Quantitative determination of total and individual flavonoids in stems and leaves of *Buddleja davidii* and *Buddleja albiflora*

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ABSTRACT

Background: *Buddleja davidii* and *B. albiflor*a are two different original plants of the famous crude medicine "Diaoyangchen." **Materials and Methods:** An ultraviolet–visible spectrophotometric method and a HPLC method were used for the determination of total and individual flavonoids (luteolin and apigenin) contents from their stems and leaves for the first time. **Results:** From the comparative evaluation, remarkable differences in flavonoids contents were observed between different origins and different parts of the samples. And content of specific flavonoid did not correspond to the total flavonoids contents in *Buddleja davidii* and *Buddleja albiflora*. **Conclusion:** With a better accuracy and precision, the methods had been proved simple, rapid, and reliable for quantitative determination of the total flavonoids and luteolin and apigenin in the two phytomedicines. Furthermore, our present study will pave the way of guidelines for the differentiation and standardization and exploitation of individual parts of this herb material.

Key words: Apigenin, *Buddleja albiflora* Hemsl., *Buddleja daviddi* Franch., luteolin, total flavonoids

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INTRODUCTION

Buddleja albiflora (BA) and *B. davidii* (BD), two members of the Genus Buddleja, are also the two different origins of crude medicine "Diaoyangchen" which is traditional used in west Hubei Province. Leaves and roots of BA and BD are used in the treatment of rheumatism, arthralgia, hemorrhaging, traumatic injury, fracture, pruritus of skin, eczema, and so on.[1] Many studies on chemical composition have shown that Buddleja mainly contain flavonoids, phenylpropanoids, terpenoids, saponins, lignins, and sterols, etc. Flavonoid is a group of compound with a lot of physiological activities widely existed in Buddleja.[2,3] Tao has found that the flavonoids in BA are luteolin, quercetin, quercetin-3-O-β-D-glucoside, apigenin, apigenin-7-O-β-D-glucos-ide, apigenin-7-O-neohesperidoside, acacetin-7-O-α-L-rham nopyranosyl-(1-6)-β-D-glucopyranoside.[4,5] A number of studies presented in literatures have proved that luteolin, quercetin and apigenin have the properties of anti-oxidant, antibacterial, antivirus, anticancer, hypotensive.[3,6-12] Besides, luteolin has functions of immunomodulation, procoagulant, antitussive, expectoranting, curing asthma, while quercetin has antithrombotic and analgesic actions.[13,14] According to the published researches, we extrapolate out of that flavonoid is one of the most important active substances. Although lots of flavonoids have been identified, the quantitative analysis has not been documented. In this test, the total flavonoids contents of stems and leaves of BA and BD were determined through ultraviolet spectrophotometry while a reversed-phase HPLC assay for simultaneous determination of the two key flavonoids (luteolin and apigenin) has been developed and validated. The methods were successfully applied to the determination of the flavonoids content in order to preliminary estimate and control the quality of the two phytomedicines.

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EXPERIMENTAL

Materials and reagents

The aerial parts of *Buddleja albiflora* and *B. davidii* were collected from Jianshi and Badong in Hubei Province, China, in July 2009 and November 2010, respectively. They were identified as *Buddeja albiflora* Hemsl. and

Buddleja daviddi Franch., respectively, by Professor Dingrong Wan, from college of pharmacy, South-central University for Nationalities, China. After air-dried in nature, the plant drugs were ground with a laboratory mill and then passed through a 20-mesh sieve.

The standards of rutin and luteolin were purchased from Chinese pharmaceutical and biological product verification station (batch numbers are 100080-200707 and 111520-200504) and apigenin (purity ≥ 98%) was obtained from aladdin reagent. Acetonitrile, HPLC grade, was purchased from Tedia Company Inc, USA; the ethanol, methanol, petroleum ether and hydrochloric acid were of analytical reagent grade.

Methods for total flavonoids determination

Preparation of standard solutions and sample

A reasonable amount of rutin (about 10 g) was weighed and dissolved in 50% ethanol and brought to the volume in a measuring flask of 50 ml. The concentration of the standard solution was 0.204 mg/ml for the establishment of calibration curve. One gram of Buddleja daviddi (BD) leaves, BD stems, *Buddleja albiflora* (BA) leaves, BA stems samples were weighed accurately and ultrasonic extracted with 40 ml of 50% aqueous ethanol for 60 min at 45ºC. The extraction was filtered and decolored by petroleum ether for several times until the supernatant become colorless. A 20 ml of each solution below (ethanol solution) was pipetted into a measuring flask of 25 ml and diluted with an appropriate extraction solvent for the future use.

Choice of the detection wavelength

According to the absorption spectra of standard and sample solutions (stems and leaves of BD), the strong absorption peak of rutin was at 510 nm while the sample solutions were both around 510 nm. So 510 nm was chosen as the detection wavelength in the determination of total flavonoids.

Procedures

A certain volume sample solutions were pipetted into a 25 ml volumetric flask and mixed with 1 ml of 5% $NaNO_2$ solution for 6 min. Then 1 ml of 10% $Al(NO_3)_3$ solution was added and reacted with the solution. Six minutes later, 10 ml of 4% NaOH solution was joined into the solution and mixed thoroughly. The final volume was adjusted to 25 ml with 50% aqueous ethanol. The mixture was incubated in the room temperature for 15 min and then the absorbance of the mixture was measured against the bank by using the ultraviolet visible spectrophotometer at 510 nm.

Methods for HPLC determination of luteolin and apigenin

Standard solution preparation and sample preparation

Appropriate amount of luteolin (7.2 g) and apigenin (11.2 g) were both dissolved in methanol and transferred into two volumetric flasks of 100 ml separately. So the concentration of them were 72 μg/ml and 112 μg/ml. Then 4 ml of each solution was pipetted accurately into a 10 ml volumetric flask and brought to the volume by methanol to prepare the standard mixture. The standard stock solution contained 28.8 μg/ml of luteolin and 48.8 μg/ml of apigenin. For sample preparation, leaf powder samples (1.0 g) of BA and BD were taken accurately and extracted with 40 ml of 50% aqueous ethanol and 10 ml of 25% HCl solution for an hour at 45ºC. After filtration, the BA leaf extraction was transferred into a 50 ml volumetric flask while the BD leaf extraction was concentrated to 3 ml and transferred to a 5 ml volumetric flask. Both were filled up to the volume by 50% aqueous ethanol. Each solution was filtered through a 0.45 μm filter before injection.

Selection of the wavelength

The absorption peak of luteolin and apigenin standards and the samples prepared for HPLC determination were around 350 nm and 330 nm and 340 nm, respectively. And the optimum separation was monitored at 340 nm. So the HPLC determination of luteolin and apigenin was performed at 340 nm.

Chromatographic analysis method of flavonoids in extracts

HPLC analysis was performed on a Dionex ultimate 3000 HPLC system (Dionex, China) combined with the YMC-Pack ODS-A (5 μm, 250 × 4.6 mm). The optimum separation of HPLC was carried out with a mobile phase composed of acetonitrile and 0.2% phosphoric acid aqueous solution (28:72, v/v) at a flow-rate of 0.8 ml/min. The volume of sample injected was 20 μl and standard solution injected was 10 μl, the detective wavelength and column temperature were set at 340 nm and 30ºC, respectively. The theoretical plate numbers of luteolin and apigenin peaks were no less than 8000 and 10 000, which met the demands of content determination by HPLC.

The identification of luteolin and apigenin peaks was carried out by comparing their retention times of the peaks with that of the standards. The concentration of each component was calculated according to the calibration curves.

RESULTS AND DISCUSSION

Determination of total flavonoids by UV-Vis spectrophotometry

Calibration curve

The calibration curve was drawn with eight standards at concentration ranged from 8.16to 65.64 μg/ml. The regression equations was calculated in the form of y = ax+b, where y and x correspond to the absorbance and

concentration. The regression equation and correlation coefficient was as follows: y = 0.0118 x–0.0071, r = 0.9998.

Optimization of extraction procedures

Flavonoids often dissolve easily in the ethanol or aqueous ethanol because of abundant hydroxyl groups. In the process of extraction, extraction time was also a significant factor. So BD leaf powder was employed as a representative to find out the optimum solvent and extraction time for sample extract. The relationships results presented in Figures 1 and 2. The results showed that flavonoid extraction yields increased among 20% ethanol to 50% ethanol and decreased sharply among 50% ethanol to 95% ethanol. As for the extraction time, flavonoid extraction yields increased and then reach a constant value after 60 min. Thus the optimal extraction parameters were 50% aqueous ethanol and 60 min extraction time.

Validation

The standard solution and BD leaf powder was used for the validation. The absorbance of the standard solution was determined by the UV-vis spectrophotometer for five times to test the precision. Repeatability tests had been carried out with BD leaf for six times. Results were listed in Tables 1 and 2. The RSD were 0.22% (n=5) and 0.63% (n=6), respectively, which indicated a high degree of precision with the UV-vis spectrophotometer and the repeatability of the method. As the stability, the absorbance of BD leaf extraction prepared was measured every 20 min from 0 min to 120 min. The results in Table 3 indicated that the solution were stable in 80 min. The RSD was 1.50% (n=6) in 120 min. In the addition, the recovery experiment was carried out to evaluate the accuracy of the method. Known amount of standard was mixed with BD leaf powder to extract and operate as those mentioned in "Preparation of standard solutions and sample" and "procedures." The absorbance was recorded separately so that standard content can be calculated. Recovery test was performed in six replicates. The standard recoveries ranged from 97.7% to 102.3%. The mean recovery was 99.6% and RSD was 1.81%, confirming the accuracy of this method [Table 4].

TF contents measurement of the samples

One gram of stem and leaf powder samples of BD and BA were taken accurately and operated as "Preparation of standard solutions and sample" mentioned before. Proper amount (1 ml of BD leaves, stems and BA leaves, 2 ml of BA stems) extractions prepared were pipptted into the volumetric flasks of 25 ml and then acted up according to the procedures. Each solution was determined in triplicate. According to the linear regression equation, total flavonoid contents of BD leaves and stems, BA leaves and BA stems can be calculated and presented in Table 5.

HPLC analysis of luteolin and apigenin

Calibration curve

The peak areas (y) of the luteolin and apigenin were measured and plotted against the amount (x) of them in different volume (from 2 μl to 20 μl) after HPLC analysis. The standard curves of luteolin and apigenin were linear

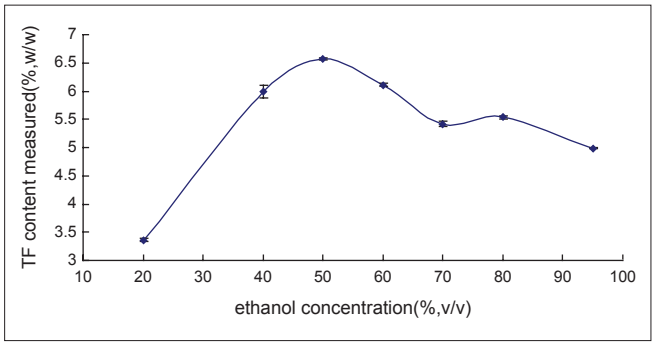

Figure 1: Content measured (in weight percentage per gram of dried sample) of total flavonoids (TF) in different ethanol concentration extract

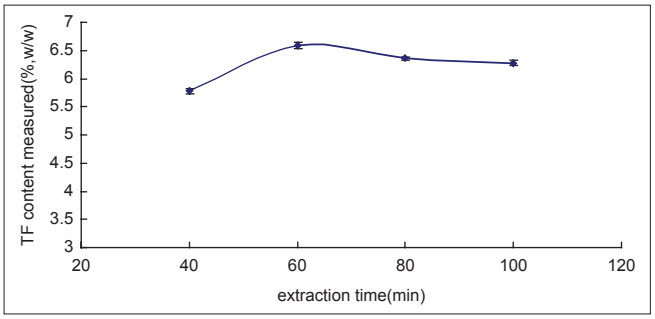

Figure 2: Content measured (in weight percentage per gram of dried sample) of total flavonoids with different extraction time of 40 min, 60 min,80 min and 100 min

Table 1: Precision experiment data of total flavonoids determination in standard solution

Absorbance	1	2	3	4	5	Mean	RSD (%)
	0.610	0.608	0.606	0.608	0.608	0.608±0.002	0.22

Mean absorbance is expressed as mean ± SD (n = 5)

Table 2: Repeatable test results of total flavonoids determination in BD leave

TF content measured (%)	1	2	3	4	5	6	Mean	RSD (%)
	6.57	6.51	6.61	6.50	6.55	6.53	6.55±0.04	0.63

Mean TF content measured is expressed as mean ± SD (n = 6)

Table 3: Stable test results of total flavonoids in 120 min

Time (min)	20	40	60	80	100	120	Mean	RSD (%)
TF content measured (%)	6.50	6.47	6.51	6.48	6.34	6.27	6.43±0.10	1.50

Mean TF content is expressed as mean ± SD (n = 6)

Table 4: Recoveries of total flavonoids in BD leaves

Sample amount (g)	Theoretical content (mg)	Added amount (mg)	Measured amount (mg)	Rescovery (%)	Mean rescovery (%)	RSD (%)
1.0183	66.7	72.2	137.3	97.8		
1.0054	65.9	64.8	132.2	102.3		
0.9987	65.4	66.5	131.4	99.2	99.6±1.8	1.81%
1.0102	66.2	64.8	131.8	101.2		
0.9958	65.2	67.0	130.7	97.7		
1.0021	65.6	64.3	129.3	99.1		

Mean recovery is expressed as mean ± SD (n = 6)

Table 5: Total flavonoids contents of BD leaf, BD stem, BA leaf and BA stem samples

Sample	Flavonoids in sample (mg)
Buddleja davidii leaf	6.55±0.04
Buddleja davidii stem	3.84±0.02
Buddleja albiflora leaf	4.68±0.04
Buddleja albiflora stem	1.85±0.03

Data are expressed as mean ± SD (n = 3)

over the range of 0.0576–0.576 μg and 0.0896~0.896 μg, respectively. The regression equations were y = 86.658 x–0.0377 (r = 0.9999) for luteolin and y = 87.132 x–0.1499 (r = 0.9998) for apigenin. The relative quantity of two flavonoids in the ethanol extracts (μg/g of the extract) was calculated from each equation.

Validation

In HPLC analysis, the mixed standard solution and BA leaf powder was used for the validation. For precision test, the same mixed standard solution was injected and determined for six times. As shown in Table 6, the RSD of precision of luteolin and apigenin were 0.99% and 0.96% (n = 6), respectively. And the repeatability was evaluated by operating BA leaves from preparation to determination simultaneously in six replicates. The RSD of luteolin and apigenin had been presented in Table 7. All the results showed that the precision and repeatability of this method were satisfied. In addition, the sample solution was assayed after 2, 4, 8, 12, 18, and 24 hours to assess the stability of luteolin and apigenin. The test results manifested that the analyte was stable in 24 hours with RSD of 0.64% and 0.61% (n = 6) [Table 8]. Six powder samples were spiked with 1 ml of standard solution containing 0.48 mg/ml of luteolin and 1.02 mg/ml of apigenin, respectively, prior to the extraction. The recoveries for luteolin and apigenin ranged from 95.3%–99.2% to 96.8%–101.6%, while RSD were 1.44% and 1.69%, demonstrating the method possessed a good accuracy [Table 9].

Assay of luteolin and apgenin

The described method was applied to assay the luteolin and apigenin in BA and BD leaf samples. Luteolin and apigenin were well separated under the present chromatographic condition [Figure 3]. Retention times were approximately 18.6 min for luteolin and 33.5 min for apigenin. The concentrations of luteolin and apigenin were calculated according to the calibration curves and presented in Table 10.

DISCUSSION

Relationship between various parts, different plants and total flavonoids content

It was obvious to see a significant relation between medicinal parts and total flavonoids content. Total amount of flavonoids was found to be much higher in leaves than stems. Its content in BD leaves was 1.7 times that in BD stems while the proportion reached 2.5 times in BA. Considering the flavonoids as the main active ingredient of "Diaoyangchen," we deduced the leaves may be the major part of the medicine. The leaves of the plant often fell off when air-dried, so it was very important to collect the fallen leaves.

Furthermore, as different origin plants of "Diaoyangchen," a remarkable difference in total flavonoids content was observed between BD and BA. Comparing the flavonoids content in leaves and stems separately, total flavonoids content of BD leaves was 1.4 times than in BA leaves, and similarly the ratio was 2.1 in stems. It was demonstrated the quality of BD may surpass the quality of BA to a certain extent. However, the plant samples used to test were harvested in different periods. Whether the

Table 6: Precision experiment results of determination of luteolin and apigenin in standard mixture

Standard	Peak area						Mean±SD	RSD (%)
	1	2	3	4	5	6		
Luteolin	25.1714	24.6987	25.1819	25.3073	25.3772	24.9581	25.1158±0.2486	0.99
Apigenin	38.8615	38.5132	39.4978	39.3018	38.9074	39.3667	39.0747±0.3751	0.96

Mean peak areas are expressed as mean ± SD (n = 6)

Table 7: Repeatable test results of contents determination of luteolin and apigenin

Component	Contents determined (μg/g)						Mean	RSD (%)
	1	2	3	4	5	6		
Luteolin	466.8	484.0	464.0	468.8	465.6	474.5	470.6±7.5	1.92
Apigenin	988.6	994.2	982.3	994.8	995.6	1003.6	993.2±7.2	1.25

Mean contents determined are expressed as mean ± SD (n = 6)

Table 8: Stable test results of luteolin and apigenin in BA leaves

Time (h)	Peak area							Mean	RSD (%)
	0	2	4	8	12	18	24		
Luteolin	16.2742	16.2101	16.3367	16.0973	16.3654	16.2752	16.1135	16.2389±0.1039	0.64
Apigenin	34.0533	34.5921	34.1334	34.5433	34.4088	34.4318	34.2076	34.3386±0.2095	0.61

Mean peak areas are expressed as mean ± SD (n = 7)

Table 9: Recoveries of luteolin and apigenin in BA leaves

Sample amount (g)	Theoretical content (μg)	Added amount (μg)	Measured amount (μg)	Rescovery (%)	Mean rescovery (%)	RSD (%)
Luteolin						
1.0298	484.0	480	919.0	95.3		
1.0159	477.5	480	949.9	99.2		
1.0067	473.1	480	913.8	95.9	96.9±1.4	1.44
0.9995	469.8	480	924.0	97.3		
0.9943	467.3	480	914.0	96.5		
1.0102	474.8	480	925.9	97.0		
Apigenin						
1.0298	1021.6	1000	1979.5	97.9		
1.0159	1007.8	1000	2039.1	101.6		
1.0067	998.65	1000	1993.0	99.7	98.8±1. 7	1.69
0.9995	991.5	1000	1964.8	98.7		
0.9943	986.35	1000	1949.2	98.1		
1.0102	1002.1	1000	129.25	96.8		

Mean recoveries *are expressed as mean* ± SD (n = 6)

distinction was related to the harvest time is subject to further study.

Relative contents of luteolin and apigenin in the total flavonoids

Considering the intimate relationship between BD and BA, we still monitored the luteolin and apigenin in BD leaves and stems although no document has been presented about the specific flavonoid compounds in BD. The HPLC analysis showed the presence of the luteolin and apigenin in BA and BD leaves as expected. Another flavonoid, quercetin, reported in the literature as BA flavonoid compound, was not checked out in our extracts because it was too little. The three kinds of flavonoids in stems extracts of BA and BD were also not checked out because we could hardly observe luteolin or apigenin or quercetin in them. Comparing the flavonoids contents in BA leaves, luteolin and apigenin were too low in BD leaves, so the extract had to be concentrated before injected and the contents of them were about one tenth about that in BA leaves. Conversely, the total flavonoids in BD leaves were much higher than BA leaves. The results indicated the the higher content of total flavonoids does not always mean higher specific flavonoid in the medicinal material of different source.

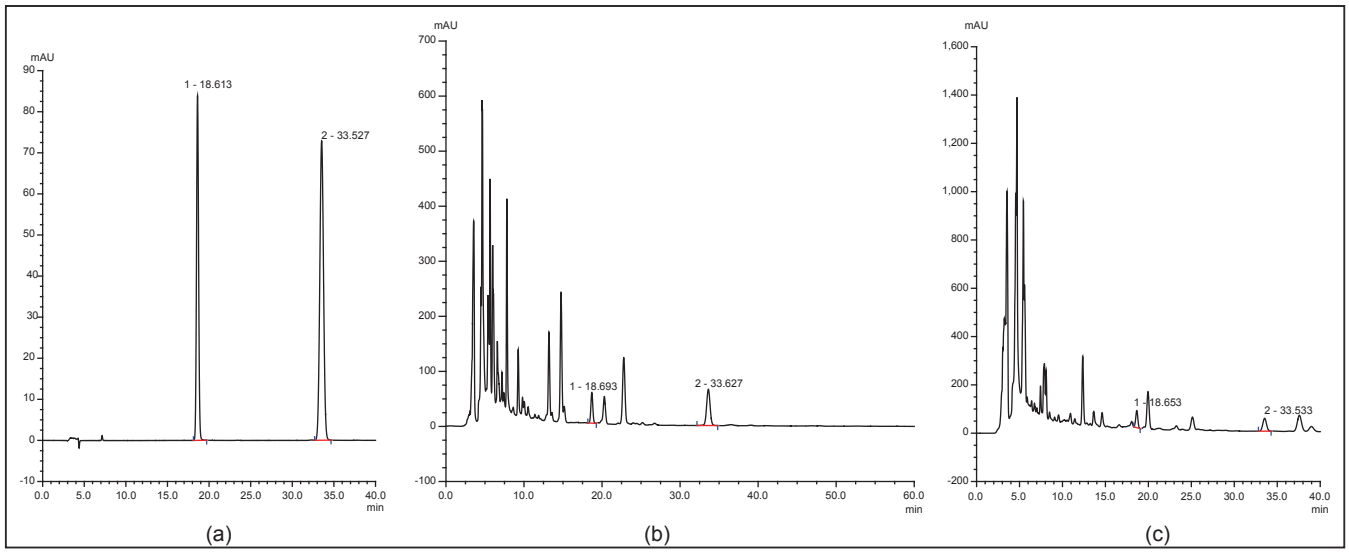

Figure 3: HPLC chromatogram of standands mixtures (a) and BA leaves (b) and BD leaves (c) with the mobile phase (28:72) acetonitrile-0.2% phosphoric acid aqueous solution; the flow rate of mobile phase is 0.8 ml/min, and the detection is 340 nm. 1-luteolin, 2-apigenin

Table 10: Luteolin and apigenin contents of BD leaf, BD stem, BA leaf and BA stem samples

Sample	Luteolin content (μg/g)	Apigenin content (μg/g)
Buddleja davidii leaf	59±2	79±2
Buddleja davidii stem	—	—
Buddleja albiflora leaf	471±3	992±4
Buddleja albiflora stem	—	—

Data are expressed as mean±SD(n=3)
"—" represents flavonoids that have not been checked out

The stability with the solvent in the determination of total flavonoids

During the stability test in total flavonoids assay, some correlation was found between the stability of the specimen under test and the solvent used to dilute the solution after chromogenic reaction. If the water was used to make the volume, the absorbance of solutions under measuring decreased continuously, especially the sample solution extracted by ethanol of high concentration. And precipitation appeared in the bottom of the cuvettes after a certain time, which may caused by the large mass of smaller polar flavonoids. Nevertheless, the phenomenon disappeared by using the same ethanol concentration that extracted the sample powder.

Relevant thinking about the content determination index of medicinal material compositions

In present a single component is often chosen as the index to evaluate the quality of the Chinese traditional medicines in most of the current quality standards. Comparing the results of total flavonoids content and the two key flavonoids contents in the two plant leaves, we think it may not so perfect because a single compound can not reflect the extensive pharmacologic actions. However, a group of substance is too complicated to accurately evaluate the quality of medicines. Therefore, it should be more reasonable to choose more indexes such as total flavonoids and some key flavonoid components in the assessment of the medicines.

CONCLUSION

Buddleja davidii and *Buddleja albiflora* are the two different plant sources of "Diaoyangchen," but the total flavonoids and luteolin and apigenin are quite different in them. If the flavonoids are considered the active ingredients or index components, the quality of leaves are much better than their stems. But the conclusion needs to be further confirmed by pharmacological research.

The rapid and reliable methods have been established for the measurement of total amount of flavonoids and individual flavonoid component quantification based on the UV-Vis spectrophotometer and HPLC. The use of spectroscopic technique in conjunction with the main analytical technique HPLC, is expected to strengthen the flavonoids assay and will be much helpful for the further study of flavonoids in *Buddleja*.

REFERENCES

1. Fang ZX, Zhao H, Zhao JH. Materia Medica of Tujia. Volume 2. Beijing: TCM Science and Technology Publishing House; 2007. p. 756.
2. Liao YH, Peter J. Houghton, J. R. S. Hoult. Novel and known constituents from Buddleja species and their activity against leukocyte eicosanoid generation. Journal of Natural Products 1999;62:1241-5.
3. Zhang HY, Pan JX, Chen YY. New researching progress in

chemical constituents and bioactivities of the Genus Buddleja. World Phytomedicines 1995;10:195-200.

4. Tao L, Huang J, Zhao Y, Li C. Chemical constituents in *Buddleja albiflora*. Zhongguo Zhong Yao Za Zhi 2009;34:3043-6.
5. Zhang HP, Tao L. Studies on the chemical constituents of *Buddleja albiflora*(II). Zhong Yao Cai 2010;33:922-4.
6. Romanová D, Vachálková A, Cipák L, Ovesná Z, Rauko P. Study of antioxidant effect of apigenin, luteolin and quercetin by DNA protective method. Neoplasma 2001;48:104-7.
7. Hirano R, Sasamoto W, Matsumoto A, Itakura H, Igarashi O, Kondo K. Antioxidant ability of various flavonoids against DPPH radicals and LDL oxidation. J Nutr Sci Vitaminol (Tokyo) 2001;47:357-62.
8. Shi HX. Studies on the structures and properties of quercetin and some other flavonoids. Master Thesis, Sichuan University: 2007. p. 23-7.
9. Adriana Basile, Simonetta Giordano, José Antonio López-Sáez, Rosa Castaldo Cobianchi. Antibacterial activity of pure flavonoids isolated from mosses. Phytochemistry 1999; 52: 1479-82.
10. Lee SH, Ryu JK, Lee KY, *et al*. Enhanced anti-tumor effect of combination therapy with gemcitabine and apigenin in pancreatic cancer. Cancer Letters 2008;259:39-49.
11. Liu HL, Jiang WB, Xie MX. Flavonoids: Recent advances as anticancer Drugs. Recent Patents on Anti-Cancer Drug Discovery 2010;5:152-64.
12. Wang HD, Liu AL, Du GH. New progress in the pharmacology of apigenin. Chinese Journal of New Drugs 2008;17:1561-5.
13. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin e-mediated mediator release from human cultured mastcells. Clinical and Experimental Allergy 2000;30:501-8.
14. Arnaldo Willain Filho, Valdir Cechinel Filho, Leonardo Olinger, Márcia Maria de Souza. Quercetin: Further investigation of its antinociceptive properties and mechanisms of action. Archives of Pharmacal Research 2000;30:501-8.

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