA) and protected by a SecurityGuard Guard system equipped with C18 cartridge (4.0 mm L. × 3.0 mm I.D.) from Phenomenex Inc. (CA, USA) under a linear gradient elution at 35°C. The mobile phases used were composed of ACN (A) and 0.30% TFA aqueous solution (B). Time program for the separation was as follows: 0 min, 15% A–85% B; 20 min, 20% A–80% B; 31 min, 30% A–70% B, followed by cleaning the column with 100% A for 5 min and then conditioning with 15% A for another 5 min. A constant flow rate was set at 1.0 mL/min throughout the entire analysis. Injection volume was 10.0 μL, the wavelength for monitoring the separation was set to 254 nm, and the contour view by DAD was plotted for an overall profile of the sample as well.

Preparation of sample solutions

The powder of the leaves, 50.0 mg, was precisely weighed into a 15 mL capped centrifuge tube; 10 mL of 20% MeOH was then transferred to the tube. The suspension was vortexed shortly prior to an ultrasonic extraction for a continuous 10 min. And, prior to injection, all the solutions were subjected to membrane filtration by 0.22 μm Minisart SRP (polytetrafluoroethylene) syringe filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Development and validation of the high-performance liquid chromatography-diode array detector method

Method development

High-performance liquid chromatography conditions, including composition of mobile phases (0.1%, 0.3% or 0.5% TFA aqueous solution), column temperature (30°C, 35°C or 40°C), injection volume (5 μL, 10 μL or 20 μL), flow rate (0.8 mL/min or 1.0 mL/min), ultraviolet wavelength, and time program, were optimized in terms of resolution, peak shape, and elution time, followed by validation for linearity, precision, accuracy, stability, and repeatability.

Linearity

Compound 1 (3.32 mg), 2 (3.67 mg), 3 (8.21 mg), and 4 (5.41 mg) were dissolved in 20% EtOH and scaled to 50 mL as stock solution of analytes (0.0664 mg/mL, 0.0734 mg/mL, 0.164 mg/mL, and 0.108 mg/mL). Six standard solutions were prepared by serial dilution from the stock solution to 33.2 μg/mL, 16.6 μg/mL, 8.30 μg/mL, 4.15 μg/mL, 2.08 μg/mL, and 1.04 μg/mL for compound 1; 36.7 μg/mL, 18.4 μg/mL, 9.18 μg/mL, 4.59 μg/mL, 2.29 μg/mL, and 1.15 μg/mL for compound 2; 82.1 μg/mL, 41.1 μg/mL, 20.5 μg/mL, 10.3 μg/mL, 5.13 μg/mL, and 2.58 μg/mL for compound 3; 54.1 μg/mL, 27.05 μg/mL, 13.5 μg/mL, 6.76 μg/mL, 3.38 μg/mL, and 1.69 μg/mL for compound 4. Subsequently, their linearity was evaluated using all of the above solutions. Each solution was then individually injected to measure the response of the analytes and calibration curves were plotted by linear regression of peak area (Y) versus the concentration of each standard (X).

Precision

The intra-day precision experiment was carried out by continuous six measurements of three mixed standard solutions (highest, middle, and lowest concentrations) and a sample solution (Zhenjiang, sample G) freshly prepared within 1 day. Relative standard deviation (RSD) of peak areas for those four analytes was then calculated.

Accuracy

Recovery test was conducted to investigate the accuracy of this developed HPLC-DAD method. In the experiments, a certain amount of reference compounds was added into 25.0 mg of *T. sinensis* leaves sample and made up to 10 mL with 20% MeOH. The sample solutions were prepared and analyzed according to the aforementioned procedures and conditions, and this test was performed at three levels (50%, 100%, and 150% of the original amount). The percentage for the recovery of each analyte was then calculated by the following equation:

Recovery % = (Detected amount − Original amount)/Spiked amount × 100%

Stability

A freshly prepared sample solution of *T. sinensis* leaves from Zhenjiang (sample G) was immediately analyzed and then stored at 25°C under shade in the autoinjector compartment for 48 h. This solution was further analyzed after storage for 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h, and the RSD of peak areas for those four analytes were calculated.

Repeatability

Six aliquots of *T. sinensis* leaves Zhenjiang (sample G) were simultaneously extracted for the preparation of six sample solutions, and which were successively loaded into the HPLC system to quantify those four compounds under the analytical conditions developed. RSD of the content for the analytes was calculated.

Determination of four flavonol glycosides

The content of those four flavonol glycosides in *T. sinensis* leaves samples from 11 production sites was determined by using this established method, and the averages of their content were calculated.

Statistical analysis

All tests and analyses were performed in triplicate except for stated, and data collected were presented in the form of mean or mean ± standard deviation. The HCA of the leaves samples was performed using SPSS software (SPSS for Windows 18.0, SPSS Inc., USA). Ward's method was applied and Squared Euclidean distance was chosen as the measurement.

RESULTS

Method development

According to the common λmax of three quercetin-derived analytes including rutinoside, quercetin-3-O-β-D-glucoside, and quercetin-3-O-α-L-rhamnoside, 254 nm was selected throughout the separation. It was found that a better performance has been achieved while either 0.3% or 0.5% TFA aqueous solution was used, and the mixture of ACN and 0.3% TFA was finally chosen, as less acid in mobile phases was more preferred, without compromising on the efficiency. And, two experimented flow rates, three column temperatures, and three injection volumes were compared, and consequently, 1.0 mL/min, 35°C, and 10 μL were employed to obtain a satisfying separation as well as a short analytical time. Figure 2 shows the typical HPLC chromatograms obtained from the standard references (L) and the 11 samples (A–K). It can be seen that a good separation of these four flavonol glycosides was achieved within about half hour under the optimized HPLC conditions.

Validation of the developed method

Linearity

High correlation coefficients ($r^2 \geq 0.9990$) of the calibration curves were obtained [Table 1], which has demonstrated that there were strong linear relationships between the peak areas and the concentrations of the four analytes.

Precision

As shown in Table 2, in the intra-day precision test, the RSDs of the peak areas were found to be no more than 3%. This indicated that the analysis of these four analytes is precise.

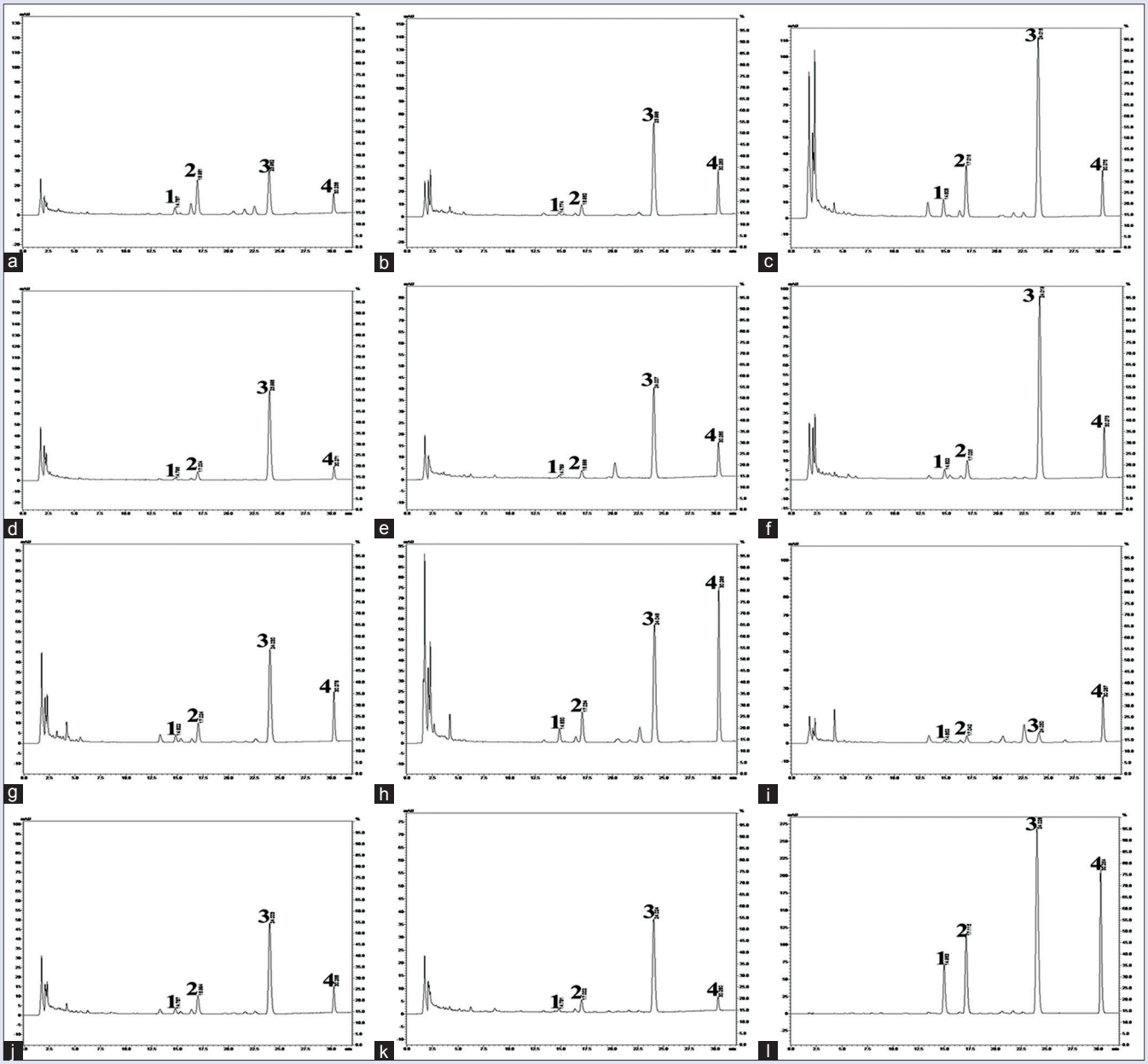

Figure 2: Typical high-performance liquid chromatography chromatograms of sample solutions from 11 origins (a-k) and mixed standards (l)

Recovery

The results obtained from recovery test have been summarized in Table 3, and briefly, the recoveries of all of these four flavonol glycosides were within the range of 96.01–108.53%, with the RSD values ranging from 0.21% to 4.21%, well verifying the high accuracy of this developed method.

Repeatability

As shown in Table 2, in the test for repeatability, the RSD of the contents of four flavonol glycosides in the natural product was found to be no more than 3.24%, thus demonstrating a good repeatability of the developed method.

Stability

In the assessment of stability [Table 2], the RSD of peak areas of compound 1, 2, 3, and 4, being acquired at eight time points as mentioned above after the sample solution was prepared, were all below 4%, which has indicated that the sample solution was stable for 48 h at room temperature under a shade condition.

Table 1: Linearity of the four flavonol glycosides

Analytes	Regression equation	r^2	Linearity range (μg/mL)
1	Y=14455120.9017X+7477.3046	0.9994	1.0375-66.400
2	Y=19584151.5883X+10045.0172	0.9996	1.1469-73.400
3	Y=23909767.1462X+32596.1379	0.9995	2.5656-164.20
4	Y=17458282.4518X+22750.1552	0.9990	1.6909-108.20

Table 2: Precision, stability, and repeatability of the analytes

Analytes	Intra-day precision (*n*=6)				Sample solution (RSD, %)	
	Standard solution (RSD, %)			Sample solution (RSD, %)	Repeatability (*n*=6)	Stability (*n*=6)
	L	M	H			
1	1.79	1.03	0.29	2.14	2.94	3.21
2	1.17	0.16	0.28	2.66	3.24	3.43
3	0.68	0.13	0.26	2.16	2.34	3.02
4	1.56	0.09	0.27	2.10	2.42	3.05

RSD: Relative standard deviation

Table 3: Recovery of the analytes (*n*=3)

Analytes	Original mean (μg)	Spiked mean (μg)	Detected mean (μg)	Recovery mean (%)	RSD (%)
1	35.37	17.70	53.92	104.36	4.21
	35.23	35.10	72.86	107.22	2.38
	35.14	53.30	92.99	108.53	1.93
2	53.94	26.97	78.92	98.53	1.32
	53.94	53.94	102.62	96.01	2.01
	53.73	80.59	131.12	100.51	1.58
3	23.61	11.80	35.82	104.60	3.18
	23.52	23.52	46.63	101.12	0.60
	23.46	35.19	58.86	102.01	1.38
4	27.83	13.91	418.68	101.41	0.21
	27.71	27.72	563.72	103.47	0.44
	27.64	41.47	722.68	106.12	1.67

RSD: Relative standard deviation

Determination of four flavonol glycosides in the samples

The content of four flavonol glycosides in 11 samples was determined thrice under the optimized HPLC analytical conditions, as mentioned previously. The HPLC chromatograms of four analytes and these analyses are illustrated in Figure 2a-l, and the average contents were tabulated in Table 4.

It could be seen that *T. sinensis* from Yantai (sample C) had the highest mean total contents of four flavonol glycosides, whereas the one from Yuncheng (sample K) had the lowest. Moreover, among the detected four analytes, quercetin-3-O-α-L-rhamnoside was found as the highest content in most of the *T. sinensis* leaves except for the samples from Danyang (H) and Jiangyin (I), both of which are in Jiangsu province; meanwhile, rutinoside was the analyte of the lowest content in all the 11 leaves samples.

Hierarchical clustering analysis

To further visualize the relationships of *T. sinensis* leaves samples harvested from various origins, a multivariate analysis technique, HCA, was performed on standardized data to investigate the similarities among these 11 samples. The clustering results of an unsupervised hierarchical analysis are illustrated as a dendrogram in Figure 3, in which two main clusters are visible. Eight samples including A, B, D, E, G, I, J, and K were observed in Cluster I, whereas samples C, F, and H were in Cluster II. It was demonstrated that the samples could not be clearly differentiated and classified by HCA in alignment with their production sites although they differed in the content of those four flavonol glycosides; in addition, there was no distinctive difference of HPLC profiles among them.

DISCUSSION

In previous studies, it has been clearly demonstrated that flavonol glycosides isolated from *T. sinensis* have a remarkable anti-oxidant ability over various oxidative models *in vitro*. The broad anti-oxidant properties of the compounds could have been responsible for the herb's effectiveness as the scavengers of superoxide and free radicals, reductive capacity, and

Table 4: Content of four flavonol glycosides in *Toona sinensis* leaves of various origins (*n*=3)

Samples	1		2		3		4		Total (mg/g)
	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	Mean (mg/g)	RSD (%)	
A	0.076	3.70	0.336	4.11	0.377	3.97	0.137	4.93	0.922
B	0.027	0.56	0.097	3.04	0.847	2.76	0.375	0.46	1.346
C	0.200	1.33	0.453	3.11	1.507	1.83	0.315	3.31	2.475
D	0.023	3.49	0.119	4.85	1.093	3.80	0.026	1.83	1.331
E	0.007	1.60	0.026	1.83	0.418	0.72	0.122	2.48	0.572
F	0.082	1.29	0.135	2.92	1.348	1.81	0.316	1.57	1.881
G	0.049	0.18	0.117	0.84	0.550	0.27	0.247	0.33	0.963
H	0.111	2.95	0.198	1.45	0.709	2.76	0.833	1.48	1.851
I	0.028	0.73	0.077	2.84	0.085	3.75	0.454	3.80	0.648
J	0.038	3.45	0.136	3.11	0.625	2.72	0.146	3.33	0.947
K	0.020	2.47	0.036	0.73	0.439	3.08	0.035	3.19	0.530

RSD: Relative standard deviation

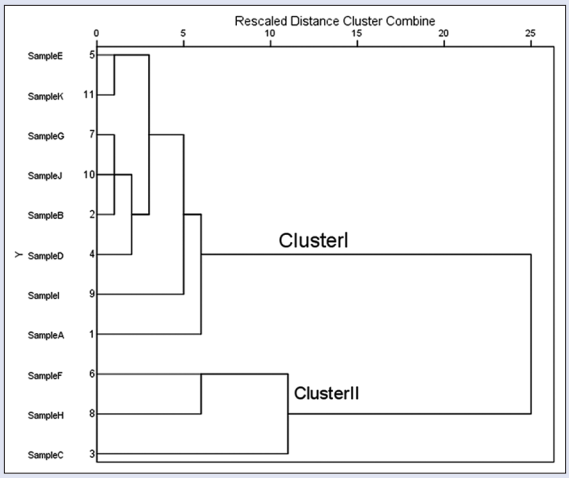

Figure 3: The dendrogram of the 11 batches of *Toona sinensis* samples by hierarchical clustering analysis

metal-chelating ability. Hence, Chinese Toon is a noteworthy material that can be consumed as a potential health supplement and an easily available resource of natural antioxidants, as well as an effective material in pharmaceutical applications. Therefore, it is necessary to conduct the investigation on the difference among the raw materials from various origins in terms of the content of bioactive flavonol glycosides.

In recent decades, HCA was often employed to elucidate the difference among various samples and distinguish similar species of medicinal material originated from different places. However, in the current study, based on the content of these four glycosides, the samples harvested from different origins were not classified into corresponding clusters according to their production provinces by using HCA, despite it was expected. And, it is supposed that in our future work, some more flavonol glycosides should be isolated and involved for the classification of Chinese Toon samples from various origins. In addition, the regular pattern on the accumulation of active flavonol glycosides is about to be explored.

Harvest time is one of the important factors affecting the efficacy of plant-sourced Chinese medicine, just as reported in a number of published papers, when Chinese medicine were harvested at different time, there are often some remarkable differences in the content of the medicinal chemical constituents, and hence regulations for a fixed harvest time are usually executed in Chinese Pharmacopoeia. In our study, all of *T. sinensis* leaves samples were collected in the autumn including July, August, and September, and there could be some variance in the content of these components, which needs to be further investigated in our subsequent study. And, there is no doubt that flavonoid glycosides accumulated in plant organism will reach the highest level at a certain time of its growing period, which could be mostly related to the existing enzymes that promote either the synthesis or the degradation of those flavonoids; in this case, it is being explored in our ongoing research.

CONCLUSION

In this study, for the first time, a simple and convenient method was optimized for the extraction of the glycosides from the plant material of *T. sinensis*, and then a fast and reliable HPLC-DAD method was established for simultaneous determination of four major flavonol glycosides including rutinoside, quercetin-3-O-β-D-glucoside, quercetin-3-O-α-L-rhamnoside, and kaempferol-3-O-α-L-rhamnoside, which was successfully applied in the subsequent assays for the samples from 11 origins. In addition, it was found that the content of those four flavonol glycosides varied much among different batches, and they may be considered as the markers to control the quality of Chinese Toon.

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Conflicts of interest

There are no conflicts of interest.

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