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ORIGINAL ARTICLE

Effect of Frost on Flavonol Glycosides Accumulation and Antioxidant Activities of Mulberry (*Morus alba* L.) Leaves

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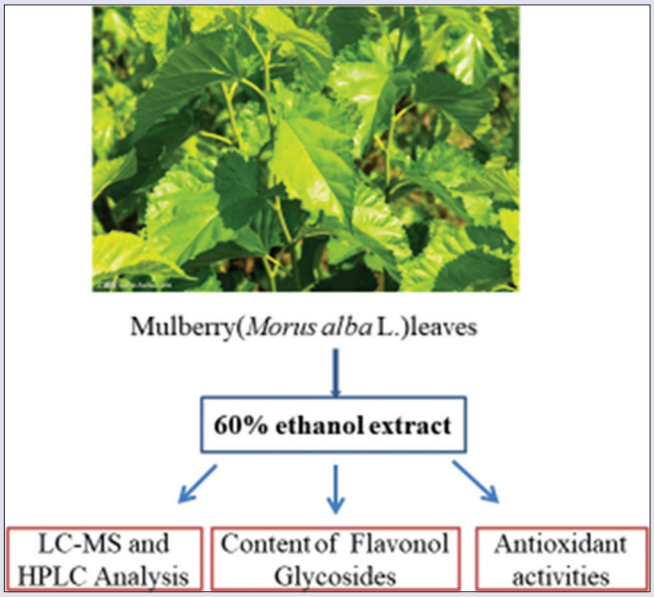
ABSTRACT

Background: Both *Pharmacopoeia of the People's Republic of China* and the ancient Chinese herbal formulas recorded that mulberry leaves collected after frost had good quality. However, the reason has not yet been fully elucidated. **Objective:** We investigated the effect of frost on the accumulation of flavonoids and antioxidant activities of mulberry leaves. **Materials and Methods:** Liquid chromatography-mass spectrometry and high-performance liquid chromatography were used to analyze chemical components and determine the content of five flavonol glycosides from mulberry leaves collected before and after frost, respectively. Antioxidant activities of the same mulberry leaves were evaluated by total antioxidant capacity (TAC), Fe^{2+} equivalent (FeE), reducing power (RP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assay. **Results:** Ten compounds were identified as flavonol glycosides exception of chlorogenic acid. Quantitative analysis showed that content of isoquercitrin, astragalin, and kaempferol-3-*O*-(6″-acetyl)-β-D-glucopyranoside reached a maximum of 3.05 mg/g, 0.70 mg/g, and 0.69 mg/g after Frost's Descent, respectively. Moreover, the lowest value of flavonol glycosides appeared in August. The antioxidant activities were also found to have the same tendency. The maximum value of TAC, FeE, RP, DPPH and ABTS were 64.3 rutin equivalent (RE) mg/g, 46.2 RE mg/g, 31.3 RE mg/g, 22.5 RE mg/g and 26.7 RE mg/g, respectively, in November. They were 1.4 times, 1.4 times, 1.6 times, 1.6 times, and 1.9 times of the minimum values, respectively, in August. There was a significantly and positively correlation between antioxidant activities and content of flavonol glycosides ($P < 0.01$). **Conclusion:** Frost is beneficial to the accumulation of flavonol glycosides and the improvement of antioxidant activities of mulberry leaves.

Key words: Antioxidant activities, correlation analysis, flavonoids, frost, mulberry leaves

SUMMARY

- The content of flavonol glycosides in mulberry leaves increased significantly after frost and showed a significantly negative correlation with climate temperature and significantly positive correlation with the antioxidant activities. The results showed that frost is beneficial to the accumulation of flavonol glycosides and the increase of antioxidant activity in mulberry leaves.



Abbreviations used: LC-MS: Liquid chromatography-mass spectrometry; HPLC: High performance liquid chromatography; Rut: rutin; IQ: Isoquercitrin; QAG: quercetin-3-*O*-(6″-acetyl)-β-D-glucopyranoside; Ast: Astragalin; KAG: kaempferol-3-*O*-(6″-acetyl)-β-D-glucopy- ranoside; TAC: Total antioxidant capacity; FeE: Fe^{2+} equivalent; RP: Reducing power; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azino-bis(3-ethylbenzoth iazoline -6-sulfonic acid.

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INTRODUCTION

Mulberry leaves are dry leaves of *Morus alba* L., which have the effect of evacuating wind-heat, clearing away Lunt-heat and moistening dryness and removing liver fire for improving eyesight.[1] Previous reports have proved that mulberry leaves have multiple biological activities such as anti-diabetic, lipid-lowering, anti-oxidation, anti-tumor, anti-bacterial, and anti-inflammatory. The active ingredients of mulberry leaves mainly include flavonoids, alkaloids, phenylpropanoids, steroids, and triterpenoids.[2-8]

According to *the Chinese Pharmacopoeia*, harvest of mulberry leaves should be after the first frost every year.[1] The reason may be dynamic changes of flavonoids and alkaloids in mulberry leaves in different growing seasons. It is consistent with our previous results regarding of flavonoids in mulberry leaves, which increased significantly after frost; while the content of alkaloids was lower.[9-11] Moreover, we also found that frost

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mulberry leaves (collected in November) had better traditional curative effect than nonfrost mulberry leaves (collected in August), while nonfrost mulberry leaves had better hypoglycemic effect than frost mulberry leaves.[12] It is known that mulberry alkaloids are active ingredients for lowering blood sugar, which have less influence on traditional efficacy. Moreover, the flavonoid compounds in mulberry leaves were reported to have antibacterial and anti-inflammatory activities,[13-15] which are related to the traditional curative effects. In addition, it was reported that flavonoid compounds of mulberry leaves had significant antioxidant activities. Quercetin and morin-3-*O*-β-D-glucopyranoside had better ABTS and DPPH free radical activities.[16] Moreover, quercetin-3-*O*-β-D-glucosyl-(1-6)-β-glucopyranose and quercetin had significant DPPH free radical scavenging activity.[3] Katsube *et al.* confirmed that rutin and quercetin-3-*O*-(6"-*O*-malonyl)-β-D-glucopyranoside were the most important antioxidant ingredients in mulberry leaves.[17] Kim and Jang[7] showed that rutin, isoquercitrin (IQ), quercetin-3-*O*-(6"-*O*-acetyl)-β-D-glucopyranose, and quercetin had the highest superoxide radical scavenging ability and stronger anti-AAPH and Cu^{2+}-induced hepatocyte oxidative damage activities. However, the effect of frost on the accumulation of flavonoids and antioxidant activities has not been reported.

In the present work, high-performance liquid chromatography-mass spectrometry (HPLC-MS) method was used to analyze the flavonoids in mulberry leaves, and the HPLC method was employed to simultaneously determine content of rutin (Rut), IQ and quercetin-3-*O*-(6"-*O*-acetyl)-β-D-glucopyranoside (QAG), astragalin (Ast), and kaempferol-3-*O*-(6"-*O*-acetyl)-β-D-glucopy ranoside (KAG) in mulberry leaves before and after frost. Total antioxidant capacity (TAC), Fe^{2+} equivalent (FeE), reducing power (RP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging abilities were evaluated for antioxidant activities of mulberry leaves before and after frost.

MATERIALS AND METHODS

Plant materials

Leaves of *Morus alba* L. were harvested from Jiangsu University mulberry plantation, located at N32°12'13.50" north longitude E119°30'45.26" east, during the growth cycle (from April 2015 to November 2015). The dried samples were grounded into powder (40–60 mesh) and kept in sealed polyethylene bag at 4°C until used.

Chemicals and reagents

Rut, IQ, and Ast standard were purchased from National Institutes for Food and Drug Control. HPLC grade acetonitrile was purchased from Merck Co (Germany). All the other reagents were analytical grade reagents and purchased from Zhenjiang Huadong chemical glass Co., Ltd (Zhenjiang, China).

Ultrasonic-assisted extraction mulberry leaves

Mulberry leaves powder (1 g, BS 110S electronic balance, Beijing Sartorius Instrument System Co., Ltd., China) was put into a 100 mL Erlenmeyer flask and soaked in 30 mL 60% ethanol for 30 min. Then, it was sonicated at 50°C by ultrasonic cleaner (KQ-250DB type, 40 KHz frequency, 250 W power, Kunshan Ultrasonic Instrument Co., Jiangsu, China) for 30 min. After extraction, the extracts were centrifuged at 10,000 rpm for 10 min and the supernatants were removed into new tubes and kept in dark at 4°C until used.

Electrospray ionization mass spectrometry (ESI-MS) analysis

Separations of mulberry extract were carried out using a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm) at 30°C and monitored with 358 nm. The mobile phase was made up of solvent A (acetonitrile) and solvent B (0.1% formic acid in water). The gradient flow was set as following: 0–12 min, 5%–9% A; 12–20 min, 9%–13% A; 20–30 min, 13%–33% A; 30–32 min, 33%–33% A; 32–42 min, 33%–43% A; 42–50 min, 43%–43% A. The injection volume is 10 μL and the flow rate is 0.8 mL/min.

The MS instrument (Thermo LXQ, USA) was operated using an ESI source in negative ionization mode with survey scans acquired from m/z 100–1000 for both MS and MS/MS. Ionization parameters were set as follows: spray voltage of ion source, 3.5 kV; capillary temperature, 325.00°C; capillary voltage, −30 V; cone voltage, −120 V. The data were collected by Xcalibur 2.0.7 SP1 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

High-performance liquid chromatography-ultraviolet analysis

HPLC analysis was performed on an Agilent 1260 HPLC instrument (Agilent Technologies, USA) equipped with an UV detector, a thermostated column compartment, an auto-sampler, and a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm) at 30°C. The analysis protocol was the same as our previous work.[18] The mobile phase consisted of acetonitrile (A) and 0.5% H_3PO_4 (B) with 0.8 mL/min flow rate. The gradient flow was set as following: 0–5 min, 10 A; 5–10 min, 10%–13.5% A; 10–15 min, 13.5%–19% A; 15–18 min, 19% A; 18–19 min, 19%–22% A; 19–35 min, 22% A; 35–40 min, 22%–30% A; 40–50 min, 30%–40% A. The injection volume was 10 μL. Contents of Rut, IQ, and Ast were analyzed using the authentic standard curve. Acetyl-IQ was quantified by the IQ's standard curve and Acetyl-Ast was determined according to the Ast's standard curve.[19]

Total antioxidant capacity assay

TAC was measured spectrophotometrically referring to the previous method.[20] One mL of sample solution at different concentration was mixed with 3 mL of reagent solution including 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The mixture was incubated at 95°C for 90 min. The absorbance was determined at 695 nm against 60% ethanol as a blank after the mixture was cooled to room temperature. The antioxidant capacity was calculated as rutin equivalents (REs) mg/g sample according to the line y = 0.0025x + 0.009 (where y is absorbance of sample, x is sample concentration). The correlation coefficient was 0.9999.

Fe^{2+} equivalent assay

FIIE was determined according to the method of Aleksandra.[21] The 1, 10-phenanthroline method was a new ferric-ion spectrophotometric method to determinate the antioxidant capacity. In brief, 0.2 mL suitably diluted sample, 1 mL of 0.2% (w/v) $FeCl_3$ solution, and 0.5 mL of 0.5% 1,10-phenanthroline solution were placed into a 10 mL volumetric flask in order and made up to 10 mL with 60% (v/v) ethanol. The mixture was kept in dark for additional 20 min reaction at room temperature. The absorbance was recorded at 510 nm and the control was using the same mixture without sample. The results were evaluated as REs mg/g sample according to the line y = 0.0062x – 0.0081 (where y is absorbance of sample, x is sample concentration). The correlation coefficient was 0.9998.

Reducing power assay

RP was determined according to the method of Pan.[22] One mL of sample solution at different concentration was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). At the same time, 1 mL of 60% ethanol was used as the control. After the mixture was incubated at 50°C for 20 min,

2.5 mL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 rpm for 10 min. The 2.5 mL of supernatant was mixed with 2.5 mL of distilled water and 2.5 mL of ferric chloride (0.1%) and then the mixture was reacted for 10 min. The absorbance was recorded at 700 nm. The RP was calculated as REs mg/g sample according to the line y = 0.0025x − 0.005 (where y is absorbance of sample, x is sample concentration). The correlation coefficient was 0.9999.

2,2-Diphenyl-1-picrylhydrazyl free radical scavenging assay

The experiment was adjusted according to Kintzios *et al.*[23] 200 μL of different concentrations of rutin solution mixed with 2.9 mL of 0.1 mmo1·L-1 DPPH solution and the absorbance was measured at 517 nm immediately after 30 min in the dark. As the same time, 60% ethanol was used as a blank control. Then the standard curve of different concentrations of rutin and the corresponding clearance rate were calculated. The experimental results showed that rutin had a good linear relationship with DPPH free radical scavenging rate in the range of 10.4–104 μmol·L-1, Y = 0.68X–0.566, (R^2 = 0.9976).

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid free radical scavenging assay

The assay was performed referring to the Wootton-Beard *et al.* method[24] with slight adjustment. An equal amount of 7 mmol·L-1 ABTS solution reacted with 2.45 mmol·L-1 potassium persulfate in the dark for 12–16 h to prepare $ABTS^+$free radicals. Then, the $ABTS^+$radical solution was diluted with 60% ethanol until the absorbance at the wavelength of 734 nm was 0.70 ± 0.02. At this time, 100 μL of the sample solution and 2.9 mL of $ABTS^+$radical solution were added in a clean test tube and well mixed. 1 mL 60% ethanol was used as a blank control. The change in absorbance value was measured at a wavelength of 734 nm.

Statistics and data analysis

The results were recorded as mean ± standard deviation. The correlation analysis was performed using IBM SPSS Statistics Version 20 (IBM Corp., Armonk, NY, USA).

RESULTS AND DISCUSSION

Qualitative analysis of high-performance liquid chromatography ESI-MS of mulberry leaves

The HPLC diagram of mulberry leaves is shown in Figure 1. There were 10 compounds identified based on retention time, fragment ions, and literature reports [Table 1]. Among them, peak 1, peak 6, peak 7, and peak 9 were confirmed by comparing the standard's retention time.

All of the 10 compounds in this article were polyphenol compounds, one of which was chlorogenic acid and the others were flavonoid glycosides with quercetin and kaempferol as parent structure. Their structural formulas are shown in Figure 2.

Peak 1 showed the $[M-H]^-$ parent ion at m/z 353.58 and secondary MS^2 ion at m/z 191.58 (quinic acid). It was identified to be chlorogenic acid according to the literatures.[6,11] Compound 2, 4, 6, 7, and 8 were identified as quercetin derivatives by the fragment ion at m/z 301[quercetin-H]$^-$, while the compound 3, 5, 9, and 10 were kaempferol derivatives defined with the fragment ion at m/z 285[kaempferol-H]$^-$ and λmax at 265 nm, typical of kaempferol moiety.[6] Peak 2 showed the $[M-H]^-$ parent ion at m/z 625.48 and secondary MS^2 ions at m/z 463.60 and 301.70 are derived from the loss of sugar moiety (MW 162). Peak 4 also showed the $[M-H]^-$ parent ion at m/z 625.54 and secondary MS^2 ions at m/z 301.60. These two compounds were isomers of quercetin diglycoside. Compound 2 and 4 were identified as quercetin-3,7-di-*O*-β-D-glucopyranoside, and quercetin-3-*O*-β-D-glucopyranosyl (1-6)-β-D-glucopyranoside according to the polarity of chemical structure and literature reports,[3,6] respectively. Peak 3, 5, and 6 showed the same $[M-H]^-$ parent ion at m/z 609, which means that they were isomers. Peak 3 had the fragment ion

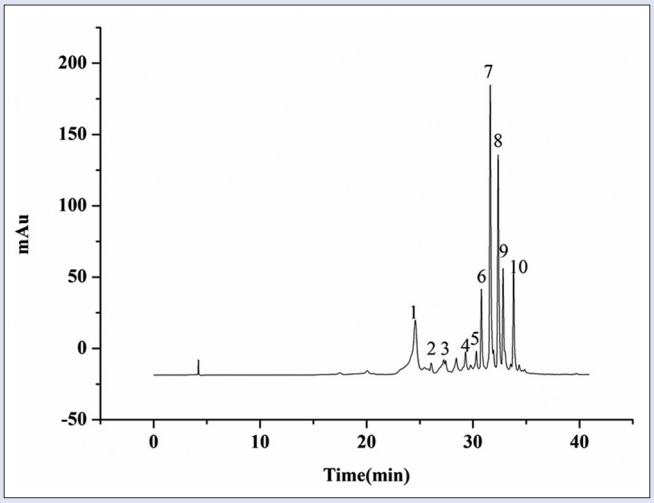

Figure 1: HPLC chromatogram of the 60% ethanol extracts from *Morus alba* L. (1) Chlorogenic acid; (2) quercetin-3,7-di-*O*-β-D-glucopyranoside; (3) kaempferol-3,7-di-*O*-β-D-glucopyranoside; (4) quercetin-3-*O*-β-D-glucopyranosyl (1-6)-β-D-glucopyranoside; (5) kaempferol-3-*O*-β-D-gludoside-(1-6)-β-D-glucopyranoside; (6) rutin; (7) isoquercitrin; (8) quercetin-3-*O*-(6-acetyl)-β-D-glucopyranoside; (9) astragalin; (10) kaempfero-3-*O*-(6-acetyl)-β-D-glucopyranoside

Table 1: High performance liquid chromatography-UV and ESI-MS compounds of *Morus alba L*

n	Rt (min)	(M-1)	fragmention	UV/Vis	Compound
1	24.57	353.58	191.58	328,302(sh)	Chlorogenic acid[6,11]
2	26.12	625.48	463.60, 301.70	351	Quercetin-3,7-di-*O*-β-D- glucopyranoside[3,6]
3	27.52	609.43	447.61, 285.73	265,315	aempferol-3,7-di-*O*-β-D- glucopyranoside[3,6,11,19]
4	29.49	625.54	301.66, 463.68	351	Quercetin-3-*O*-β-D-glucopyranosyl (1-6)-β-D- glucopyranoside[3,6]
5	30.56	609.66	285.68	265,315	Kaempferol-3-*O*-β-D- glucopyranosyl (1-6)-β-D- glucopyranoside[3,6,11,19]
6	31.02	609.51	301.06	254,352	Rutin[3,6,11,19]
7	31.86	463.64	301.69	253,355	Isoquercitrin[3,6,11,19]
8	32.65	505.52	301.64	253,355	Quercetin-3-*O*-(6″-acetyl)-β-D-glucopyranoside[3,11,19]
9	33.05	447.59	285.66	265,346	Astragalin[3,6,11,19]
10	34.06	489.65	285.68	265,344	Kaempferol-3-*O*-(6″-acetyl)-β-D-glucopyranoside[3,11,19]

ESI-MS: Electrospray ionization mass spectrometry; UV: ultraviolet

at m/z 447.61and 285.73 and peak 5 had the ion at 285.68, derived from the loss of the glucose fragment, which suggested the compound 3 and 5 were the kaempferol diglycosides. They were assigned as kaempferol-3, 7-di-*O*-β-D-glucopyranoside, and kaempferol-3-*O*-β-D-glucopyran osyl (1-6)-β-D-glucopyranoside. These two compounds were already identified in mulberry leaves by Dugo *et al.*[6] and Thabti *et al.*,[19] respectively. Peak 6, generating MS^2 ion at m/z 301.06, was identified as rutin based on reports from the previous publications.[3,6,11,19] Peak 7, with $[M-1]^-$ parent ion at m/z 463.64 and secondary MS^2 ion at m/z 301.69, was produced by losing the sugar moiety (dehydrated glucose). Therefore, compound 7 was deduced to be quercetin-3-*O*-β-D-gluco pyranoside (IQ), in accordance with the literatures.[3,6,11,19] Peak 8 was detected as $[M-1]^-$ parent ion at m/z 505.52 and gave a fragment ion at m/z 301.63, derived from the loss of acetylated sugar moiety.[3,11,19] Thus, compound 8 was identified as quercetin-3-*O*-(6-acetyl)-β-D-gl ucopyranoside. Peak 9, with $[M-1]^-$ ion at m/z 447.59 and secondary MS^2 ion at m/z 285.66, was assigned as kaempfrol-3-*O*-β-D-glucopyr anoside (Ast), which already was identified in mulberry leaves.[3,6,11,19] Meanwhile, the less polar compound 10, having $[M-1]^-$ ion at m/z 489.65 and secondary MS^2 ion at m/z 285.68, was kaempferol-3-*O*-(6-a cetyl)-β-D-glucopyranoside described in the literatures.[3,11,19]

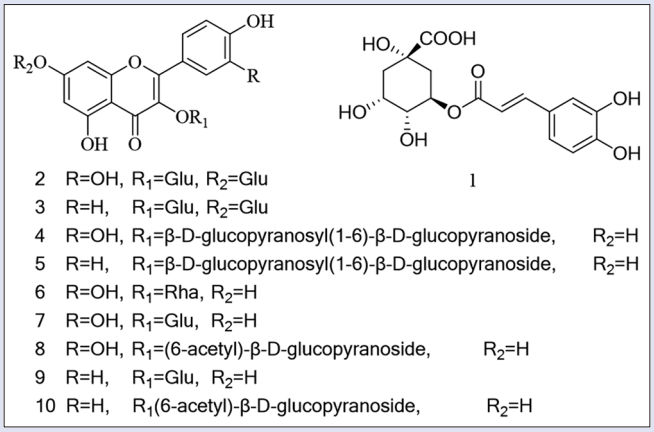

Figure 2: Chemical structure of identified compounds in mulberry leaves

Quantitative analysis of five flavonoid glycosides components in mulberry leaves before and after frost by high-performance liquid chromatography

Mulberry leaves from May to November in 2015 were collected to determinate the content of Rut, IQ, Acetyl-IQ, Ast, and Acetyl-Ast. Their accurate contents were calculated according to the regression equations [Table 2].

Results showed that the content of five flavonoids is higher at lower temperature in May, beginning to fall with an increasing temperature in June, till the lowest at higher temperature in August and gradually increased when the temperature became lower in September. The content of flavonol glycosides reached the maximum after Frost's Descent (October 23, a term marks the time when frost starts to descend across China in the Chinese lunar calendar) until November. This conclusion verified the rationality of harvesting mulberry leaves after the first frost in accordance with You and Wan.[25] Content of IQ, Ast and KAG reached a maximum of 3.05 mg/g, 0.70 mg/g, and 0.69 mg/g, respectively, in November.

Correlation analysis between temperature and five flavonol glycosides in mulberry leaves

Temperature may be one of the key factors causing the change of chemical composition in mulberry leaves before and after frost. Therefore, SPSS 20.0 software was used to analyze the correlation between the average climatic temperature and content of flavonol glycosides in mulberry leaves [Table 2] so as to discuss the effect of frost on the accumulation of flavonoids in mulberry leaves. The temperature for May to November 2015 is listed in Figure 3. The correlation analysis results are shown in Table 3.

It could be seen from the above table that there was a negative correlation between mean temperature and flavonoid content. In which, temperature was significantly and negatively correlated with the content of IQ and Ast ($P < 0.01$) and negatively correlation with content of KAG and sum ($P < 0.05$). The results showed that lower temperature was beneficial to the accumulation of flavonoids. Relevant literature reports were consistent with this conclusion. Caldwell *et al.*[26] reported that isoflavone content in *Glycine max* (L.) Merr decreased by 65% from 18°C to 23°C and decreased to about 90% at 2°C. The

Table 2: The content of Rut, IQ, Acetyl-IQ, Ast, and Acetyl-Ast of mulberry leaves picked in different date

Date	Rut	IQ	Acetyl-IQ	Ast	Acetyl-Ast	Sum
05/15	2.83±0.05	1.24±0.02	2.85±0.026	0.34±0.008	0.34±0.027	8.26±0.12
05/25	1.88±0.06	0.99±0.04	3.54±0.031	0.31±0.009	0.44±0.028	6.37±0.09
06/05	2.14±0.05	1.01±0.07	2.49±0.041	0.44±0.004	0.34±0.021	7.57±0.18
06/15	1.59±0.05	0.73±0.03	2.81±0.030	0.25±0.004	0.43±0.022	5.40±0.07
06/25	1.79±0.04	0.92±0.03	2.61±0.026	0.26±0.004	0.36±0.024	6.22±0.08
07/05	1.70±0.03	0.96±0.04	2.01±0.068	0.24±0.006	0.31±0.035	5.87±0.08
07/15	1.12±0.03	0.60±0.03	2.73±0.033	0.13±0.003	0.41±0.026	4.16±0.15
07/25	1.50±0.06	0.78±0.03	1.68±0.123	0.24±0.003	0.25±0.025	5.66±0.14
08/05	1.40±0.08	0.57±0.09	1.77±0.034	0.17±0.006	0.29±0.022	5.07±0.31
08/15	1.01±0.04	0.91±0.02	0.70±0.032	0.12±0.006	0.11±0.020	4.11±0.12
08/25	0.51±0.04	0.51±0.04	2.79±0.035	0.06±0.004	0.56±0.029	1.89±0.06
09/05	1.41±0.06	0.88±0.06	2.72±0.033	0.23±0.005	0.59±0.023	5.87±0.13
09/15	1.31±0.04	1.78±0.04	2.09±0.067	0.37±0.004	0.43±0.024	6.78±0.08
09/25	0.96±0.06	1.88±0.05	2.57±0.045	0.19±0.006	0.57±0.028	5.56±0.18
10/05	1.28±0.06	2.90±0.05	2.20±0.029	0.66±0.004	0.48±0.041	7.99±0.15
10/15	1.05±0.05	2.60±0.04	1.65±0.035	0.52±0.006	0.36±0.011	6.85±0.11
10/25	0.97±0.03	2.37±0.08	2.37±0.059	0.48±0.015	0.60±0.017	5.83±0.06
11/05	0.99±0.03	2.67±0.09	2.78±0.056	0.59±0.014	0.69±0.020	7.22±0.21
11/15	1.10±0.02	2.89±0.02	2.15±0.025	0.70±0.003	0.47±0.040	8.16±0.05
11/25	1.08±0.09	3.05±0.06	3.48±0.029	0.61±0.005	0.38±0.018	7.36±0.09

Ast: Astragalin; Rut: Rutin; IQ: Isoquercitrin

literature[27] also showed that ripening grapes caused a significant decrease in anthocyanin and flavonoid content at elevated temperatures. Becker *et al.*[28] reported that cultivation of garden lettuce (*Lactuca sativa*) at low temperatures promoted an increase of its main component, cyanidin-3-*O*-(6"-*O*-malonyl)-glucoside.

Antioxidant activities of mulberry leaves picked in different date

The change trend of antioxidant activities was basically the same in mulberry leaves collected in different date by five methods. When the temperature was lower in May, antioxidant activities were higher. In June, the activities began to decrease with an increase in temperature and the activities were lowest when the temperature was higher in August. The minimum values for TAC, FeE, RP, DPPH, and ABTS were 45.3 RE mg/g Sample, 31.9 RE mg/g Sample, 19.1 RE mg/g Sample, 13.8 RE mg/g Sample, and 14.1 RE mg/g Sample, respectively. The activities gradually increased when the temperature became lower in September. The activities reached its maximum after Frost's Descent to November. The maximum values for TAC, FeE, RP, DPPH, and ABTS were 64.3 RE mg/g Sample, 46.2 RE mg/g Sample, 31.3 RE mg/g Sample, 22.5 RE mg/g Sample, and 26.7 RE mg/g Sample [Table 4].

Correlation analysis between temperature and antioxidant activities of mulberry leaves before and after frost

In this work, SPSS 20.0 software was used to analyze the correlation between average climate temperature [Figure 3] and antioxidant activities of mulberry leaves [Table 4]. The results showed that there was a significantly negative correlation ($P < 0.05$) [Table 5].

Correlation analysis between antioxidant activities and content of flavonoids in mulberry leaves

The results of five antioxidant activities tests were significantly and positively correlated with content of IQ, Ast and sum [Table 6]. Wang *et al.*[29] also found that when content of flavonoids and polyphenols were the highest in the ethanol extract of mulberry leaves, the DPPH free radical activities were also the highest. These were also consistent with Guo *et al.*[30]

CONCLUSION

It is required that mulberry leaves should be collected after frost since ancient times. The timely collection of Chinese medicinal

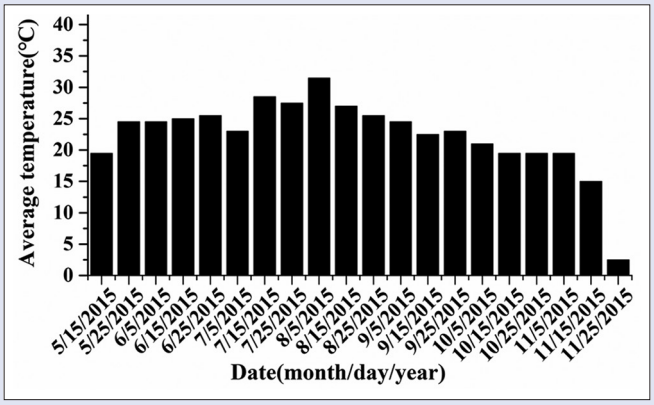

Figure 3: Temperature for May to November 2015

Table 3: Correlation analysis between average temperature and flavonoids accumulation in mulberry leaves picked in different dates

Relativity	Rut	IQ	Ast	QAG	KAG	Sum
Temperature						
Pearson	0.073	−0.709**	−0.714**	−0.373	−0.488*	−0.463*
Correlation significance	0.528	0.000	0.000	0.096	0.029	0.040

*Correlation is significant at the 0.05 level (two-tailed);**Correlation is significant at 0.01 level (two-tailed). Ast: Astragalin, Rut: Rutin, IQ: Isoquercitrin

Table 4: The antioxidant activities of mulberry leaves picked in different date (*n*=3)

Date	TAC (RE mg/g Sample)	FeE (RE mg/g Sample)	RP (RE mg/g Sample)	DPPH (RE mg/g Sample)	ABTS (RE mg/g Sample)
5/15	60.7±0.7	42.9±0.5	30.5±0.3	21.4±0.3	25.1±0.2
5/25	57.6±1.1	45.2±0.3	29.2±0.6	20.9±0.5	23.4±0.3
6/5	56.5±0.4	41.5±0.3	26.9±0.5	18.2±0.2	19.9±0.3
6/15	56.3±1.2	40.7±0.5	26.9±0.3	18.9±0.4	21.6±0.5
6/25	57.3±0.7	39.6±0.4	25.5±0.4	18.3±0.4	21.3±0.3
7/5	55.5±1.1	42.2±0.4	25.4±0.3	18.2±0.5	21.3±0.3
7/15	49.9±0.7	40.4±0.5	27.1±0.7	17.5±0.6	21.1±0.3
7/25	48.6±0.8	38.5±0.3	23.2±0.6	15.8±0.4	18.8±0.3
8/5	45.3±1.9	35.6±0.3	20.3±0.4	13.9±0.5	16.8±0.2
8/15	47.1±0.9	31.9±1.0	19.1±0.4	13.8±0.3	16.9±0.1
8/25	52.5±0.5	34.2±0.6	19.9±0.5	14.1±0.2	14.1±0.2
9/5	56.3±0.5	37.3±0.4	22.4±0.7	15.2±0.3	17.2±0.3
9/15	60.2±0.5	40.2±0.4	25.3±0.4	17.3±0.5	20.6±0.4
9/25	60.9±0.4	43.1±0.6	26.5±0.3	18.1±0.3	21.1±0.2
10/5	60.9±0.5	42.7±0.4	27.9±0.3	18.9±0.2	21.9±0.3
10/15	63.5±0.9	42.0±0.2	28.5±0.4	20.4±0.5	24.1±0.4
10/25	63.8±0.7	43.7±0.5	30.5±0.4	21.9±0.3	25.9±0.3
11/5	64.3±0.6	45.8±0.7	31.3±0.6	22.5±0.2	26.7±0.3
11/15	61.2±0.8	46.2±0.8	30.7±0.5	21.5±0.3	25.5±0.3
11/25	57.7±1.4	42.7±1.8	30.6±0.3	20.9±0.6	24.9±0.4

TAC: Total antioxidant capacity, FeE: Fe^{2+} equivalent, RP: Reducing power, RE: Rutin equivalent, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid

Table 5: Correlation analysis between average temperature and antioxidant activities of mulberry leaves picked in different dates

Relativity	TAC	FeE	RP	DPPH	ABTS
Temperature					
Pearson correlation	−0.586**	−0.542*	−0.552*	−0.660**	−0.657**
Significance	0.007	0.014	0.012	0.002	0.002

*Correlation is significant at the 0.05 level (two-tailed);**Correlation is significant at 0.01 level (two-tailed). TAC: Total antioxidant capacity, FeE: Fe^{2+} equivalent, RP: Reducing power, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid

Table 6: Coefficients of Pearson's Correlation analysis between antioxidant activities and flavonoids accumulation

	Rut	IQ	Acetyl-IQ	Ast	Acetyl-Ast	Sum
TAC Pearson Correlation	0.028	0.635**	0.358	0.706**	0.622**	0.632**
Significance	0.907	0.003	0.121	0.001	0.003	0.003
FeE Pearson Correlation	0.207	0.571**	0.494*	0.715**	0.459*	0.722**
Significance	0.382	0.009	0.027	0.000	0.042	0.000
RP Pearson Correlation	0.126	0.589**	0.308	0.754**	0.363	0.689**
Significance	0.596	0.006	0.187	0.00	0.116	0.001
DPPH Pearson Correlation	0.204	0.604**	0.469*	0.749*	0.393	0.700**
Significance	0.389	0.005	0.037	0.000	0.087	0.001
ABTS Pearson Correlation	0.175	0.656**	0.345	0.752**	0.301	0.721**
Significance	0.459	0.002	0.137	0.000	0.197	0.000

*Correlation is significant at the 0.05 level (two-tailed), **Correlation is significant at 0.01 level (two-tailed). TAC: Total antioxidant capacity, FeE: Fe^{2+} equivalent, RP: Reducing power, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, IQ: Isoquercitrin, Ast: Astragalin

materials is a prerequisite for the efficacy. This study shows that mulberry leaves have better antioxidant activities after frost, which may be caused by an increase in the content of flavonol glycosides. The results provide a theoretical basis for the appropriate picking time of mulberry leaves.

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Conflicts of interest

There are no conflicts of interest.

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