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## A novel estimation method of total flavonoids in edible medicinal mulberry leaves by ultrasound-assisted hydroalcohol-acid extraction and HPLC-DAD

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### Summary

Mulberry leaves have been widely used to produce various health products. In this paper, an efficient procedure of ultrasound-assisted hydroalcohol-acid extraction (UAHAE) was established to estimate the total flavonoids content in mulberry leaves by quantifying their resulting aglycones (quercetin and kaempferol) using HPLC-DAD. Effective hydrolysis of glycosides to aglycones was achieved in an ethanol-HCl-water (7/2/1, v/v/v) solution at 75 °C by ultrasound (40 kHz) for 60 min. The average contents of quercetin and kaempferol were 7.12 mg/g and 2.13 mg/g, respectively, in the variety of cultivar MC308, and their recoveries were 105.48% and 105.81%, respectively. The total flavonoid content of most varieties was between 4-10 mg/g, accounting for 80% in 86 species of mulberry leaves. With the increase in leaf maturity, the general trend of the change in flavonoid content was a decrease at first and then an increase. The highest total flavonoid content in commercial mulberry tea (CT 2) is 4.76 mg/g, and the DPPH radical scavenging activities were similar to the distribution of total flavonoids measured by UAHAE in three types of mulberry tea. These findings provide a basis for industrial-scale manufacturing of edible medicinal mulberry leaf products.

**Key Words:** mulberry leaves; total flavonoids; aglycones; quercetin; kaempferol; ultrasound; hydrolysis

### Introduction

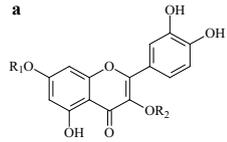
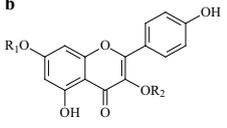
Mulberry (*Morus alba* L.) is an industrial crop with a high economic value (ZHANG et al., 2016). Its fruit, leaves, branches and roots are good sources of bioactive compounds, and it has a long history of use in traditional Chinese medicine. Mulberry leaf is the main product of the mulberry tree, accounting for 64% of the ground part of the tree (WANG et al., 2005). It is nutritious and nontoxic and is usually used as feed for silkworms in most Asian countries. Some researchers have found that mulberry leaves are rich in phenylpropanoids

(HUNYADI et al., 2012), flavonoids (WANYO et al., 2011), polysaccharide (THIRUGNANASAMBANDHAM et al., 2015), alkaloids (SUGIYAMA et al., 2016) and many other bioactive compounds. As a herbal medicine, extracts of mulberry leaves possess medicinal benefits against obesity (LIM et al., 2013), diabetes (REN et al., 2015), atherosclerosis, hypertension (LEE et al., 2011) and tumours (YANG et al., 2012). Because of their significant bioactivities, mulberry leaves have been widely used to produce various health products, such as beverages, health food and mulberry-leaf tea (ZHANG et al., 2016).

Flavonoids and flavonoids glycosides are the most important components in mulberry leaves. The total flavonoid content accounted for 1.0-3.0% of the mulberry leaf dry weight, which is higher than in other plants (LI et al., 2012). The major type of flavonoid in mulberry leaves is glycoside, with mainly quercetin and kaempferol aglycones (Tab. 1). KIM et al. identified nine flavonoids from mulberry leaves, kaempferol-3-O-β-D-glucopyranoside; kaempferol-3-O-(6''-O-acetyl)-β-D-glucopyranoside; quercetin-3-O-(6''-O-acetyl)-β-D-glucopyranoside; quercetin-3-O-β-D-glucopyranoside; kaempferol-3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside; quercetin-3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside; quercetin-3-O-β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside; quercetin-3,7-di-O-β-D-glucopyranoside; and quercetin (KIM et al., 1999). KAYO et al. purified and identified two new flavonoids from a butanol extract of mulberry leaves, isopentenyl flavanone and isopentene flavanone glycoside (DOI et al., 2001). Rutin, hesperetin, quercetin, kaempferol, naringenin, hydroxyflavin (CHANG et al., 2016) and kaempferol (YANG et al., 2012) have also been isolated from mulberry leaves. Many researchers have confirmed that flavonoids extracted from mulberry leaves possess anti-hyperlipidemic (KOJIMA et al., 2000), anti-diabetic (KOBAYASHI et al., 2010), anticancer (DEEPA et al., 2013), hepatoprotective (KALANTARI et al., 2009), and anti-Alzheimer's disease (NIIDOME et al., 2007) activities as well as have a synergistic effect on regulating the blood sugar level with alkaloids and polysaccharides in mulberry leaves.

The level of total flavonoids in mulberry leaves is conventionally estimated by a colorimetric method using rutin as a standard. How-

**Tab. 1:** Selected flavonoids with quercetin and kaempferol aglycones isolated from mulberry leaves

	R1 = H, R2 = H	Quercetin	CHANG et al., 2016
	R1 = H, R2 = glc	Quercetin 3-O-β-D-glucopyranoside (Isoquercitrin)	KIM et al., 1999
	R1 = H, R2 = 6''-acetyl-glc	Quercetin 3-O-(6''-O-acetyl)-β-D-glucopyranoside	KIM et al., 1999
	R1 = H, R2 = rha-glc	Quercetin 3-O-β-D-rutinoside (Rutin)	CHANG et al., 2016
	R1 = glc, R2 = H	Quercetin 7-O-β-D-glucopyranoside	WANG et al., 2013
	R = glc, R2 = glc	Quercetin 3,7-di-O-β-D-glucopyranoside	KIM et al., 1999
	R1 = H, R2 = H	Kaempferol	YANG et al., 2012
	R1 = H, R2 = glc	Kaempferol 3-O-β-D-glucopyranoside (Astragalinn)	KIM et al., 1999
	R1 = H, R2 = rha-glc	Kaempferol 3-O-β-D-rutinoside	WANG et al., 2013

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ever, due to the interference of other coexisting substances, such as chlorogenic acid and protocatechuic acid, in medicinal herbs and extracts, the total flavonoids determined by the colorimetric method is higher than the actual value. Furthermore, most flavonoids standards are not commercially supplied, and there are difficulties in measuring the content of various flavonoids in mulberry leaves. After oral administration, flavonoid glycosides will be transferred to their aglycones by the action of hydrolytic enzymes and microorganisms. Therefore, the effective components of oral flavonoid glycosides are their aglycones (LIU et al., 2007).

Ultrasound-assisted hydroalcohol-acid extraction (UAHAE) was developed in this study to estimate the content of flavonoids in mulberry leaves and mulberry tea. Quercetin and kaempferol, the major aglycones in mulberry leaves, were quantitatively detected by high performance liquid chromatography with a diode array detector (HPLC-DAD). Then, the total levels of the two aglycones were used to estimate or express the total flavonoids and their bioactivities in biosamples, especially mulberry leaves and tea. In this study, the flavonoid contents of 86 varieties of mulberry leaves and 3 species of mulberry leaves tea were examined by UAHAE.

## Materials and methods

### Chemicals and materials

Different species of mulberry leaves were picked from the Mulberry Garden of Soochow University in May 2016. Then the mulberry leaves were freeze-dried on a lyophilizer (Modulyod, Thermo Electron Corporation, USA). The resulting powder was stored at  $-4^{\circ}\text{C}$  and further used for the following experiments. Commercial tea 1 of mulberry leaves (CT1) and MC792 mulberry leaves were provided from Shandong Sericulture Institute. Commercial teas 2 and 3 of mulberry leaves (CT2 and CT3) were purchased from Hangzhou Qingxin and Anhui Cunyutang Co. Ltd. (China). Rutin and chlorogenic acid were purchased from TCI Shanghai Co. Ltd (China). Quercetin, 5, 6-dihydroxycoumarin, isoquercitrin, astragaline and kaempferol standards were purchased from Sigma-Aldrich Co. Ltd. All other chemicals and solvents were of analytical grade, except those used for HPLC analysis, such as acetonitrile and methanol, which were of HPLC grade.

### To optimize hydrolysis conditions

The extraction procedure was measured using Zhao's method (ZHAO et al., 2016) with some modifications. 250 mg of powder was suspended in 10 ml of an ethanol-HCl-water (V/V/V: 7/2/1) solution and kept at  $75^{\circ}\text{C}$  and 40 kHz (Ultrasonic Bath Jiemeng Instruments, China) for different times for the hydrolysis. For comparison, 250 mg of powder was suspended in 10 ml of an ethanol-water (V/V: 7/3) solution and kept at  $75^{\circ}\text{C}$  and 40 kHz for one hour. Then, the supernatant was used in the following analysis.

### Reproducibility analysis

A 250-mg sample powder [leaf of mulberry cultivar 308# (shortly MC308), from middle position of the branch] was suspended in 10 ml of an ethanol-HCl-water (V/V/V: 7/2/1) solution and kept at  $75^{\circ}\text{C}$  and 40 kHz for 1 h. Five repeated extractions of the samples were measured by HPLC-DAD to calculate the relative standard deviation (RSD).

### Recovery assay

Known levels of quercetin and kaempferol were added to the sample solution. Then, the mixture was extracted. The levels of quercetin and kaempferol were quantitatively detected by HPLC-DAD. The re-

coveries of the two aglycones were calculated by the linear equation produced from the standard curves.

### HPLC-DAD analysis

HPLC was performed with a Shimadzu HPLC system (Shimadzu, Japan), which consisted of a pump (LC-20AT), diode array detector (DAD, SPD-M20A), C18 column (Agilent  $250 \times 4.6$  mm) and LC-solution system manager program. The mobile phase was methyl alcohol-water-acetic acid at a ratio of 500/500/0.4 (v/v/v). The flow rate was 1 mL/min, and the eluate absorbance was monitored at 370 nm using a scanning range of 200 nm to 600 nm. The injection volume of the extract was 10  $\mu\text{L}$ .

HPLC analytical condition: mobile phase solvent A: acetonitrile, and solvent B: acetic acid / water (4:96, v/v). The flow rate was 1.0 mL/min. The gradient for separation was 95% solvent B at 0 min, 25% solvent A and 75% solvent B at 40 min, 40% solvent A and 60% solvent B at 50 min, 47% solvent A and 53% solvent B at 60 min, and 60% solvent A and 40% solvent B at 65 min. The eluate absorbance was monitored at 370 nm using a scanning range of 200 nm to 600 nm. The injection volume of the extract was 10  $\mu\text{L}$ .

### Determination of various samples

Mulberry leaves and tea were collected and immediately turned into powder through freeze-drying. Different leaf positions were collected from Husang 32. The mulberry branches were divided into 1-10 from apical to basal. The extraction method was UAHAE.

### Assay of total flavonoids by colorimetric method

For this section, 0.4 mL of the 70% ethanolic plant extract was mixed with 4.6 ml of ethanol. Then, 0.3 ml of 5%  $\text{NaNO}_2$  (for 5 min), 0.3 ml of  $\text{Al}(\text{NO}_3)_3$  (for 6 min), 4 mL of 4% NaOH and 0.4 mL of 70% ethanol were added to the mixture solution. The mixture was incubated for 10 min at room temperature. Then, the absorbance was measured at 510 nm (Hitachi UV-3000, Japan). The flavonoid content of the extracts was compared against the rutin standard calibration curve, which was plotted at 0, 0.01, 0.02, 0.04, 0.06 and 0.08 mg/mL.

### DPPH assay

The 70% ethanolic extracts of cultivar Lu-792 and three commercial mulberry teas (CT1, CT2 and CT3) were concentrated in a vacuum and freeze-dried (Thermo, America). The DPPH radical scavenging activities of these extracts were measured using a previously reported method (ZHAO et al., 2014).

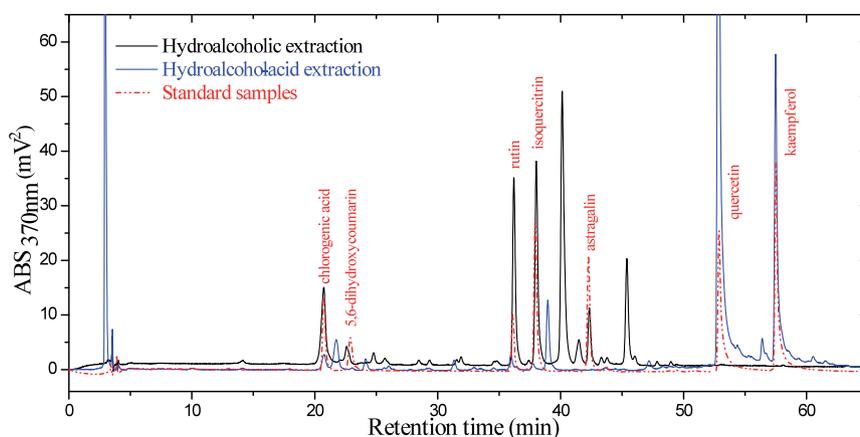
### Statistical analysis

The data are expressed as the mean  $\pm$  SD. Comparisons were made using one-way ANOVA with Origin 9.1 software.  $P < 0.05$  was considered statistically significant.

## Results and discussion

### Comparison of hydroalcoholic and hydroalcohol-acid extraction

High performance liquid chromatography (HPLC) was used for qualitative and quantitative research. The chromatogram showed several peaks; these peaks were identified by comparing the retention times of various standards (Fig. 1). Chlorogenic acid, rutin, isoquercitrin, and astragaline were identified from hydroalcoholic extraction, but hardly any quercetin or kaempferol was detected. However, high levels of quercetin (52.88 min) and kaempferol (57.50 min) were identified from hydroalcohol-acid extraction, and the levels of ru-



**Fig. 1:** HPLC chromatogram of hydroalcoholic (black line) and hydroalcohol-acid (blue line) extracts. Hydroalcoholic Extraction: extracted by ethanol-water (V/V: 7/3) solution. Hydroalcohol-acid Extraction: extracted by ethanol-HCL-water (V/V/V: 7/2/1) solution

tin, isoquercitrin, and astragalgin significantly decreased. This result means that flavonoid glycosides transformed to their resulting aglycones (quercetin and kaempferol).

On a C18 reverse-phase column, quercetin and kaempferol showed good retention behaviour. The concentrations and area under the chromatographic peak showed a good linear relationship. The linear regression equations for the two standard samples were as follows: quercetin,  $y = -149539.64 + 2100898.95x$ ,  $R^2 = 0.9954$ , and kaempferol,  $y = -136852.43 + 2674291.47x$ ,  $R^2 = 0.9963$ . The levels of the two aglycones, quercetin and kaempferol, detected by HPLC-DAD were individually calculated by two linear regression equations in the following experiment.

#### Optimisation of acid hydrolysis

Different extraction times (10, 30, 50, 60, and 70 min) were investigated to obtain all of the flavonoid aglycones. The levels of the quercetin and kaempferol aglycones were approximately 6.6 mg/ml and 1.9 mg/ml with the increase in extraction time from 10-70 min (40 kHz ultrasonic). The maximum extraction levels of flavonoid aglycones were  $6.91 \pm 0.16$  mg/mL and  $2.06 \pm 0.05$  mg/mL at 60 min, and the flavonoid concentration decreased with a longer extraction time. At 30 and 50 min, the levels of the quercetin and kaempferol aglycones were also high, but the higher error line indicated that hydrolysis was not stable. Therefore, the best extraction condition was to suspend 250 mg of the sample powder in 10 mL of an ethanol-HCL-water (V/V/V: 7/2/1) solution and kept at 75 °C and 40 kHz for 60 min.

#### Reproducibility of aglycone extraction by UAHAE-HPLC

The mulberry leaf sample was extracted five times; then, these samples were measured by HPLC-DAD (Tab. 2). According to the individual linear equations, the levels of quercetin and kaempferol were calculated to be an average of 7.12 mg/g and 2.13 mg/g with relative standard diversity (RSD) values of 6.44% and 6.86%, respectively.

These data indicate that this method for measuring the contents of quercetin and kaempferol from mulberry leaves has good reproducibility.

#### Recovery of aglycone extraction by the UAHAE method

To evaluate the accuracy of this analytical method, a recovery study of the extracts from mulberry leaves was performed by adding known levels of quercetin and kaempferol standards. The mean recovery was 105.48% for quercetin and 105.81% for kaempferol (Tab. 3). Recovery percentages higher than 100% could be explained by the interference of the sample matrix. The relative standard deviation (RSD) determined the accuracy and stability of the methods. The RSD of the average recovery was 2.99% for quercetin and 2.62% for kaempferol. The recovery test showed that the UAHAE method for the hydrolysis and extraction of quercetin and kaempferol from mulberry leaves had very good precision.

#### The content of total flavonoids at different leaf positions

The mulberry branches were divided into several parts, from apical to basal (P1-P10). The total flavonoid levels of different mulberry leaves from the same branch also exhibited significant differences. At position 1 (P1), the highest level of total flavonoid content was 13.86 mg/ml (Fig. 2). Then, a significant decrease was observed from P2 to P5 (6.37 mg/g). From P5 to P7, the total flavonoid content increased to 9.63 mg/mL, and no significant differences were observed until P10. With the increase in leaf maturity, the general trend of the change in flavonoid content was a decrease at first and then an increase. The same results were found in bilberry leaves. A previous study reported that higher levels and increased expression of flavonoid pathway genes were observed in bilberry apical leaves exposed to direct sunlight compared to basal leaves that received limited sunlight (JAAKOLA et al., 2004). The increase in the flavonoids from P5 to P7 may be due to synthesis and accumulation in basal leaves (MARTZ et al., 2010). The difference between leaf positions may result from

**Tab. 2:** Reproducibility for the determination of the two aglycones by the UAHAE method

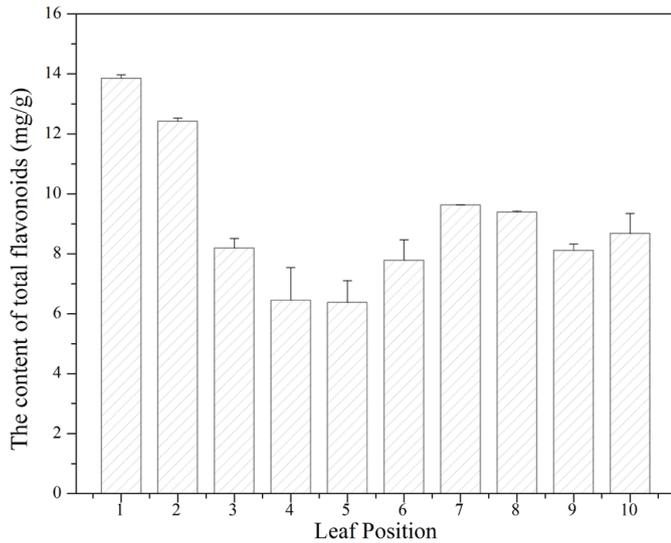
Aglycones	Repeated measurements (mg/g)					Means (mg/g)	± SD	RSD (%)
	1	2	3	4	5			
Quercetin	7.73	7.19	6.96	7.26	6.48	7.12	0.46	6.44
kaempferol	2.35	2.13	2.08	2.16	1.94	2.13	0.15	6.86

Experimental materials: The mulberry leaf variety is MC308.

**Tab. 3:** Recoveries for the extraction of the two aglycones by the UAHA method

Aglycones	Sample content (mg/g)	Dosage (mg)	Theoretical value (mg/g)	Measured value (mg/g)	Recovery (%)	RSD (%)
Quercetin	7.72	3.87	7.73	8.16 ± 0.24	105.48 ± 3.15	2.99
kaempferol	2.34	1.17	2.34	2.48 ± 0.07	105.81 ± 2.77	2.62

Experimental materials: The mulberry leaf variety is MC308.



**Fig. 2:** The content of total flavonoids at different leaf positions 1-10: from apical to basal of the branch

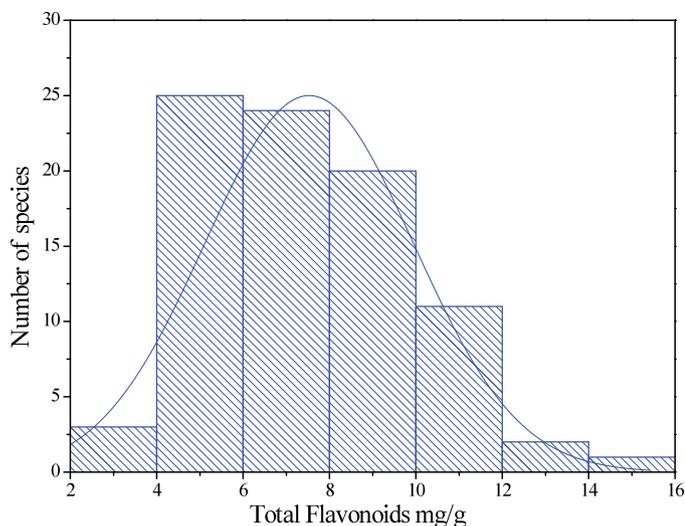
different accumulation and allocation due to a variety factors that are responsive to the interaction between intrinsic, soil nutrient content and, environmental stimuli, including light, pathogens, temperature (VAGIRI et al., 2015).

**The content of total flavonoids in different mulberry varieties**

The content of total flavonoids was estimated by the sum of quercetin and kaempferol. As shown in Tab. 4, the content of total flavonoids in different mulberry leaves ranged from 2.25-14.33 mg/g, which had a broad range for different varieties of mulberry leaves. This result also suggested that the total flavonoids had significant genotypic differences in different mulberry leaves. The minimum content of total flavonoids was found in LiuYe (2.25 mg/g) of all tested varieties. In the Lu-10 variety, the maximum flavonoid content (14.33 mg/g), was six-fold higher than in LiuYe. The distribution of flavonoids in mulberry leaves also showed a normal distribution (Fig. 3). Most varieties were distributed between 4-10 mg/g, accounting for 80% of the total determination. Additionally, 16% of mulberry leaves were higher than 10 mg/g, and several of the highest varieties were LU-9, G60, LU10, indicating that they were potential resources for new product development.

**Tab. 4:** Total flavonoids in 86 varieties of mulberry leaves

Mulberry cultivars	Total flavonoids (mg/g)						
MC206	5.76	T1	8.61	LU-10	14.33	G60	13.71
MC302	11.74	T10	10.68	LU-11	7.86	Boluo	6.18
MC303	8.56	T2	7.48	LU-12	5.19	CanZhuan4	7.53
MC304	7.2	T3	6.84	LU-14	8.3	DuoHu	9.38
MC307	5.39	T4	7.78	LU-2	5.47	FengWeiAnDu	5.45
MC308	9.61	T5	10.91	LU-3	6.61	FuYang	8.83
MC403	6.89	T6	11.78	LU-4	6.44	GaoGan	2.96
MC406	7.12	T7	10.03	LU-5	4.08	Gui 94	5.87
MC842	6.06	T8	9.56	LU-6	7.1	Gui D50	3.98
MC843	5.47	T9	11.31	LU-7	7.26	Gui Y50	11.57
MC3071	5.73	BeiDong2	8.78	LU-8	7.94	HeTian	8.53
D05	10.05	BeiDong 3	8.99	LU-9	12.87	HeiYou	5.99
G04	9.98	BeiDong 7	11.26	NO1	6.88	HongDing	7.04
HongQiao7	6.39	KangHan6	4.65	WoLong	8.73	HongPiMao	8.53
HongPi	7.61	JiuWenLong	7.68	HuSang4	8.77	RiZhou 9	6.86
Bei L6	10.12	GuaPiao	4.38	HuSang7	5.77	Sha2	6.92
LiuYe	2.25	HuSang 1	8.66	HuangBanSang	5.66	SuZhou	4.24
BingWU	8.94	HuSang32	6.38	NanD 7	9.32	Tang10	6.75
BaiPi	5.05	NO3	8.23	NiuJin	5.2	Yu71-1	5.48
WuHai	8.48	NanD1	9.07	Nong	10.51	YuanTouHeYe	4.25
WuLong	5.67	NanD2	5.79	QingPiGuaPiao	5.27	NanD 3	5.38
QingPiWaSang	5.16	NanD 4	4.49				

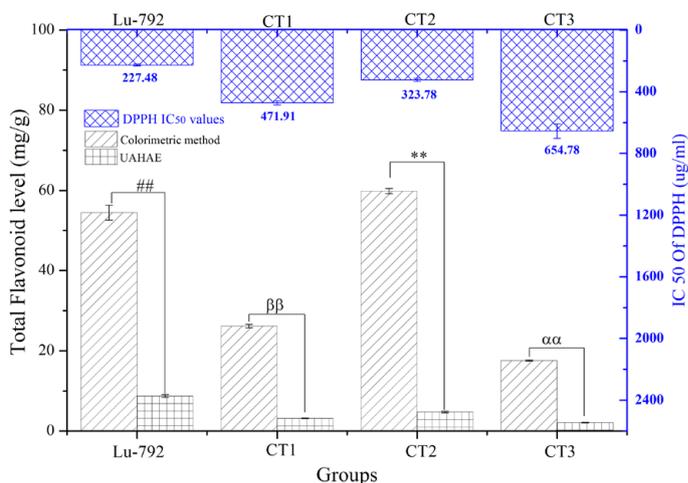


**Fig. 3:** Distribution diagram for the total flavonoid content in mulberry leaves

### Total flavonoids in mulberry tea

Mulberry leaf preparations are commonly used in commercial beverages (especially mulberry tea) and health foods. Because mulberry leaf tea does not contain tannic acid and caffeine, it cannot cause an increase in blood pressure or nervous excitation. It has become a popular functional beverage in some countries, such as China, Japan, and Korea. The quantitative analysis results revealed substantial diversity in the total flavonoid contents in different species of mulberry tea. We measured three commercial teas of mulberry leaves (CT1, CT2, and CT3 (dark colour)) by the traditional colorimetric method and UAHAE. The total flavonoid content measured by the traditional colorimetric method was 26.14 mg/ml in CT1; however, the total flavonoid content measured by the UAHAE method was 3.15 mg/ml (Fig. 4). It was also found in mulberry tea that the contents measured by the traditional colorimetric method were much higher than those measured by UAHAE. Recently, Guo et al. had doubts about the traditional colorimetric method, which uses rutin as a standard to estimate the total flavonoid levels in bio-samples. Their results showed that non-flavonoids, such as caffeic acid, protocatechuic aldehyde, and chlorogenic acid, had a maximum absorption at 510 nm (GUO et al., 2002). Chlorogenic acid (SÁNCHEZ-SALCEDO et al., 2015) and protocatechuic acid (WU et al., 2013) have been identified from mulberry leaves, and these compounds react with  $\text{NaNO}_2$ ,  $\text{Al}(\text{NO}_3)_3$ , and  $\text{NaOH}$  with a maximum absorption at 507 nm. Anna also found that the procedure in the presence of  $\text{NaNO}_2$  in alkaline medium seemed to be specific for rutin, luteolin and catechins, but phenolic acids also exhibited considerable absorbance at 510 nm. (PEKAL et al., 2014). These reasons can lead to an increase in the total flavonoid content measured by the colorimetric method. Commercial mulberry leaf CT1 tea is made from Lu-792. The content of total flavonoids in Lu-792 is 8.74 mg/g; however, 3.15 mg/g is observed in CT1. For the Lu-792 ethanolic extract, the IC<sub>50</sub> value of the DPPH radical scavenging abilities was 227.45  $\mu\text{g/ml}$ , which was significantly better than that of CT1 (470.24  $\mu\text{g/ml}$ ). Drying is one of the most critical processes in tea manufacturing, but this process may damage the antioxidant and nutritional qualities of the product (WANYO et al., 2011). KUMAR et al. found that a long drying time may cause degradation in food quality. In addition, this method results in undesirable qualities of the product, such as a dark colour and decreased antioxidant activity (PRAVEEN KUMAR et al., 2005).

Flavonoids have been demonstrated to have good antioxidant activity (PARHIZ et al., 2015). To evaluate the relationship between the flavo-



**Fig. 4:** Determination of total flavonoids by the colorimetric method and UAHAE in mulberry tea extracts and their DPPH radical scavenging activities. Commercial mulberry leaves tea (CT1, CT2, CT3), Lu-792: the source of C1, ##  $p < 0.01$ , \*\*  $p < 0.01$ ,  $\alpha$   $p < 0.01$ ,  $\beta$   $p < 0.01$  compared to the colorimetric method

noid content measured by the UAHAE method and antioxidant activity, tea extracts were used to analyse the DPPH scavenging property. As shown in Fig. 4, the CT2 extract showed the maximum content (4.76 mg/g), while CT3 had the lowest content (2.13 mg/g). The IC<sub>50</sub> values of the DPPH radical scavenging abilities of each mulberry tea extract were 470.24  $\mu\text{g/ml}$ , 338.44  $\mu\text{g/ml}$  and 651.39  $\mu\text{g/ml}$ . The total flavonoid content of CT2 (59.84 mg/g) measured by the traditional colorimetric method was higher than Lu-792 (54.45 mg/g), but the content measured by UAHAE was reversed in the two samples. The IC<sub>50</sub> values of DPPH radical scavenging abilities of Lu-792 (227.45  $\mu\text{g/ml}$ ) were lower than CT2 (338.44  $\mu\text{g/ml}$ ), which means that UAHAE more accurately reflects the actual flavonoid content. The radical scavenging activities were similar to the distribution of total flavonoids measured by UAHAE in three types of mulberry tea.

### Conclusion

Mulberry leaves have become a health product, especially mulberry tea, in some countries, such as China, Japan, and Korea. The established UAHAE method is efficient for flavonoid extraction and quantification in mulberry leaves and tea. The optimal hydrolysis of flavonoid glycosides and extraction of the resulting aglycones occur in an ethanol-HCl-water (7/2/1, V/V/V) solution at 75 °C (40 kHz ultrasonic) over 1 h. The validated method is precise and stable. In this study, mulberry leaves of 86 varieties and mulberry leaf tea of 3 species were examined for their flavonoid content. These findings will provide a basis for better industrial-scale manufacture of mulberry tea and for a wide range of similar food products.

### Compliance with Ethical Standards

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**Conflict of Interests** Jin-Ge Zhao declares that he has no conflict of interest. Yu-Qing Zhang declares that he has no conflict of interest.

### References

CHANG, Y.C., YANG, M.Y., CHEN, S.C., WANG, C.J., 2016: Mulberry leaf polyphenol extract improves obesity by inducing adipocyte apoptosis

- and inhibiting preadipocyte differentiation and hepatic lipogenesis. *J. Funct. Foods* 21, 249-262.
- DEEPA, M., SURESHKUMAR, T., SATHEESHKUMAR, P.K., PRIYA, S., 2013: Antioxidant rich *Morus alba* leaf extract induces apoptosis in human colon and breast cancer cells by the downregulation of nitric oxide produced by inducible nitric oxide synthase. *Nutr. Cancer* 65, 305-310.
- DOI, K., KOJIMA, T., MAKINO, M., KIMURA, Y., FUJIMOTO, Y., 2001: Studies on the constituents of the leaves of *Morus alba* L. *Chem. Pharm. Bull.* 49, 151-153.
- GUO, Y.J., FAN, L., WANG, X.Q., ZHANG, L.Z., 2002: Discussion about  $\text{NaNO}_2$  -  $\text{Al}(\text{NO}_3)_3$ - $\text{NaOH}$  colorimetry for determination of total flavonoids. *Chin. J. Pharm. Ana. L.* 22, 97-97.
- HUNYADI, A., MARTINS, A., HSIEH, T.J., SERES, A., ZUPKÓ, I., 2012: Chlorogenic acid and rutin play a major role in the in vivo anti-diabetic activity of *Morus alba* leaf extract on type II diabetic rats. *PLoS One* 7(11). e50619.
- JAAKOLA, L., MAÄTTÄ-RIIHINEN, K., KÄRENLAMPI, S., HOHTOLA, A., 2004: Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus* L.) leaves. *Planta* 218, 721-728.
- KALANTARI, H., AGHEL, N., BAYATI, M., 2009: Hepatoprotective effect of *Morus alba* L. in carbon tetrachloride-induced hepatotoxicity in mice. *Saudi. Pharmaceut. J.* 17, 90-94.
- KIM, S.Y., GAO, J.J., LEE, W.C., RYU, K.S., LEE, K.R., KIM, Y.C., 1999: Antioxidative flavonoids from the leaves of *Morus alba*. *Arch. Pharm. Res.* 22, 81-85.
- KOBAYASHI, Y., MIYAZAWA, M., KAMEI, A., ABE, K., KOJIMA, T., 2010: Ameliorative effects of mulberry (*Morus alba* L.) leaves on hyperlipidemia in rats fed a high-fat diet: induction of fatty acid oxidation, inhibition of lipogenesis, and suppression of oxidative stress. *Biosci. Biotechnol. Biochem.* 74, 2385-2395.
- KOJIMA, T., FUJIMOTO, Y., 2000: Mulberry leaf extract inhibits the oxidative modification of rabbit and human low density lipoprotein. *Biol. Pharm. Bull.* 23, 1066-1071.
- LEE, Y.J., CHOI, D.H., KIM, E.J., KIM, H.Y., KWON, T.O., KANG, D.G., LEE, H.S., 2011: Hypotensive, hypolipidemic, and vascular protective effects of *Morus alba* L. in rats fed an atherogenic diet. *Am. J. Chin. Med.* 39, 39-52.
- LI, M.C., YANG, D., GUO, Y., FAN, G.W., WANG, H., 2012: Advances in studies on chemical constituents from mulberry flavonoids and its pharmacological effects. *Liaoning J. Tradit. Chin. Med.* 39, 377-380.
- LIM, H.H., LEE, S.O., KIM, S.Y., YANG, S.J., LIM, Y., 2013: Anti-inflammatory and antiobesity effects of mulberry leaf and fruit extract on high fat diet-induced obesity. *Exp. Biol. Med.* 238, 1160-1169.
- LIU, Y., HU, M., 2002: Absorption and metabolism of flavonoids in the caco-2 cell culture model and a perused rat intestinal model. *Drug Metab. Dispos.* 30, 370-377.
- MARTZ, F., JAAKOLA, L., JULKUNEN-TIITTO, R., STARK, S., 2010: Phenolic composition and antioxidant capacity of bilberry (*Vaccinium myrtillus*) leaves in Northern Europe following foliar development and along environmental gradients. *J. Chem. Ecol.* 36, 1017-1028.
- NIIDOME, T., TAKAHASHI, K., GOTO, Y., GOH, S., TANAKA, N., KAMEI, K., SUGIMOTO, H., 2007: Mulberry leaf extract prevents amyloid beta-peptide fibril formation and neurotoxicity. *Neuroreport.* 18, 813-816.
- PARHIZ, H., ROOHBAKHSH, A., SOLTANI, F., REZAEI, R., IRANSHAHI, M., 2015: Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: an updated review of their molecular mechanisms and experimental models. *Phytother. Res.* 29, 323-331.
- PEKAL, A., PYRZYNSKA, K., 2014: Evaluation of aluminium complexation reaction for flavonoid content assay. *Food Anal. Method* 7, 1776-1782.
- PRAVEEN KUMAR, D.G., UMESH HEBBAR, H., SUKUMAR, D., RAMESH, M.N., 2005: Infrared and hot-air drying of onions. *J. Food Process. Preserv.* 29, 132-150.
- REN, C.J., ZHANG, Y., CUI, W.Z., LU, G.B., WANG, Y.W., HUANG, L., MU, Z.M., 2015: A polysaccharide extract of mulberry leaf ameliorates hepatic glucose metabolism and insulin signaling in rats with type 2 diabetes induced by high fat-diet and streptozotocin. *Int. J. Biol. Macromol.* 72, 951-959.
- SÁNCHEZ-SALCEDO, E.M., MENA, P., GARCÍA-VIGUERA, C., HERNÁNDEZ, F., MARTÍNEZ, J.J., 2015: (Poly) phenolic compounds and antioxidant activity of white (*Morus alba*) and black (*Morus nigra*) mulberry leaves: Their potential for new products rich in phytochemicals. *J. Funct. Foods* 18, 1039-1046.
- SUGIYAMA, M., KATSUBE, T., KOYAMA, A., ITAMURA, H., 2016: Effect of solar radiation on the functional components of mulberry (*Morus alba* L.) leaves. *J. Sci. Food Agr.* 96, 3915-3921.
- THIRUGNANASAMBANDHAM, K., SIVAKUMAR, V., MARAN, J.P., 2015: Microwave-assisted extraction of polysaccharides from mulberry leaves. *Int. J. Biol. Macromol.* 72, 1-5.
- VAGIRI, M., CONNER, S., STEWART, D., ANDERSSON, S.C., VERRALL, S., JOHANSSON, E., RUMPUNEN, K., 2015: Phenolic compounds in blackcurrant (*Ribes nigrum* L.) leaves relative to leaf position and harvest date. *Food Chem.* 172, 135-142.
- WANG, F., LI, J.R., 2005: Research Progress on Chemical Constituents, Physiological Function and Application of Mulberry Leaves. *Food Sci.* 26, 111-116.
- WANG, Y., XIANG, L., WANG, C., TANG, C., HE, X., 2013: Antidiabetic and antioxidant effects and phytochemicals of mulberry fruit (*Morus alba* L.) polyphenol enhanced extract. *Plos One* 8(7). e71144.
- WANYO, P., SIRIAMORNUN, S., MEESO, N., 2011: Improvement of quality and antioxidant properties of dried mulberry leaves with combined far-infrared radiation and air convection in Thai tea process. *Food Bioprocess. Process* 89, 22-30.
- WU, C.H., CHEN, S.C., OU, T.T., CHYAU, C.C., CHANG, Y.C., WANG, C.J., 2013: Mulberry leaf polyphenol extracts reduced hepatic lipid accumulation involving regulation of adenosine monophosphate activated protein kinase and lipogenic enzymes. *J. Funct. Foods* 5, 1620-1632.
- YANG, T.P., LEE, H.J., OU, T.T., CHANG, Y.J., WAN, C.J., 2012: Mulberry leaf polyphenol extract induced apoptosis involving regulation of adenosine monophosphate-activated protein kinase/fatty acid synthase in a p53-negative hepatocellular carcinoma cell. *J. Agr. Food Chem.* 60, 6891-6898.
- YANG, Z., WANG, Y., WANG, Y., ZHANG, Y., 2012: Bioassay-guided screening and isolation of  $\alpha$ -glucosidase and tyrosinase inhibitors from leaves of *Morus alba*. *Food Chem.* 131, 617-625.
- ZHANG, D.Y., WAN, Y., XU, J.Y., WU, G.H., LI, L., YAO, X.H., 2016: Ultrasound extraction of polysaccharides from mulberry leaves and their effect on enhancing antioxidant activity. *Carbohydr. Polymer* 137, 473-479.
- ZHAO, J.G., ZHANG, Y.Q., 2016: A new estimation of the total flavonoids in silkworm cocoon sericin layer through aglycone determination by hydrolysis-assisted extraction and HPLC-DAD analysis. *Food Nutr. Res.* 60.
- ZHAO, J.G., YAN, Q.Q., XUE, R.Y., ZHANG, J., ZHANG, Y.Q., 2014: Isolation and identification of colourless caffeoyl compounds in purple sweet potato by HPLC-DAD-ESI/MS and their antioxidant activities. *Food Chem.* 161, 22-26.

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