Revista Brasileira de Farmacognosia 28 (2018) 151–155

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original article

Optimization of baicalin water extraction process from *Scutellaria baicalensis* (a traditional Chinese medicine) by using orthogonal test and HPLC

Huilin Ni[a,1], Zhiyong Wu[a,1], Ishfaq Muhammad[a], Ziyin Lu[b], Jichang Li[a,*]

[a] *Heilongjiang Key Laboratory for Animal Disease Control and Pharmaceutical Development, College of Veterinary Medicine, Northeast Agricultural University, Xiangfang District, Harbin, PR China*
[b] *Department of Animal Production, College of Life Science Engineering, Shenyang Institute of Technology, Economic Developing District, Fushun, PR China*

ARTICLE INFO

Article history:
Received 22 November 2017
Accepted 7 February 2018
Available online 22 February 2018

Keywords:
Extraction process
Optimization
Baicalin
Orthogonal test
HPLC
Uterus suppositories

ABSTRACT

In this study, we optimized the baicalin water extraction process from *Scutellaria baicalensis* Georgi, Lamiaceae (a traditional Chinese medicine). Orthogonal test design $L9(3)^4$ was used to analyze the optimization of water extraction process of baicalin from *S. baicalensis*. The effect of solid–liquid ratio, extraction time and soaking time on the yield of baicalin were investigated and optimized by orthogonal test. High-performance liquid chromatography was employed for the determination of extraction yield of baicalin. Analysis of variance was carried out to study the effects of the above three factors. The results showed that solid–liquid ratio plays a significant role in attaining maximum extraction yields of baicalin. However, the other two factors had some effect (not statistically significant) on the extraction yield of baicalin. Conclusively, the optimum experimental conditions such as the solid–liquid ratio (1:12), extraction time (30 min) and soaking time (1 h) for the water extraction of baicalin were proposed which can provide the maximum extraction yield of baicalin. In addition, the score based on the content of baicalin and total solid residues yield were used as evaluation indexes for baicalin uterus suppositories evaluation.

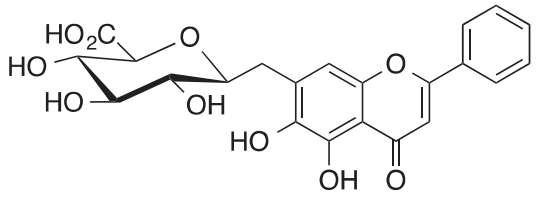
Introduction

Baicalin (baicalein 7-*O*-β-D-glucuronic acid) (**1**) known as huangqin in Chinese traditional medicine, is the principal flavonoid derivative found in the roots of *Scutellaria baicalensis* Georgi, Lamiaceae (*Scutellariae radix*) (Lu et al., 2011). Baicalin have excellent biological actions and become more and more popular in pharmaceutical and food industries, cosmetics, and displays anti-inflammatory (Meng et al., 2009), anticancer activity, effective against bacterial infections and oxidative stress-related diseases (Izui et al., 2016). Previously, different extraction methods such are heat reflux extraction (HRE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) were reported to isolate and purify baicalin (Wang et al., 2010). However, little is known about the optimization of baicalin water extraction process. Endometritus is an inflammatory condition of the uterus common in cows after parturition. It is mostly caused by bacteria and leads to severe economic losses such as culling, treatment cost, reproductive inefficiency, milk discard, and increased risk of antibiotic residues in food products. Antibiotics such as cephalosporins, penicillin or a combination of ampicillin with oxytetracycline or cloxacillin are commonly used for the treatment of endometritus (Klugman et al., 1997). While, plant-derived antibacterial (baicalin) could be a promising alternative to avoid antibacterial resistance. Baicalin has shown synergistic effects with oxytetracycline and tetracycline (Novy et al., 2011) and potent against inflammation. Therefore, traditional Chinese medicine, *i.e.* baicalin uterus suppositories, will be an effective therapy against endometritus. Based on these considerations, we optimized the water extraction process of baicalin uterus suppositories to ensure effective treatment of endometritus and to reduce the risk of antibacterial resistance and drug residues in food, especially in milk. Many previous studies used orthogonal test (OT) for the optimization of analytical extraction procedures. It can save time and lesson the number of assays (Zhao et al., 2011). Therefore, in the present study, OT (a chemometric method) was used to optimize the baicalin water extraction process through high performance liquid chromatography (HPLC), and to evaluate the factors which mainly influence the quantitative yield

* Corresponding author.
E-mail: lijichang@neau.edu.cn (J. Li).
[1] These authors contributed equally to this work and should be considered co-first authors.

https://doi.org/10.1016/j.bjp.2018.02.001

of baicalin. The effects of extraction time, solid–liquid ratio and soaking time on baicalin yield were studied by orthogonal test L9(3)[4]. Baicalin uterus suppositories were evaluated based on baicalin content and total solid residues yield values. In addition, this work will provide a basis for further studies to have a check on the quality control of baicalin uterus suppositories preparation.



1

Materials and methods

Samples and chemicals reagents

Baicalin standard (batch number: Z0271504) were obtained from Chinese veterinary medicine supervision center and the roots of *Scutellaria baicalensis* Georgi, Lamiaceae (batch number: 15062501) were bought from Runhe Chinese medicine processing plant Ltd. (Harbin, China). Methanol and phosphoric acid (HPLC grade) were obtained from Kermel Chemical Reagent Company (Tianjin, China). Deionized water provided by Wahaha Company Ltd. (Hangzhou, China).

Baicalin extraction protocol and sample preparation

The roots of *S. baicalensis* crushed by a grinder (AK-1000A, Wenling, China) into powder form of particle size less than 150 μm and soaked in water for 1 h. It is then heated in water at 100 °C and concentrated to a density of 1.05–1.15 g/cm^3. The extract is placed in a vacuum chamber for drying at 50 °C for 5 h. The extract was ground into powder and 0.1 g of powder dissolved in 25 ml methanol. The solution is ultrasonicated (KQ3200 Kunshan Ultrasonic Instrument Co., Ltd., China) for 30 min and the precipitate is removed by centrifugation. Finally, the solution was analyzed by HPLC.

Orthogonal test design

Orthogonal test was designed to include three factors and three levels to optimize the water extraction process of baicalin including the solid–liquid ratio, soaking time, and extraction time. Table 1 shows orthogonal test L9(3)[4] parameters. Each treatment was performed in triplicates.

HPLC identification and quantification

A High Performance Liquid Chromatography (HPLC) Acquity (Waters Alliance, USA) equipped with a 2998-UV detector (Waters, USA) was used. The system was computer controlled installed with Empower2 software employed for the analysis of data. The chromatographic column (Venusil XBP C18 (L) (5 μm, 250 mm × 4.6 mm), Bonna-Agela Technologies, China) was used for the separation of baicalin. An isocratic flow rate of 1 ml/min maintained through the column at an injection volume of 10 μl. The experiments were performed at a detection wavelength of 280 nm and at a column temperature of 25 °C. Mobile phase consisted of methanol–water–phosphoric acid (ratio 47:53:0.2 (v/v/v)).

Table 1
Factors and levels of orthogonal test.

Levels	Extraction time (min) A	Solid–liquid ratio (w/v) B	Soaking time (min) C
1	30	10	60
2	60	12	90
3	90	14	120

Analysis of data

The experiments were performed three times unless otherwise mentioned. Analysis of data was performed by SPSS (version 17.0, Chicago, USA) software using multivariate analysis of variance (MANOVA). The value of p was chosen <0.05.

Results

Validation of the extraction method

In order to optimize the proposed extraction method, the level of linearity, precision, stability, reproducibility and recovery of experiments were performed. The results showed that the extraction process was fully validated in terms of the above parameters. The chromatograms obtained for baicalin standard substance and *S. baicalensis* are shown in Fig. 1. Baicalin peak appears at a retention time of approximately 12 min.

Linear range and standard curve determination

Baicalin standard substance containing baicalin content 95.9% was precisely weighed (8.0 mg) and dissolved in 10 ml methanol in a brown volumetric flask to obtain stock solution (contain baicalin 767.20 μg/ml). The stock solution was then diluted to the required concentrations (15.34, 38.36, 153.4, 306.88, 767.20 μg/ml) to draw the standard curve under the chromatographic conditions as stated above. Linear regression was applied to calculate the standard curve. The coefficients of determination (R^2) value (0.9999) revealed a good linearity over the selected range for baicalin (15.34–767.20 μg/ml) as shown in Fig. 2.

Precision and stability experiments

The precision of the method was determined for the same standard substance six times. The coefficient of variation (%RSD) value (0.54%) showed (Table 2) that the method is precise. The stability of the optimized extraction process was checked at 0, 2, 4, 8, 12 and 24 h using the same standard substance kept at room temperature. In order to determine the stability, same chromatographic condition was employed as mentioned in "Materials and methods" section. The %RSD value (0.56%) displayed in Table 2 indicates that the samples were in good stability within 24 h.

Reproducibility and recovery of baicalin

The reproducibility of the method was determined for *S. baicalensis* (same batch number). The process was repeated six times and 0.96% RSD value showed that the repeatability of the method was good (Table 2). The recovery of baicalin content was calculated for the same six samples (repeated six times) shown in Table 3. The recovery of baicalin obtained was 0.3069 mg after adding baicalin (0.2612 mg). The recovery rate was calculated by the following formula.

$$\text{Recovery rate}\,(\%) = \frac{(\text{detected value} - \text{added value})}{\text{original value}} \times 100$$

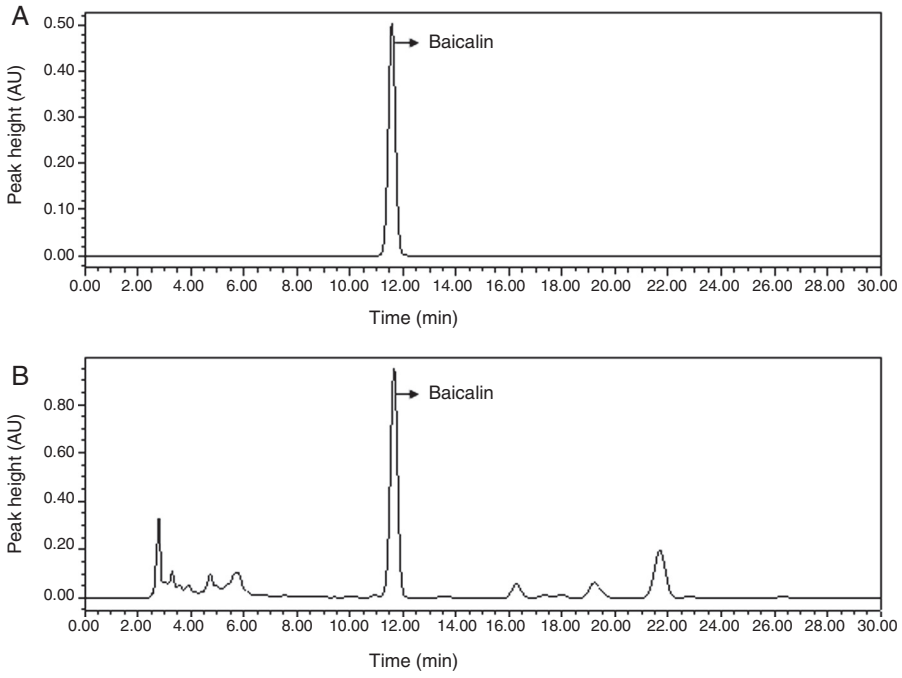


Fig. 1. HPLC chromatograms of baicalin. (A) Standard solution of baicalin and (B) sample of *Scutellaria baicalensis* extract (whereas $n = 3$).

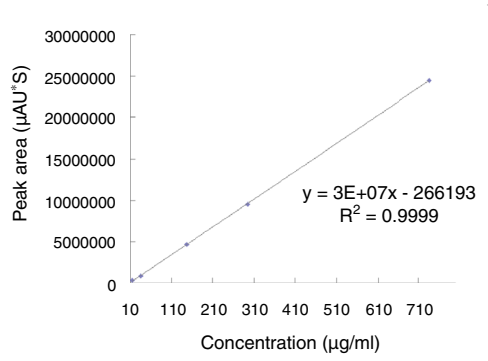


Fig. 2. Standard curve of baicalin. Horizontal axis represents concentration of baicalin in μg/ml and vertical axis represents peak area (μAU (absorption unit) × S (time in sec)).

Table 2
Precision and stability experiments.

	Precision	Stability	Reproducibility
Peak area (μAU × S)	4,729,420 ± 255	4,734,606 ± 265	17,692,820 ± 486
RSD (%)	0.54	0.56	0.96

Note: Results indicated mean ± SD, where $n = 6$.

The recovery of the sample was 99.3% and the RSD was 1.55%, which indicated that the recovery rate of the method was good and the method was feasible (Table 3).

Effect of solid–liquid ratio, extraction time and soaking time on the yield of baicalin

In this study, we investigated the effect of solid–liquid ratio, extraction time and soaking time on the extraction yield of baicalin. Fig. 3B displays the effect of solid–liquid ratio on baicalin content. Solid–liquid ratio was fixed at 1:6, 1:8, 1:10, 1:12 and 1:14. It has been noted that at 1:10–1:14, the extraction yield of baicalin increased and reached a maximum value (32.7 mg/g) when solid/liquid ratio was 1:12. The yield of baicalin decreased after solid–liquid ratio exceeded 1:12. The results showed that the appropriate solid–liquid ratio was in between 1:10 and 1:14, therefore, the solid–liquid ratio (1:10, 1:12 and 1:14) was included in the orthogonal test. The effect of extraction time and soaking time on extraction yield of baicalin from *S. baicalensis* are shown in Fig. 3A and C. All other extraction conditions (*e.g.* soaking time and solid–liquid ratio) were fixed at 1 h and 1:12 for investigation of extraction time effect on baicalin yield. The results revealed that the maximum extraction yield of baicalin was obtained at 60 min (Fig. 3A). Similarly, the effect of soaking time on the extraction yield of baicalin is shown in Fig. 3C. The results showed that the appropriate soaking time was between 30 and 90 min, therefore, the soaking times (30, 60 and 90 min) were included in the orthogonal test.

Optimization of the extraction parameters

The orthogonal test was used to optimize the operating conditions for the extraction of baicalin which is a scientific and systematic method. Orthogonal test has many advantages such as to reduce the testing treatments and to analyze scientific results. In the present study, all the selected factors were evaluated using an orthogonal $L9(3)^4$ test design. Orthogonal test results, K and R values are displayed in Table 4, and analysis of variance is shown in Table 5. It has been noted that the mean extraction yields of baicalin was good at A1 (extraction time; 30 min), B2 (solid–liquid ratio; 12) and C1 (60 min). In addition, analysis of variance showed that solid–liquid ratio was the most important factor in extraction process, and other factors (soaking time and extraction time) have non-significant interactions with extraction yield of baicalin. It can be seen from Table 4 that the influence level of extracting conditions on baicalin yield varies in order; B > C > A. Total solid yield was calculated by the following formula.

$$\text{Total solid yield}\,(\%) = \frac{(M - m)}{W} \times 100\%$$

where M is the weight (g) of the total solid residues after extraction, m is the weight (g) of evaporating dish; and W is the weight of the sample (root of *S. baicalensis*). The content of baicalin and total solid residues yield were used as evaluation indexes for baicalin

Table 3
Result of recovery of baicalin in medicinal materials.

No	Original (mg)	Added (mg)	Detected (mg)	Recovery (%)	Mean value (%)	RSD (%)
1	0.3069	0.2612	0.5638	98.35		
2	0.3069	0.2612	0.5682	100.04		
3	0.3069	0.2612	0.5678	99.89	99.30	1.55
4	0.3069	0.2612	0.5598	96.82		
5	0.3069	0.2612	0.5714	101.26		
6	0.3069	0.2612	0.5667	99.46		

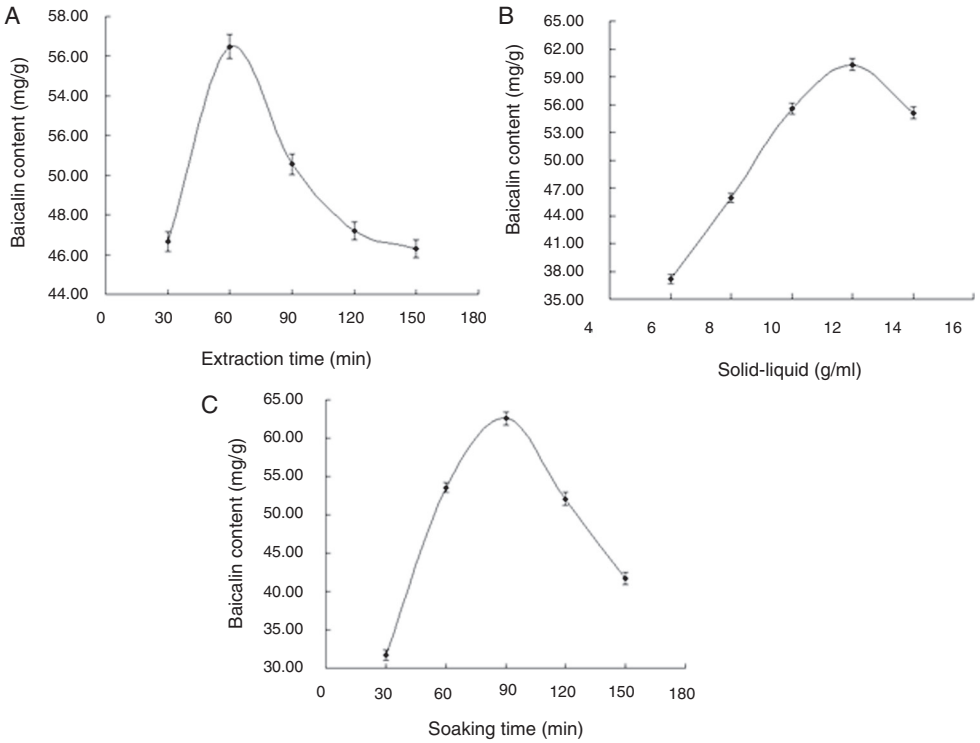


Fig. 3. The effect of (A) extraction time, (B) solid–liquid ratio, and (C) soaking time on baicalin content (whereas $n = 3$).

Table 4
Results of L9(3^4) orthogonal test.

Level	A	B	C	Total baicalin (mg)	Total solid yield (%)	Overall score
1	1	1	1	57.35	33.91	97.52
2	1	2	2	55.45	35.57	96.55
3	1	3	3	47.65	34.70	87.03
4	2	1	2	40.9	34.17	77.66
5	2	2	3	52.1	36.43	93.16
6	2	3	1	52.45	36.96	94.02
7	3	1	3	35.35	29.57	67.15
8	3	2	1	55.4	36.00	96.84
9	3	3	2	57.35	36.70	99.78
K_1	281.10	242.33	288.38			
K_2	264.84	286.55	273.99			
K_3	263.77	280.83	247.34			
R	17.23	44.22	41.04			

uterus suppositories, and the calculated score was used for the evaluation of uterus suppositories. Most importantly, the relationship between the solids content yield and the baicalin content is crucial for the purpose of calculating the index in relation to the uterus suppositories evaluation. The solid content may contain other flavones except baicalin as obvious from Fig. 1B. While, baicalin is the main content of uterus suppositories, therefore to evaluate uterus suppositories in terms of quality, baicalin content should be determined. The overall score (shown in Table 4) was calculated by the equation:

$$\text{Overall score} = \left\{100 \times \left(\frac{\text{baicalin content}}{\text{maximum baicalin content}}\right) \times 0.7\right\} + \left\{100 \times \left(\frac{\text{total solid residues yield}}{\text{maximum total solid residues yield}}\right) \times 0.3\right\}$$

whereas 0.3 is the weight coefficient of solid yield rate and 0.7 is the weight coefficient of baicalin content.

Table 5
Analysis of variance.

Factor	Sum of squares	Freedom	Mean square error	*F*	*p*
A	727.009	2	363.505	0.214	[b]
B	33.100	2	16.550	19.209	[a]
C	64.410	2	32.205	8.468	[b]
Error	126.724	2	63.362		

Note: $F(0.1) = 9$, $F(0.05) = 19$.
[a] $p < 0.05$
[b] $p > 0.05$.

Table 6
Optimum scale-up test.

	R1	R2	R3
Scutellaria baicalensis Georgi (g)	500	500	500
Baicalin content (mg/g)	62.32	60.29	59.68
Total solid yield (%)	32.53	33.40	33.25

Optimization of process amplification

In order to investigate the stability of the extraction process, the optimum conditions according to the above results were chosen (extraction time, 30 min; solid–liquid ratio, 1:12 and soaking time, 60 min) and the yield of baicalin and total solid residues yield were calculated. The results (Table 6) are stable and feasible at 50 times more of the sample tested under the above optimum extracting conditions which confirmed that the optimized extraction process is stable and precise.

Discussion

The optimization for accurate, sensitive and reliable inexpensive methods is very important in the development of highly accurate methods (Elerath and Pecht, 2009). Our data revealed that the method is validated in terms of the level of linearity, stability, precision, recovery and reproducibility of experiments. The level of linearity is checked by linear regression model as stated in previous reports. Previous reports demonstrated that extraction time and extraction temperature are the major factors in the optimization of an extraction process. Additionally, several factors were taken into consideration while optimizing an extraction method. Among these factors, to choose a good, easily available and cheap extraction solvent is a fundamental factor (Ekberg et al., 2008). Some researcher used ethanol for extraction of flavonoids from *Scutellariae radix* (Song and Meng, 2010). In this study, we chose water as an extraction solvent because of its easy availability and cheap in price than other solvents. In our study, the effects of three experimental factors such as extraction time, solid–liquid ratio and soaking time on baicalin yield from the root of *S. baicalensis* were studied by orthogonal test $L9(3)^4$ design. Orthogonal test is a scientific method allowed to select the best experimental conditions for water extraction process of the substance (Kong et al., 2013). Previous reports also demonstrated that orthogonal test is a concise chemometric test with good analysis of results and reduce testing treatment amounts. A study reported the isolation and purification of baicalin along with wogonin and oroxylin A from the medicinal plant *S. baicalensis* and obtained good peak resolution for the target compounds (Li et al., 2014). Similarly, we also obtained good peak resolution for baicalin. The chromatogram for baicalin as standard substance and *S. baicalensis* extract appears at a retention time of 12 min. Furthermore, we verified the stability of the optimized extraction process by choosing the optimum conditions such as extraction time, 30 min; solid–liquid ratio, 1:12 and soaking time, 60 min which confirmed that the optimized extraction process is stable and precise.

Conclusion

In summary, orthogonal test $L9(3)^4$ results revealed the optimum extracting conditions for baicalin isolation including solid–liquid ratio (1:12), extraction time (30 min) and soaking time (1 h). The results showed that the extraction process was reliable, stable, inexpensive and feasible. Total solid residues yield and baicalin content were used as evaluation indexes for the uterus suppositories preparation process to ensure the standard and efficacy of baicalin uterus suppositories and to have a check-up on the quality of industrial production and application.

Ethical statement

All the experiments protocols were performed in accordance with guidelines and approved protocols of Northeast Agricultural University.

Authors contribution

JL designed and supervised experiments; HN and ZW performed experiments; ZL provided help during experiments, and IM contributed to the writing of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

This study was supported by the Natural Science Fund of Heilongjiang Province (grant C201424).

References

Ekberg, C., Fermvik, A., Retegan, T., Skarnemark, G., Foreman, M.R.S., Hudson, M.J., Englund, S., Nilsson, M., 2008. An overview and historical look back at the solvent extraction using nitrogen donor ligands to extract and separate An(III) from Ln(III). Radiochim. Acta 96, 225–233.

Elerath, J., Pecht, M., 2009. A highly accurate method for assessing reliability of redundant arrays of inexpensive disks (RAID). IEEE Trans. Comput. 58, 289–299.

Izui, S., Sekine, S., Maeda, K., Kuboniwa, M., Takada, A., Amano, A., Nagata, H., 2016. Antibacterial activity of curcumin againsti periodontopathic bacteria. J. Periodontol. 87, 83–90.

Klugman, K., Goldstein, F., Kohno, S., Baquero, F., 1997. The role of fourth-generation cephalosporins in the treatment of infections caused by penicillin-resistant streptococci. Clin. Microbiol. Infect. 3, s48–s60.

Kong, F., Bi, Y., Yan, C., Zeng, Z., 2013. Orthogonal test design for optimization of the ultrasonic extraction of naringin from *Citrus grandis tomentosa*. J. Med. Plants Res. 7, 720–726.

Li, T., Feng, Z., Yao, M., Liao, Q., Zhao, Z., Zhang, L., 2014. Comparative pharmacokinetic and tissue distribution study of baicalin, baicalein, wogonoside, wogonin and oroxylin-A after oral administration of component compatibility of Sht and total flavonoids fractions of *Radix Scutellariae* to rats. Anal. Methods UK 6, 5799–5807.

Lu, Y., Joerger, R., Wu, C., 2011. Study of the chemical composition and antimicrobial activities of ethanolic extracts from roots of *Scutellaria baicalensis* Georgi. J. Agric. Food Chem. 59, 10934–10942.

Meng, W., Xiaoliang, R., Xiumei, G., Vincieri, F.F., Bilia, A.R., 2009. Stability of active ingredients of traditional Chinese medicine (TCM). Nat. Prod. Commun. 4, 1761–1776.

Novy, P., Urban, J., Leuner, O., Vadlejch, J., Kokoska, L., 2011. In vitro synergistic effects of baicalin with oxytetracycline and tetracycline against *Staphylococcus aureus*. J. Antimicrob. Chemother. 66, 1298–1300.

Song, D.G., Meng, Q.G., 2010. Different sources of ethanol extract of *Scutellaria* and anti-inflammatory effect of aqueous extract comparative study. Chin. Arch. Tradit. Chin. Med. 9, 1852–1854.

Wang, L., Qin, P., Hu, Y., 2010. Study on the microwave-assisted extraction of polyphenols from tea. Front. Chem. Eng. China 4, 307–313.

Zhao, Y., Cimpoia, R., Liu, Z., Guiot, S.R., 2011. Orthogonal optimization of *Carboxydothermus hydrogenoformans* culture medium for hydrogen production from carbon monoxide by biological water-gas shift reaction. Int. J. Hydrogen Energy 36, 10655–10665.