Extraction of Oleanolic Acid from *Clematis mandshurica* and Protective Effect of the Acid on Neural Function and Early Brain Injury after Subarachnoid Haemorrhage

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ABSTRACT

This paper aims to design a simple and rapid way to extract and determination of oleanolic acid from *Clematis mandshurica* Rupr. (*C. mandshurica*), and to disclose the protective effect of the acid on neural function and early brain injury after subarachnoid haemorrhage. To this end, an orthogonal test was performed to examine the effects of ultrasonic-assisted extraction (UAE) variables (i.e. liquid-material ration, time, temperature and frequency) on the extraction of oleanolic acid from *C. mandshurica*. Then, the oleanolic acid from *C. mandshurica* was quantified and analysed by liquid chromatography with photodiode array detection (LC-DAD), and the fraction of oleanolic acid was collected by automatic fraction collector. Finally, the author investigated how oleanolic acid influences the neural function and early brain injury after subarachnoid haemorrhage. Through the analysis, the highest oleanolic acid yield of 0.7192% was obtained with the extraction temperature of 70°C, extraction frequency of 90kHz, extraction time of 10min and liquid-material ratio of 30:1. Meanwhile, significant improvement of neural function was observed in the oleanolic acid group of 20mg/kg, and the model group suffered from much more serious neural function injuries than the sham-operated group. This means the oleanolic acid group of 20mg/kg can effectively reduce the cerebral vascular permeability caused by subarachnoid haemorrhage. This research reveals the protective mechanism of oleanolic acid on protection against early brain injury after subarachnoid haemorrhage in rats.

Key Words: Neural Function, Brain Injury, *Clematis mandshurica*, Oleanolic Acid, Orthogonal Test, Liquid Chromatography with Photodiode Array Detection (LC-DAD)

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Introduction

Clematis mandshurica Rupr. (*C. mandshurica*) is a species of Clematis growing in North-eastern China. It has officially listed in the Pharmacopoeia of the People's Republic of China (PPRC) as an important herbal medicine (Pharmacopoeia Commission of the Ministry of Health of the PRC, 2015). Most of the pharmacological effects of *C. mandshurica* originate from its primary active component, the oleanolic acid, such as the treatment of rheumatic arthralgia, limb numbness, difficulty in flexion and extension, inflammation and neuro damages (Córdova *et al*., 2014; Zhao *et al*., 2008). The structure of oleanolic acid is illustrated in Figure 1.

It is of great interest to find a reasonable method to extract oleanolic acid from *C. mandshurica*. Chemical synthesis is a potential way, but its yield is too low and processes are too complex. In most cases, oleanolic acid is extracted from *C. mandshurica* through conventional means like macro-porous resin extraction, Soxhlet

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extraction and reflux extraction (Hao *et al*., 2014; Yang *et al*., 2006). Unfortunately, all of these methods require lots of time and labour and consume many toxic organic solvents, which are costly to dispose of after extraction. Against this backdrop, it is meaningful to develop a new environmental-friendly extraction method that can be scaled up for commercial production. One of the viable options is ultrasonic-assisted extraction (UAE). Capable of enhancing yield and avoiding thermal damage, the UAE has been extensively applied to extract natural products and improve solvent extraction (Chen *et al*., 2017; Chen *et al*., 2018; Hilbig *et al*., 2018). The excellent performance is attributable to the desirable solvent penetration into plant materials, a mechanical effect of cavitation bubble collapse (Hsieh *et al*., 2014; Paniwnyk *et al*., 2009; Zhang *et al*., 2018). In general, the UAE is a highly efficient method with reduced solvent- and time-consumption.

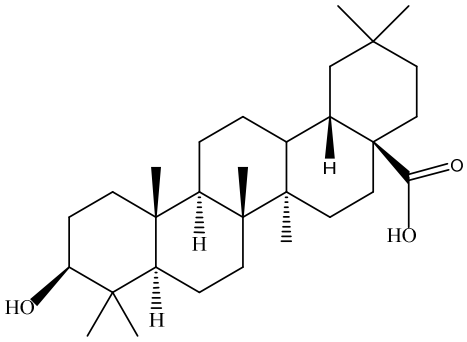

Figure 1. Chemical structure of oleanolic acid in *C. mandshurica*

This paper aims to design a simple and rapid way to extract and determination of oleanolic acid from *C. mandshurica*, and to disclose the protective effect of the acid on neural function and early brain injury after subarachnoid haemorrhage. To this end, an orthogonal test was performed to examine the effects of UAE process variables (i.e. liquid-material ration, time, temperature and frequency) on the extraction of oleanolic acid from *C. mandshurica*. Then, the oleanolic acid from *C. mandshurica* was quantified and analysed by liquid chromatography with photodiode array detection (LC-DAD), and the fraction of oleanolic acid was collected by automatic fraction collector. This is the first report to combine the UAE with the LC-DAD for the extraction and quantification of oleanolic acid from *C. mandshurica.* Finally, the author investigated how oleanolic acid influences the neural function and early brain injury after subarachnoid haemorrhage. In this way, this research reveals the protective mechanism of oleanolic acid on protection against early brain injury after subarachnoid haemorrhage in rats.

Methods

Materials, standards and reagents

The roots of biennial *C. mandshurica* were harvested from the Medicinal Plant Farm of Jilin Agricultural University (Jilin, China), and identified by Dr. Limin Yang (College of Chinese Medicinal Materials, Jilin Agricultural University). The roots were ground into near-homogeneous herbal powder and then sieved through 40-mesh screen (0.42 mm). The sieved powder was dried at 60°C until reaching a constant weight and blended well before use. The standards for oleanolic acid, whose purity is above 98% w/w, were purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). The analytical grade ethanol was purchased from Beijing Chemical Works (Beijing, China). The HPLC-grade methanol was purchased from Fisher Chemical (US). The double-distilled water was prepared in our lab.

UAE

The UAE was conducted in an ultrasonic bath (DL-800E, Zhi Xin instrument Co., Ltd., China). A 1.0g powder sample was placed in a volumetric flask and added with ethanol and hydrochloric acid (9:1). After the extraction, the filtrate and the retentate were separated by suction filtration. Then, the filtrate was filtered by a 0.45μm filter for further experiments.

Immersion extraction

After UAE optimization, the conventional immersion extraction was carried out to serve as a contrast. A 1.0g *C. mandshurica* powder was mixed with 60mL extraction solution. Then, the mixture was placed in a water bath at 70°C for 10min and 24h, respectively. After extraction, the filtrate and the retentate were separated by suction filtration. Then, the filtrate was filtered by a 0.45μm filter for further experiments.

Standard preparation

The reference substance of oleanolic acid was weighed accurately and dissolved in methanol to prepare a standard stock solution. This solution was stored in a refrigerator at 4°C. Before

experiments, the solution was diluted with methanol to obtain calibration solutions, and quantified through external calibration. The calibration curve was constructed by plotting the peak area (y) versus the standard analyte concentration (x, μg): y=528.999287×x+12.36802, R^2=0.99903.

UAE condition optimization

The effects of UAE extraction variables on the yield of oleanolic acid from *C. mandshurica* were investigated through single-factor experiments. The variables include liquid-material ratio (5:1~50:1mL/g), extraction time (10~80min), extraction temperature (20~80°C), and extraction frequency (30, 50, 70 and 90kHz). Next, an orthogonal test was designed to optimize the extraction variables according to the results of the above experiments.

Analysis of oleanolic acid in the extract

The oleanolic acid from *C. mandshurica* was quantified by rapid resolution LC-DAD on an Agilent 1260 system (Agilent Technologies, US). The system consists of a quaternary pump, a degasser, an auto-sampler, a DAD detector and an automatic fraction collector and a thermostatic column compartment.

The extract was analysed by LC using an Agilent HC-C_{18} chromatographic column (4.6mm× 250mm I.D., 5.0μm) (Agilent Technologies, US) at 25°C. The mobile phase includes 85% methanol and 0.4% ammonium acetate aqueous solution, and the flow rate was kept constant at 1.0mL/min. The peaks of interest were monitored at 210nm by the DAD detector. 10μL sample solution was directly injected into the chromatographic column. The chromatographic peak of oleanolic acid was confirmed by comparing their retention time with the reference standard, and the fraction of oleanolic acid was collected by the automatic fraction collector. To obtain oleanolic acid groups (10 and 20mg/kg), the fraction was dried by a rotary evaporator, and then was accurately weighed and dissolved in water.

Experimental animals and grouping

72 male pathogen-free Sprague Dawley rats (weight: 300~360g each) were purchased from Shenyang Pharmaceutical University. These rats were feed with free food and water in an incubator at 18~22℃ with 12h/12h light and dark cycles. The animal experiments were carried out according to the *Guide for the Care and Use of Laboratory Animals* (National Research Council), aiming to reduce the pain and number of the animals.

The 72 rats were divided evenly into four groups: sham-operated, model, and oleanolic acid groups (10 and 20mg/kg). Before operation, these rats were only feed with no food but water. Within 1 hour after the model establishment, the rats were administered with drugs. After 24h, the rats were given neurological scores concerning spontaneous activity, quadrilateral symmetry, arm stretching, climbing, touch reaction and tentacles reaction, and given the scores from 0-3, the total point was sum of them, and the low total points indicate that the Neurological damage is more serious.

Modelling and determination of cerebral vascular permeability

After the single intraperitoneal injection of 10% chloral hydrate, the rats were cut open longitudinally in the middle of the neck, followed by the separation of the common, external and internal carotid arteries. Specifically, the external carotid artery was distended, while the common and internal carotid arteries were temporary blocked by operation line. The line of fish was inserted into the external carotid through the small orifice of the external carotid artery and reached the branch of anterior cerebral artery and the central artery in the rat brain. Then, the arteries were broken by the line to simulate the subarachnoid haemorrhage, while the arteries were not break in the sham-operated group (Bederson *et al*., 1995).

2% Evans blue (EB) solution (5mL/kg) were intravenously injected in the tail and systematically circulated for 1h. the brain tissue were taken out after the perfusion of saline. Then, the left brain, right brain and epencephalon were separated and weighed individually. The tissues were put into 10mL/g methanamide and lixiviated for 24h in an incubator at 60℃. The filtrate was later filtered and absorbance was measured at 620nm by a microplate reader.

Results and Discussion

Effects of extraction variables on the yield of oleanolic acid

Liquid-material ratio

The impact of liquid-material ratio on the yield of oleanolic acid (Figure 2A) was investigated under

ultrasonic irradiation at the extraction time of 10min, extraction frequency of 70kHz and extraction temperature of 70°C. In general, the target components are dissolved more effectively by a larger solvent volume, resulting in enhanced yield of oleanolic acid (Dong *et al*., 2010; Li *et al*., 2005; Valachovic *et al*., 2001). As shown in Figure 2A, the yield increased significantly increased as the liquid-material ratio grew from 5mL/g to 30mL/g; any further growth of the ratio could not induce obvious changes to the yield. Thus, the volume of 30mL is sufficient for extraction.

Extraction time

The impact of extraction time on the yield of oleanolic acid (Figure 2B) was studied by changing the time from 1min to 30min. The other extraction variables are as follows: the extraction temperature was set to 70°C, the extraction frequency to 70kHz, and the liquid-material ratio to 20:1. As shown in Figure 2B, the yield of oleanolic acid soared when the extraction time increased from 1min to 10min but remained basically unchanged when the time changed from 10min to 30min. This is because more target substances were destroyed over the time (Shimaoka *et al*., 1975; Sun *et al*., 2010). Therefore, the extraction time should not last more than 10min.

Extraction temperature

The impact of extraction temperature on the yield of oleanolic acid (Figure 2C) was explored at six different temperatures (20, 30, 40, 50, 60, 70 and 80°C). The other extraction variables are as follows: the extraction time was set to 10min, the extraction frequency to 70kHz, and the liquid-material ratio to 20:1. Figure 2C demonstrates an obvious influence of temperature increase on the extraction efficiency. The yield of oleanolic acid was on the rise when the temperature grew from 20 to 60°C, but declined when the latter continued to increase from 60 to 80°C.

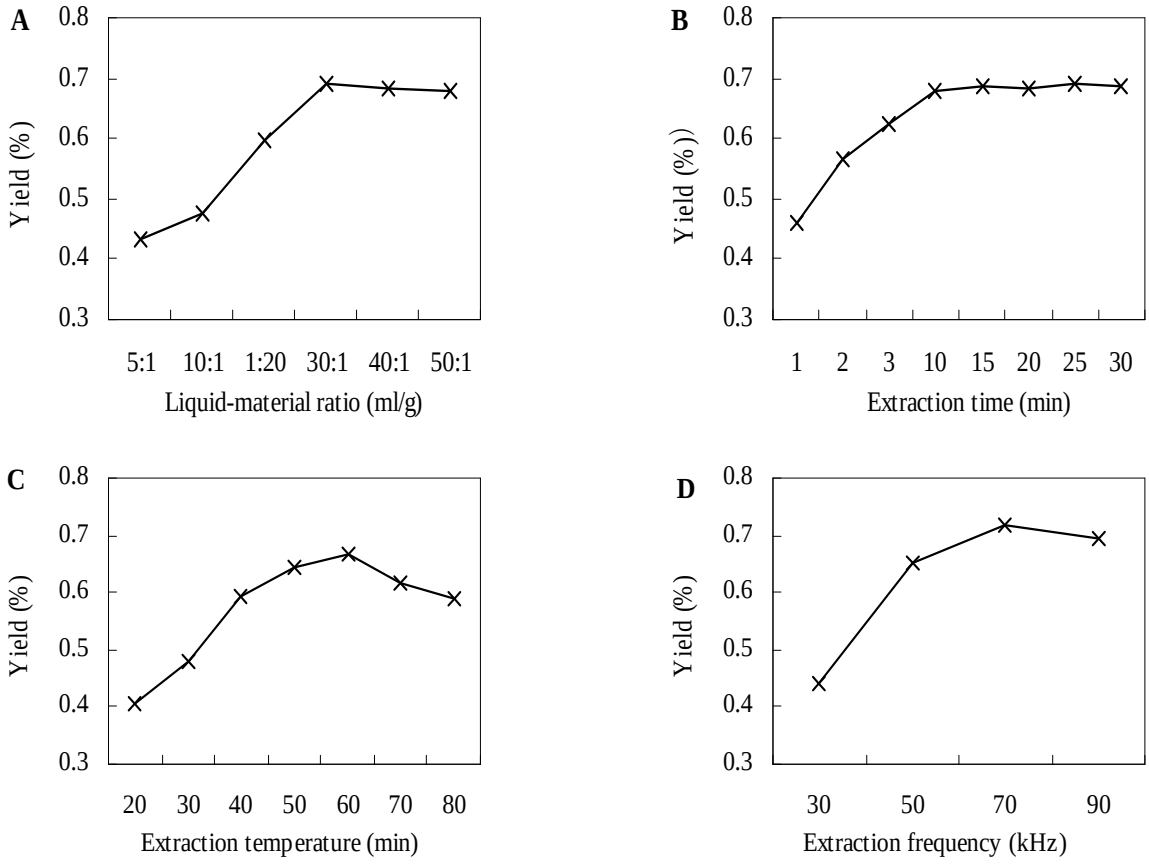

Figure 2. The effects of liquid-material ratio (A), extraction time (B), extraction temperature (C) and extraction frequency (D) on the yield of oleanolic acid extracted from *C. mandshurica*

This trend can be explained as follows. The temperature rise of the extraction medium enhances the diffusivity of the solvent into the cells and promote the desorption and solubility of the target compounds of the cells, leading to the dissolution of the components (Dong *et al*., 2010; Xia *et al*., 2012). However, when the extraction temperature surpasses a certain threshold (50 to 60°C), the yield will start to decrease because fewer acoustic cavitation bubbles are created by the ultrasound and the thermal degradation of oleanolic acid. In addition, the growing extraction temperature may accelerate the solvent volatilization, push up the energy cost and promote the extraction of impurities (Zhang *et al*., 2009). Thus, the highest yield can be obtained at the extraction temperature of 60°C.

Extraction Frequency

The impact of extraction frequency on the yield of oleanolic acid (Figure 3D) was investigated under ultrasonic irradiation at the extraction time of 10min, extraction temperature of 70°C, and liquid-material ratio of 20:1. It can be seen from Figure 2D that the maximum yield appeared at the frequency of 70kHz; the yield did not increase much with any further increase of the frequency.

Optimization by orthogonal test and further discussion

Considering the effects of the above variables on the UAE, it is critical to optimize the experimental conditions. Therefore, an orthogonal test was designed for the UAE of oleanolic acid based on results from the above single factor experiments, aiming to optimize the combination of variables. The optimization targets four variables: liquid-material ratio, extraction time, extraction temperature and extraction frequency (Table 1). A total of 16 tests are needed to examine the four variables at three levels each. The tests were performed in a random sequence to ensure the validity of the results. All tests were carried out in in triplicate.

Table 1. Variables and levels of the $L_9(3^4)$

Level	A The ratio of liquid to materials	B Extraction time (min)	C Extraction temperature (℃)	D Extraction frequency (KHz)
1	20:1	5	50	50
2	30:1	10	60	70
3	40:1	15	70	90

The mean yield for the variables at each level was calculated and recorded in Table 2. The data on oleanolic acid extraction (Table 2) were analysed by DPS 12.01 (Hangzhou, China) to evaluate the effect of each variable on the optimization criteria. According to the *R* values, the four variables were ranked as extraction temperature>extraction frequency>extraction time > liquid-material ratio by their impact on the yield of oleanolic acid. The total score of each variable on three levels reveals the variation in oleanolic acid yield with the variable levels. All four variables exhibited major impacts on the yield, among which the extraction temperature boasted the greatest impact.

Table 2 Results of $L_9(3^4)$ orthogonal test

No.	Factors				Extraction yield (%)
	A	B	C	D	
1	1	1	1	1	0.4353
2	1	2	2	2	0.6104
3	1	3	3	3	0.7011
4	2	1	2	3	0.6044
5	2	2	3	1	0.6947
6	2	3	1	2	0.5865
7	3	1	3	2	0.6972
8	3	2	1	3	0.6104
9	3	3	2	1	0.5518
K1	1.7468	1.7369	1.6322	1.6818	-
K2	1.8856	1.9155	1.7666	1.8941	-
K3	1.8594	1.8394	2.093	1.9159	-
k1	0.5823	0.579	0.5441	0.5606	-
k2	0.6285	0.6385	0.5889	0.6314	-
k3	0.6198	0.6131	0.6977	0.6386	-
R	0.0462	0.0595	0.1536	0.078	-

Table 3. ANOVA table for the UAE of oleanolic acid by $OA_9(3^4)$

Source of variance	Sum of square	df	Mean square	*F*-value	*Signi-ficant*
A	0.0109	2	0.0054	30.3642	*
B	0.0161	2	0.008	44.8727	*
C	0.1123	2	0.0561	313.3535	**
D	0.0334	2	0.0167	93.2943	*
Error	0.0032	18	0.0002	-	-
Total	0.0109	-	-	-	-

*df:Degree of freedom; F0.01 (2,18) = 99.4 F0.05 (2,18) = 19.4; A: Liquid-materials ratio; B: Extraction time; C: Extraction temperature; D: Extraction frequency; **: p < 0.01 (F > F0.01 (2,18)); *: p < 0.05 (F > F0.05 (2,18))*

Through extreme difference analysis, the optimal extraction conditions were concluded as $C_3D_3B_2A_2$: the extraction temperature of 70°C, extraction frequency of 90kHz, extraction time of 10min and liquid-material ratio of 20:1. Although this combination was not included in the orthogonal test, the extraction temperature, extraction time and liquid-material ratio were on the optimal levels in the fifth test. Thus, the optimal conditions of $C_3D_3B_2A_2$ were tested again. In the test, 1.0g sample was extracted and the oleanolic acid yields were 0.7214%, 0.712% and 0.7242%, respectively.

Comparison between UAE and immersion extraction

Table 4 compares the results obtained by the UAE and immersion extraction. After 10min extraction, the oleanolic acid yield of the UAE at 70°C with 30mL extraction solution and liquid-material ratio of 30:1 was 0.7192%, about 3.9 times higher than that by immersion extraction (0.1826%) at 70°C with 60mL extraction solution. It was not until 24h that the immersion extraction reached the same level of recovery (95.23%). Thus, the UAE is much more efficient in time, solvent and energy than immersion extraction. The high efficiency may be ascribed to the ultrasound destruction of the cells of *C. mandshurica*. The broken cells facilitate the dissolution of oleanolic acid in the solvent. Suffice it to say that the UAE is a suitable way to extract oleanolic acid from *C. mandshurica*.

Table 4 Comparison of UAE and immersion extraction.

Extraction Method	Ultrasonic-Assisted Extraction	Immersion Extraction	
Extraction temperature (°C)	70	70	70
extraction frequency	90KHz	-	-
Extraction time (h)	2/3	2/3	24
Ratio of liquid to materials (mL/g)	30:1	60:1	60:1
Oleanolic acid yield(%)	0.7192	0.1826	0.7145

Table 5 Effects of oleanolic acid on neural function and brain EB content in rats after subarachnoid haemorrhage

Group	Neurological function score (Scores)	Evans blue solution(μg/kg)		
		Left brain	Right brain	epencephalon
Sham group	19.8	0.75	0.82	0.62
Model	7.75###	1.93###	1.95###	1.78###
10 mg/kg of oleanolic acid	9.37	1.61	1.58	1.47
20mg/kg of oleanolic acid	13.1**	1.33**	1.29**	1.19**

*### P < 0.001 vs sham group; **P < 0.01 vs model group*

Effects of oleanolic acid on neural function

Compared with sham-operated group, the model group suffered from serious injury of neural function (P<0.001); significant improvement of neural function was observed in the oleanolic acid group of 20mg/kg (P<0.01), but not in the oleanolic acid group of 10mg/kg (Table 5). The EB content increased significantly after the subarachnoid haemorrhage rat model, indicating the growth of the cerebrovascular permeability. However, the EB content plunged in the oleanolic acid group of 20mg/kg (P<0.01). This means the oleanolic acid can significantly improve the cerebrovascular permeability (Table 5).

Conclusions

In this paper, an efficient UAE is employed to extract oleanolic acid from *C. mandshurica*. Then, an orthogonal test was designed to determine the optimal extraction variables. The test results show that the optimal extraction conditions are the extraction temperature of 70°C, extraction frequency of 90kHz, extraction time of 10min and liquid-material ratio of 30:1. The statistical analysis indicate that all four variables exhibited major impacts on the yield, among which the extraction temperature boasted the greatest impact. The highest oleanolic acid yield was obtained using the optimized variables. Compared with immersion extraction, the UAE can shorten the extraction time and reduce solvent consumption. Therefore, the UAE is a suitable way to extract oleanolic acid from *C. mandshurica.*

In addition, it is discovered that the oleanolic acid group of 20mg/kg can significantly improve neural function in rats, but the model group suffered from much more serious neural injuries than the sham-operated group. The EB permeability of rat brain increased obviously after 24 h of subarachnoid haemorrhage, but plunged in the oleanolic acid group of 20mg/kg. This means oleanolic acid can effectively reduce the cerebral vascular permeability caused by subarachnoid haemorrhage. The research findings reveal the protective mechanism of oleanolic acid against early brain injury after subarachnoid haemorrhage in rats.

References

Bederson JB, Germano IM, Guarino L. Cortical blood flow and cerebral perfusion pressure in a new noncraniotomy model of subarachnoid hemorrhage in the rat. Stroke 1995; 26(6):1091-92.

Chen C, Wang L, Wang R, Luo XH, Li YF, Li J, Li YN, Chen ZX. Ultrasound-assisted extraction from defatted oat (*Avena sativa* L.) bran to simultaneously enhance phenolic compounds and β-glucan contents: Compositional and kinetic studies. Journal of Food Engineering 2017; 222: 1-10.

Chen GJ, Bu F, Chen XH, Li CF, Wang SS, Kan JQ. Ultrasonic extraction, structural characterization, physico-chemical properties and antioxidant activities of polysaccharides from bamboo shoots (*Chimonobambusa quadrangularis*) processing by-products. International Journal of Biological Macromolecules 2018; 112: 656-60.

Córdova C, Gutiérrez B, Martínez-García C, Martín R, Gallego-Muñoz P, Hernández M, Nieto ML. Oleanolic acid controls allergic and inflammatory responses in experimental allergic conjunctivitis. PloS one 2014; 9(4): e91282.

Chinese Pharmacopoeia Commission. Pharmaco-poeia of the People's Republic of China (vol. I). Beijing: China Medical Science Press, 2015.

Dong J, Liu Y, Liang Z, Wang W. Investigation on ultrasound-assisted extraction of salvianolic acid B from *Salvia miltiorrhiza* root. Ultrasonics Sonochemistry 2010; 17(1): 61-65.

Hao N, Yu Y, Zhang MX, Wang Y, Li YH, Lv WW, Li HB. Extraction process of total flavone and saponin from Weilingxian. Journal of Chinese Medicinal Materials 2014; 37(5): 884-89.

Hilbig J, Alves VR, Müller CM, Micke GA, Vitali L, Pedrosa RC, Block JM. Ultrasonic-assisted extraction combined with sample preparation and analysis using LC-ESI-MS/MS allowed the identification of 24 new phenolic compounds in pecan nut shell [*Carya illinoinensis* (Wangenh) C. Koch] extracts. Food Research International. 2018; 106: 549-57.

Hsieh CW, Cheng JY, Wang TH, Wang HJ, Ho WJ. Hypoglycaemic effects of Ajuga extract in vitro and in vivo. Journal of Functional Foods 2014; 6(1): 224-30.

Li H, Chen B, Yao S. Application of ultrasonic technique for extracting chlorogenic acid from *Eucommia ulmodies* Oliv.(*E. ulmodies*). Ultrasonics Sonochemistry 2005; 12(4): 295-300.

Paniwnyk L, Cai H, Albu S, Mason TJ, Cole R. The enhancement and scale up of the extraction of anti-oxidants from *Rosmarinus officinalis* using ultrasound. Ultrasonics Sonochemistry 2009; 16(2): 287-92.

Shimaoka A, Seo S, Minato H. Saponins isolated from *Bupleurum falcatum* L.; components of saikosaponin b. Journal of the Chemical Society, Perkin Transactions 1 1975(20): 2043-48.

Sun Y, Wei L, Wang J, Bi J, Liu Z, Wang Y, Guo Z. Optimization of supercritical fluid extraction of saikosaponins from *Bupleurum falcatum* with orthogonal array design. Journal of Separation Science 2010; 33(8):1161-16.

Valachovic P, Pechova A, Mason TJ. Towards the industrial production of medicinal tincture by ultrasound assisted extraction. Ultrasonics Sonochemistry 2001; 8(2):111-17.

Xia EQ, Yu YY, Xu XR, Deng GF, Guo YJ, Li HB. Ultrasound-assisted extraction of oleanolic acid and ursolic acid from *Ligustrum lucidum* Ait. Ultrasonics Sonochemistry 2012; 19(4):772-76.

Yang L, Pei J, Wan D. Content measurement of triterpenoidal sapogenin oleanolic acid in traditional chinese medicine Wei Ling Xian by HPLC. Natural Product Research and Development 2006; 18(2): 313-15.

Zhao YQ, Yang LX, Zhang XM, Qiu MH. Research progress of ingredient, pharmacological activity and clinical application of Radix Clematidis. Journal of Chinese Medicinal Materials 2008; 31(3): 465-70.

Zhang HF, Yang XH, Zhao LD, Wang Y. Ultrasonic-assisted extraction of epimedin C from fresh leaves of Epimedium and extraction mechanism. Innovative Food Science & Emerging Technologies 2009; 10(1): 54-60.

Zhang Y, Prawang P, Li C, Meng X, Zhao Y, Wang H, Zhang S. Ultrasonic assisted extraction of artemisinin from *Artemisia Annua* L. using monoether-based solvents. Green Chemistry 2018; 20(3); 713-23.