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Full Length Research Paper

Study on the optimization of the technology for the extraction and purification of total flavone in *Scutellaria baicalensis* and its antibacterial activity

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The objective of this research is to study the technology for the extraction of flavone in *Scutellaria baicalensis* and its antibacterial activity; to adopt macroporous absorption resin method to investigate the technology for purification of total flavone in *S. baicalensis* through orthogonal experiment, and adopt the improved tube double dilution method to study its antibacterial activity. The final determined optimal purification technology is A2B1C3, that is, the resin column diameter-height ratio is 1:5, eluent flow rate is 0.5 ml/min, and eluent consumption is 9BV. The average content of total flavone purified by resin reaches 89.67%. The MIC of this extract to *Staphylococcus aureus* and *Klebsiella pneumonia* is 1.56 and 3.13 mg/ml respectively. Macroporous absorption resin technology is the efficient method to extract flavone in *S. baicalensis*, which has strong antibacterial activity.

Key words: *Scutellaria baicalensis*, total flavone extraction, purification, antibacterial activity.

INTRODUCTION

Scutellaria baicalensis is the dry root of *S. baicalensis* Georgi, the plant in the family Labiatae (State Pharmacopeia Committee of China, 2010), it has a long medicinal history in our country, the earliest documentation is in the "Sheng Nong's herbal classic", with other names of Fuchang, Huangwen, Jingqin, etc (Huang, 1984), and thereafter many ancient medicinal books supplement and improve, to make its application more and more comprehensive. *S. baicalensis* has the efficacy of clearing heat and drying damp, purging intense heat and detonicating hemostasis and tocolysis (Song et al., 2001), and its main indications are damp-warm syndrome, summer-warm disease, chest tightness, vomiting, lung heat, cough, blood-heat bleeding, hyperpyrexia, polydipsia, carbuncle, boils and fetal irritability.

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Modern pharmacological studies show that flavonoids are the main active ingredients of *S. baicalensis.* Domestic and foreign researchers have separated more than 40 kinds of flavonoids from *S. baicalensis*, in which four main ingredients have higher contents and significant pharmacological effects, namely baicalin, baicalein, wogonin and wogonoside. In recent years, the studies show that baicalin is the main antibacterial ingredient in *S. baicalensis*, the studies on its antibacterial effect are many (Qu, 2002; Liu et al., 2001, 2002; Zhang et al., 2006; Gao et al., 1999) some other flavonoids also have the antibacterial effect, so this study take the total flavone as the study object. Macroporous absorption resin is a new type of non-ionic macromolecular adsorbent, with the advantages of large adsorption capacity, wide application range, easy regeneration, reusability, etc, and is widely used in the separation and purification of active ingredients in Chinese herbal medicine (Zhu et al., 1998; Lu et al., 2002). This study adopts macroporous absorption resin to

investigate the purification of total flavone in *S. baicalensis*, to achieve the best purification technology conditions, and it studies the antibacterial activity of enriched and purified total flavone.

EXPERIMENTAL MATERIALS

Instruments

UV2100 ultraviolet and visible spectrophotometer (USA UNICO (Shanghai) Instrument Co. Ltd); Analytical balance FA2004 (Shanghai Balance Instrument Plant); Rotary evaporator RE-52AA (Shanghai Yarong Bio-chemical Instrument Plant); High speed refrigerated centrifuge GL-21M (Changsha Xiangyi Centrifuge Instrument Co. Ltd); Micro vortex mixer XW-80A (Shanghai Huxi Analytical Instrument Plant); SW-CJ-2F clean bench (Henan Tianchi Instrument and Equip-ment Co. Ltd); DHP9162 electro-heating standing-temperature cultivator (Shanghai Qixin Scientific Instrument Co. Ltd); Glass resin column (Beijing Xinweier Glass Instrument Co. Ltd).

Drugs and reagents

Scutellaria baicalensis decoction (Weifang Shenzhou Tiancheng Chinese Herbal Decoction Co. Ltd); *S. baicalensis* reference product (National Institute for the Control of Pharmaceutical and Biological Products); MH broth (Shanghai Microorganism Reagent Co. Ltd); Agar (Shanghai Microorganism Reagent Co. Ltd); Macro-porous absorption resin (Cangzhou Baoen Adsorbing Material Technology Co. Ltd); other reagents are analytically pure.

Bacteria

Saphylococcus aureus ATCC25923 and *Klebsiella pneumoniae* ATCC700603 are preserved and cultured by this laboratory.

EXPERIMENTAL METHODS

Preparation of total flavone in *Scutellaria* baicalensis

In this study, an appropriate amount of *S. baicalensis* extract was crushed and weighed according to the optimal technology in the literature (Zeng and Liu, 2009). The ultrasonic extraction power was 235 W, solid-liquid ratio was 1: 20, ethanol was 60% and extraction time was 9 min. After extract filtration, the ethanol was recycled from the filtrate, and 50% ethanol was added to it to settle for later use.

Pretreatment of macroporous resin

The new macroporous resin of six models was soaked in 95% ethanol for 24 h, and was made to fully swell, after which it was added to the column by wet method. The effluent was washed out with 95% ethanol, after which an appropriate amount of water (effluent : water = 1:5) was added to it without white turbidity, and then it was repeatedly rinsed with distilled water till there was no smell of ethanol. Finally, for the acid-base treatment, 4BV 5% hydrochloric acid solution was used through the resin layer at the flow rate of 5BV/h, soaked for 3 h, and washed with distilled water at the same flow rate till the water pH was neutral. Afterwards, 4BV 5% sodium hydroxide solution was used through the resin layer at the flow rate of 5BV/h, soaked for 3 h, and washed with distilled water at the same flow rate till the water pH was neutral. At last, it was soaked in distilled water for later use.

Measuring of the content of total flavone in *Scutellaria baicalensis*

Preparation of reference product solution

5.00 mg baicalin reference product was accurately weighed with 50% ethanol in a 50 ml measuring flask as the reference product solution.

Drawing of standard curve

0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml reference product solution were accurately measured and added to a 25 ml measuring flask, respectively, after which 50% ethanol was added to the scale and shaken evenly. The absorbance was measured at the wavelength of 279 nm, before the standard curve was drawn with the absorbance as the vertical axis and the concentration as the horizontal axis (Figure 1).

Screening of macroporous resin

Due to different polarity, specific surface area, pore size and pore volume of macroporous resin, the adsorption and separation effect of the experiment was very different and as such, the models were screened.

Static adsorption and elution experiment

Measuring of adsorption rate

Total flavone was extracted from *Scutellaria baicalensis* with the optimal extraction technology into 5.0 mg/ml test solution, and was accurately measured to 30 ml and added into a 100 ml conical flask with a stopper pre-added to the processed 1.0 g macroporous resin (the resin did not drop water after filtration). The resin was oscillated on a constant-temperature oscillator at 100 r/min for 24 h and filtered after full adsorption. The remaining content of the total flavone in the filtrate was measured, and the adsorption capacity and adsorption rate of all kinds of resin were calculated according to Formula 1 and 2:

$$\text{Adsorption capacity (mg/g)} = (m0 - m1)/m \quad (1)$$

$$\text{Adsorption rate (\%)} = (m0 - m1)/m0 \times 100\% \quad (2)$$

in which, m0 is the mass (mg) of the total flavone contained in the test solution before adsorption; m1 is the mass (mg) of the total flavone contained in the effluent after adsorption; and m is the mass (g) of all kinds of dry resin.

Measuring of resolution rate

30 ml 80% ethanol was added to the saturated resin and oscillated on a constant-temperature oscillator at 100 r/min for 24 h, before it was filtered after full resolution. The content of total tannin in the filtrate was measured, and the resolution rate of all kinds of resin was calculated according to Formula (3):

$$\text{Resolution rate (\%)} = m2/(m0 - m1) \times 100\% \quad (3)$$

where, m0 is the mass (mg) of the total flavone contained in the test solution before adsorption; m1 is the mass (mg) of the total flavone contained in the effluent after adsorption; and m2 is the mass (mg)

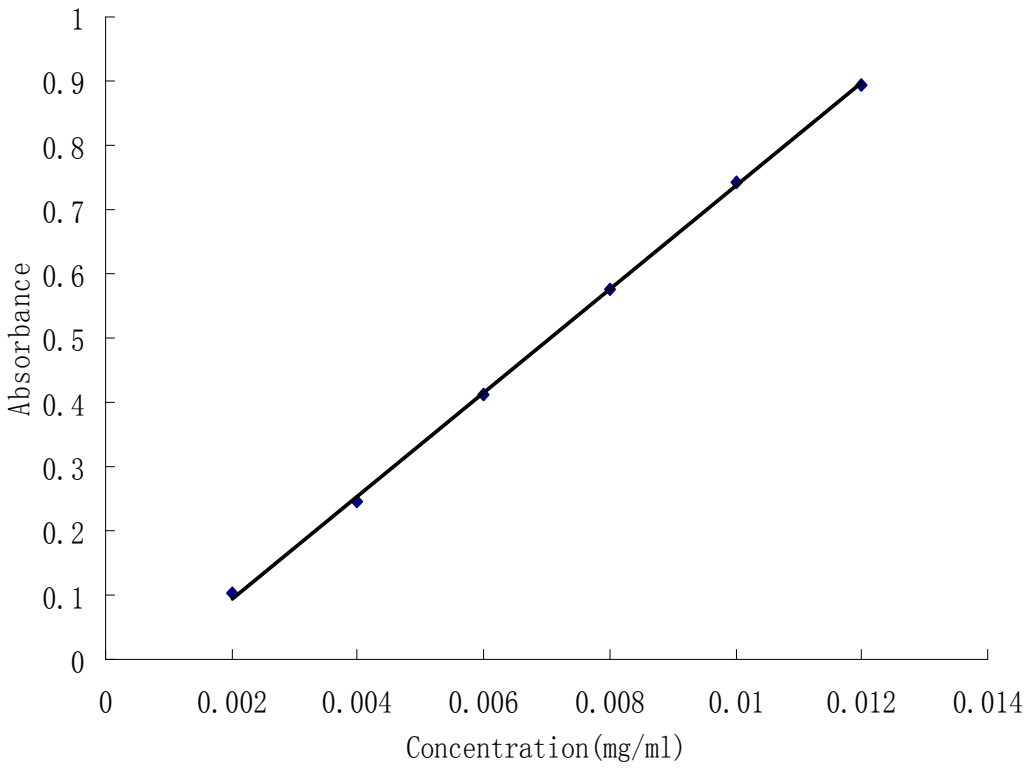

Figure 1. Standard curve of the measuring of the content of total flavone in *Scutellaria baicalensis.*

Table 1. Levels of adsorption factors of macroporous absorption resin.

Level	Factor		
	A Diameter-height ratio	B Elution flow rate (ml/min)	C Eluent consumption
1	1：3	0.5	5BV
2	1：5	1	7BV
3	1：7	1.5	9BV

of the total flavone contained in the resolution solution.

Dynamic adsorption experiment

Leakage curve

10 g of processed HPD10 resin was weighed, and added to the resin column, after which 200 ml of 10 mg/ml upper column was added to the solution to make a dynamic adsorption at the flow rate of 1 ml/4 min. A tube of effluent per 5 ml was collected and the content of total flavone was measured. The leakage curve was drawn with the content corresponding to the sample volume before the mutation point was used as the sample volume.

Selection of effluent concentration

10 g of pre-processed HPD100 resin was accurately weighed and added to the column by wet method, after which 50 mL upper column solution (10 mg/mL) was added to it. After complete pre-adsorption, it was eluted with water and 30, 50 and 70% ethanol respectively, before it was rinsed with 4BV for each concentration. The eluent was settled in a 50 ml measuring flask, and the content of total flavones in *S. baicalensis* was measured by ultraviolet spectrophotometry.

Investigation of resin diameter-height ratio, elution flow rate and eluent consumption

The main factors influencing the adsorption property of macroporous absorption resin are resin diameter-height ratio, elution flow rate and eluent consumption, and each factor has three levels used to select the optimal elution conditions (Table 1).

Based on the levels of the foregoing factors, the L_9 (3^4) orthogonal test table was selected, and the results of the influence of these three factors were investigated. An appropriate amount of

Table 2. Macroporous resin screening results.

Resin model	Adsorption capacity (mg/g)	Adsorption rate (%)	Resolution rate (%)
NKA	95.28	63.52	51.32
D101	112.70	75.13	55.76
DA201	90.65	60.43	47.13
HPD100	137.96	91.97	85.36
HPD400	128.66	85.77	81.24
HP20	117.48	78.32	74.65
AB-8	124.32	82.88	78.55

HPD100 resin was accurately measured and added to the upper column solution for pre-adsorption for 30 min, after which 50% ethanol was added to it for elution. The eluent was collected, while the content of total flavone was measured for comprehensive scoring, before the optimal purification technology was determined.

Verification of the optimal extraction and purification technology for the total flavone in *Scutellaria baicalensis*

In order to verify the aforementioned optimal extraction and purification technology, 3 batches of it were tested according to the optimal extraction and purification technology, in order to calculate the content of total flavone.

Drug sensitivity test

For this test, 0.2 ml of the activated test bacterial solution was taken and diluted to 1/2 Mcfarland Standard, and was evenly coated and inoculated in sterile nutrient broth agar medium. The drug paper was pasted on the agar plate inoculated by means of the test bacteria, with 6 pieces in equidistance on each plate, and 3 parallels were made for each test. After inverted culture, the drug paper was taken out from the incubator at 37℃ for 2 4 h, after which the diameters of all the inhibition zones were read with a vernier caliper three times for each inhibition zone, and the mean value was taken (Tao et al., 2009).

Measuring of minimal inhibitory concentration (MIC)

MIC of the total flavone extracted from *S. baicalensis* was measured with the optimal extraction and purification technology. The improved tube double dilution method was adopted to dilute the original bacterial solution diluted to 1/2 Mcfarland Standard with nutrient broth by 1:1 000, to obtain the diluted bacterial solution. For this test, 12 sterile tubes were used. 1.00 ml sterile culture medium was added to each tube, except tube 1; 1.6 ml culture medium and 0.4 ml 0.5 g/ml total flavone extract were added to tube 1, and 1.00 ml was taken from tube 1 and added to tube 2 after mixing. 1.00 mL was taken from tube 2 and added to tube 3 after mixing. It was done continuously and double diluted to tube 10. After mixing, 1.00 ml was removed from tube 10. No drug was added to tube 11 since it was used as the control for bacteria growth observation, while 1 ml sample but not bacteria was added to tube 12 for observing whether or not the test extract was contaminated. 0.1 ml diluted bacterial solution was added to tubes 1 and 11, mixed evenly, and placed in 37℃ incubator for 24 h, after which the r esults were observed. Observation of the results and comparison of tubes 11 and 12 was done with the naked eye, and the highest drug dilution factor without turbidity changes was the MIC value of this drug (Mo et al., 2009).

Killing curve

For this curve, 1/2, MIC, 2, 4, 8 and 16 MIC drug concentration was used in the tubes, after which bacterial solution was added to it and made at a concentration of 105 CFU/ml. Then the samples were fully mixed, taken and diluted at 0, 2, 4, 6, 8, 12, 18 and 24 h. The number of viable bacteria was counted with the plate method, and the bacteria-time curve was drawn afterwards (Woo et al., 1994).

EXPERIMENTAL RESULTS

Standard curve of the measuring of the content of total flavone in *S. baicalensis* is shown in Figure 1. The regression equation of total flavone content measurement is y = 80.3 × -0.0669 (R2 = 0.9995), indicating that the total flavone content in 2 to 12 µg/ml has good linear relationship.

Macroporous resin screening results

Macroporous resin screening results are shown in Table 2. The experimental results of the comprehensive analysis on static adsorption show that HPD100 macroporous resin is the best resin extracted and purified in this experiment.

Leakage curve

Leakage curve is shown in Figure 2. The horizontal axis is effluent volume, the vertical axis is total flavones content, and it can be known from the leakage curve that when the upper column solution volume is 50 ml, the solution starts to leak, so the adsorption volume of 10 g resin should be 50 ml, 10 mg/ml upper column solution.

Eluent concentration selection result

Eluent concentration selection result is shown in Table 3. It can be known from the aforementioned table that 50% ethanol is the best eluent concentration. Orthogonal test

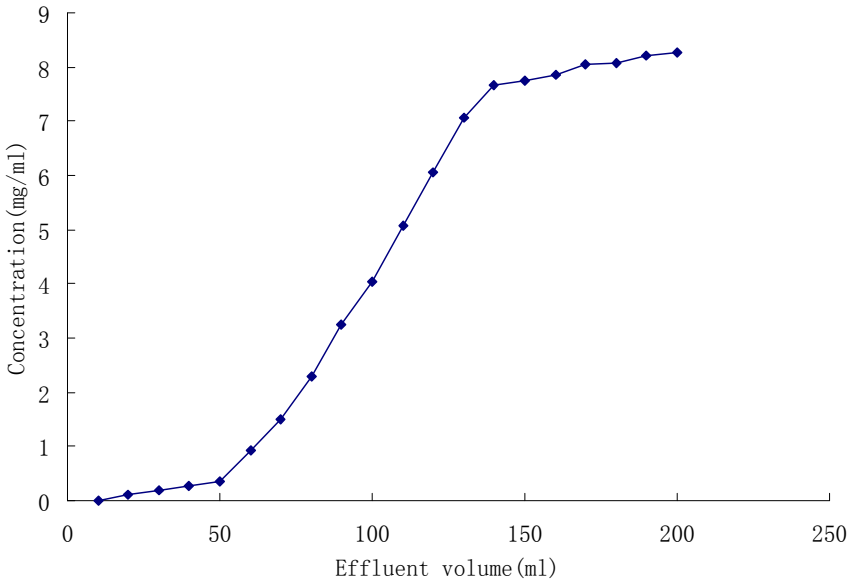

Figure 2. Leakage curve investigation results.

Table 3. Different eluent concentration investigation results.

Eluent concentration	Total flavone content (%)
Distilled water	0.71
30% ethanol	18.5
50% ethanol	50.7
70% ethanol	25.9

result is shown in Table 4. Intuitive analysis shows that the sequence of the factors influencing macroporous resin purification technology is C>A>B, that is, eluent consumption has the greatest influence, and the second is the resin column diameter-height ratio; variance analysis results show that: eluent consumption P<0.05, with the significant difference. The finally determined optimal purification technology is A2B1C3, that is, the resin column diameter-height ratio is 1:5, the eluent flow rate is 0.5 ml/min, and eluent consumption is 9BV.

Verification test results

This is shown in Table 5. It can be known from the verification test results that the average content of total flavone after resin purification reaches 89.67%, and this technology is stable, reasonable and feasible.

Drug sensibility test results

According to the determination standard for in vitro antibacterial results, total flavone in *S baicalensis* is highly sensitive to *Klebsiella pneumoniae*, while the most antibacterial to *Staphylococcus aureus*. The test results of the drug sensitivity of the extract of total flavone in *S. baicalensis* to the two pathogenic bacteria are shown in Table 6. The result as earlier stated is the mean value of the three repeated tests.

MIC value of the extract of total flavone in *S. baicalensis*

MIC value of the extract of total flavone in *S. baicalensis* is shown in Table 7. It can be seen from the aforementioned table that the MIC values of *Staphylococcus aureus* and *Klebsiella pneumoniae* are 1.56 and 3.13 mg/ml respectively. The results of killing curves are shown in Figure 3.

DISCUSSION

Currently, the main methods to enrich and purify baicalin

Table 4. Investigation result of the orthogonal test on macroporous resin purification technology.

Level		Factor				
		A diameter - height ratio	B Elution flow rate	C Eluent consumption	Blank	Total flavone content (%)
	1	1	1	1	1	55.72
	2	1	2	2	2	61.81
	3	1	3	3	3	67.36
	4	2	1	2	3	73.62
	5	2	2	3	1	79.34
	6	2	3	1	2	61.12
	7	3	1	3	2	82.46
	8	3	2	1	3	57.22
	9	3	3	2	1	57.54
Intuitive analysis	K_1	61.630	70.600	58.020	64.200	
	K_2	71.360	66.123	64.323	68.463	
	K_3	65.740	62.007	76.387	66.067	
	R	9.730	8.593	18.367	4.263	
Variance analysis	sum of squares of deviation from mean	143.149	110.833	522.590		
	Degree of freedom	2	2	2		
	F value	5.224	4.044	19.070		

Table 5. Technology verification test results.

Test times	Total flavone content after ultrasonic extraction (%)	Total flavone content after resin purification (%)
1	10.24	89.54
2	10.63	90.12
3	10.44	89.36

Table 6. Antibacterial effect of the extract of total flavone in *S. baicalensis*.

Test bacteria	Inhibition zone diameter (mm)
Staphylococcus aureus	15.8
Klebsiella pneumoniae	13.6

Table 7. Result of MIC value of the extract of total flavone in *S. baicalensis*.

Final drug concentration (mg/ml)	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20
Staphylococcus aureus	−	−	−	−	−	−	−	+	+	+
Klebsiella pneumoniae	−	−	−	−	−	−	+	+	+	+

"+" indicates that growth is observed, while "-" indicates that growth is not observed.

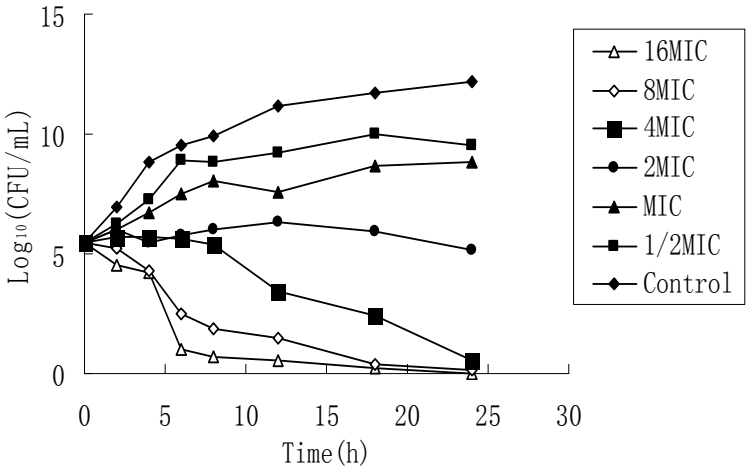

Figure 3. Results of the killing curves for different concentrations.

from the leachate of *S. baicalensis* are conventional water boiling and acid precipitation, ethanol extraction and ultrafiltration etc, but these methods have the disadvantages of long extraction time, low baicalin extraction rate, large solvent consumption, difficult filtration and high cost etc. Macroporous resin is a "pure polymers" containing gap structure, with the advantages of large specific surface area, large adsorption capacity, good selectivity, easy regeneration process, high adsorption speed, etc. It is not influenced by inorganic salts, so it is particularly suitable for the separation and purification of compounds from water solution, and in recent years, it has received wide attention in drug extraction and separation. In this study, the combination of ultrasonic extraction and macroporous resin purification is adopted, with the advantages of short extraction time and high efficiency, and the total flavone content is higher than that in the past. HPD-100 macroporous resin has better baicalin enrichment and purification effect than other types of resin, which is basically the same as the conclusion in the literature (Bi et al., 2003). After analysis, the polarity of compounds directly affects the macroporous resin separation effect. In general, the molecules with larger polarity are suitable for separation on medium-polar and polar resin, while the molecules with smaller polarity are suitable for separation on non-polar resin. Flavonoids generally have phenolic hydroxyl and glycosidic bonds, with certain polarity and hydrophilicity and strong ability to generate hydrogen bonds, in favor of the adsorption of weakly polar and polar resin. In this study, baicalin is a flavonoid connected with the structure of glucuronic acid, with certain hydrophilicity and lipid solubility, suitable for the adsorption of weakly polar resin, such as HPD. HPD-100 macroporous resin used in this experiment is weakly polar resin, and 50% ethanol should be used. Liu et al. (2002) studied the factors influencing macroporous resin to adsorb and separate herbal chemical ingredients, and the reported results are consistent with the conclusion in this experiment. Flavonoids are a large class of active ingredients in medicinal plants, with a variety of biological activities, and the main function is bacteriostasis or sterilization. The experimental results show that total flavone in *S. baicalensis* is a natural antibacterial agent and is expected to be used for food preservation, anti-bacterial and anti-inflammatory functions, etc, opening up a new way for the comprehensive application of *S. baicalensis.*

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