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8(1), pp. 1-8, 8 January, 2014
DOI: 10.5897/AJPP2013.3939
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Orthogonal test design for optimizing the extraction of total flavonoids from *Flos pueraria*

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Accepted 16 December, 2013

The conditions for extraction of the total flavonoids from *Flos pueraria* was optimized and the purification and separation process were conducted to identify the main constituents of the total flavonoids as well. The solvent for extraction and its concentration, the solid-to-liquid ratio, extraction duration, temperature and ultrasonic frequency were investigated through a single-factor experiment. An orthogonal design (L9 (3^4)) was constructed to achieve the best extraction conditions. The crude extract was then purified sequentially by petroleum ether, ethanol and chloroform, n-butyl alcohol, and eluted gradually with mixed mobile phase of methanol-chloroform solution in the silica gel column system. The ingredients were further separated by color reaction, ultraviolet spectrophotometry, high performance liquid chromatography, infrared and mass spectral analysis. The optimum extraction condition for the total flavonoids from *F. pueraria* was as follows: extraction by 50% (v/v) methanol solution, the solid-to-liquid ratio at 1:30, extraction duration 2.0 h, the temperature of 70°C, ultrasound 3 times and 30 min each time. Five isoflavones were separated and identified as irisolidone, genistein, daidzein, kakkalide and puerarin, respectively. Under these optimal conditions, the yield of total flavonoids in the extracts was up to 17.5%, while the output rate previously reported is about 8% in general. Our study provided means for further development and utilization of the bioactive components from *F. pueraria*.

Key words: *Flos pueraria*, flavonoids, orthogonal design, purification, ultrasound-assisted extraction.

INTRODUCTION

Flos puerariae, a well-known Chinese medicine compound is the dry bud of *Pueraria lobata* which is a plant in the genus *Pueraria* in the pea family Fabaceae, subfamily Faboideae and it is widely cultivated in East Asian countries. It has been traditionally used for headache, dizziness, polydipsia and vomiting associated with alcoholism since ancient time. Previous studies have shown that *F. pueraria* possesses extensive pharmacological effects such as antioxidant (Xiong et al., 2010), antidiabetic (Choi et al., 2004), antivirus (Tsuchihashi et al., 2009), antitumor, liver protective (Lee et al., 2003), detoxification of alcohol (Lee et al., 2001) and estrogen-like action. There are a lot of bioactive components in *F. pueraria* and of these, flavonoids are the most important class of compounds with 2-benzene chromone nucleus. They are widely distributed throughout the plant kingdom and possess a variety of biological activities. Flavonoids intake is reported to be negatively correlated with the incidence of some chronic diseases including cardiovascular diseases (Koon et al., 2011), type II diabetes (Jung et al., 2006), neurodegenerative diseases (Ramassamy, 2006), cancers (Lin et al., 2009) etc. Conventional flavonoid extraction methods include Soxhlet extraction, maceration and heat reflux extraction.

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These methods generally result in limited output due to oxidation and hydrolysis. It is known that the extraction conditions such as temperature, the solvent, the solid-to-liquid ratio, and the duration all contribute to the output efficiency. Additionally, ultrasound-assisted extraction (UAE) has been widely used for extraction of the bio-active substances from food and medicinal plant research industries, and compared with conventional extraction methods, UAE has many advantages such as less solvent consumption, reduced time, higher extraction rates and greater environmental protection. It is previously reported that the output rate of total flavonoids from *F. pueraria* is about 8% in general (Zhu et al., 1995).

In this study, we tried to optimize the procedure for extraction of the flavonoids from *F. pueraria* with the assistance of ultrasonic treatment which has not been reported before. An orthogonal array is employed to analyze the interaction among these operating factors.

MATERIALS AND METHODS

Reagent and drug

The flowers of *P. lobata* (*F. pueraria*) was acquired from Green Mountain *Pueraria mirifica* powder factory in Hengfeng city of Jiangxi province. 200 mesh column chromatography silica gel was obtained from Sinopharm Chemical Reagent Co. Ltd. The standard Rutin was obtained from Shanghai shunbo biotech co., Ltd. Other chemicals were of analytical grade and purchased from local markets.

Equipment

SB-5200 ultrasonic cleaning device (Ningbo xinzhi bio-tech Co.,Ltd.); SBS-100 digital meter automatic collectors (Shanghai huxi Analysis Instrument Factory co., Ltd); RE-5299 rotary evaporation instrument (Yingyuyuhua Apparatus Factory of Gongyi city); SHB-β water circulating multi-purpose vacuum pump (Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd); 50-Conc. UV-Vis spectrophotometric meter (VARIAN Corporation in the United States); LCQ Deca XP-type mass spectrometer (Thermo Finnigan companies in the United States); VECTOR22 fourier transform near infrared spectrometer (Bruker Corporation in Germany); RD-3 melting point apparatus (Tianjin Chuangxin electronic equipment manufacturing Co., Ltd).

Ultrasound-assisted extraction (UAE)

10 g *F. pueraria* was air-dried to constant weight at room temperature. It was mixed with 300 ml of 50% (v/v) of varied solvent (methanol, ethanol or n-butanol); varying solvent concentrations from 0 to 100% (v/v); varying solid-to-liquid ratios from 1:10 to 1:50. The extraction temperature was varied from 20, 30, 40, 50, 60, 70 to 80°C. The extraction duration was varied from 0, 0.5, 1.0, 1.5, 2.0 to 2.5 h. The ultrasound was set at 40 KHz, 30 min each time and varied from 1 to 3 times. At the end of each process, the extract solution was cooled to room temperature and complemented to 300 ml if there is any loss by evaporation. After that, 1 ml of the final extract solution was drawn and centrifuged at 4,000 rpm for 15 min. 0.1 ml of the supernatant was diluted 50-fold with 90% ethanol analyzed using an ultraviolet spectrophotometer. The absorbance was set at 265 nm and the content of total flavonoids was calculated from the standard curve. The blank control was set with solvents.

Optimization design

In order to optimize of the UAE conditions for *F. pueraria*, an orthogonal array ($L9(3^4)$) was constructed to evaluate the effects of the following factors: solvent concentration (A), solid-to-liquid ratio (B), extraction temperature (C), and extraction duration (D). Factors and experimental data are displayed in Table 1.

Purification, separation and structure identification

Besides flavonoids, the extract of *F. pueraria* also contains other substances such as proteins, carbohydrates, nucleic acids, pigments, volatile oils and so on. In order to purify the flavonoids, petroleum ether, absolute alcohol, chloroform and n-butanol was further used to remove lipids, polysaccharides, proteins and others. The purified extracts were then separated by silica gel column chromatography. The separation condition was as follows: The column was 7.0 × 50 cm. The column temperature was set at 25°C. The sample volume is 10 ml and gradients sequentially eluted by chloroform-methanol mixture (v/v = 50:1, 20:1, 10:1, 5:1, 4:1, 3:1, 2:1, 1:1, respectively). The average velocity was 3 ml/min. A tube was collected every 2 min and each gradient was collected for 25 tubes. UV spectrophotometry was used for track scanning and high performance liquid chromatography, infrared spectrum and mass spectrum were used for identification of the isolated monomer compounds. The conditions for high-*performance liquid chromatography* (HPLC) to identify the isolated monomer compounds in the final extract were as follow: Shimadzu Labsolution2010-UV; column: Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm); mobile phase consisted of A (acetonitrile) and B (1‰ phosphoric acid in water); 1 to 20 min (14% A: 86% B); 20 to 30 min (25% A: 75% B); 30 to 40 min (28% A: 72% B); 40 to 50 min (30% A: 70% B); 50 to 60 min (40% A: 60% B); flow rate: 0.8 ml/min; UV wavelength: 270 nm; column temperature: 30°C.

Statistical analysis

Values are presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to determine the significant differences among the groups, and values less than 0.05 were considered significant. Date analysis was performed using the statistical program for social science (SPSS) 13.0.

RESULTS AND DISCUSSION

Calibration and linearity of the rutin

90% ethanolic solution containing rutin standard (0.021 mg/ml) was prepared. This solution was then diluted in 90% ethanol to obtain concentrations corresponding to 0, 0.0042, 0.0084, 0.0168, 0.0336 and 0.0672 mg/ml. All solutions were stored at 4°C. λ_{max} 265 nm was obtained in the ultraviolet scanning from 200 to 500 nm, and standard curve was drawn as $Y = 30.812X + 0.0017$, $R^2 = 0.9999$. There is a good linearity in the range of 4.2 to 67.2 μg/ml (Figure 1A and B).

Effect of suitable solvent and its concentration on flavonoid yield

Solvents with varied concentration possess different

Table 1. Orthogonal design factors and levels.

Factors	Concentration (%) A	Solid-to-liquid ratio (g/ml) B	Temperature (°C) C	Duration (h) D
Level 1	40	1/20	50	1.0
Level 2	50	1/30	60	1.5
Level 3	60	1/40	70	2.0

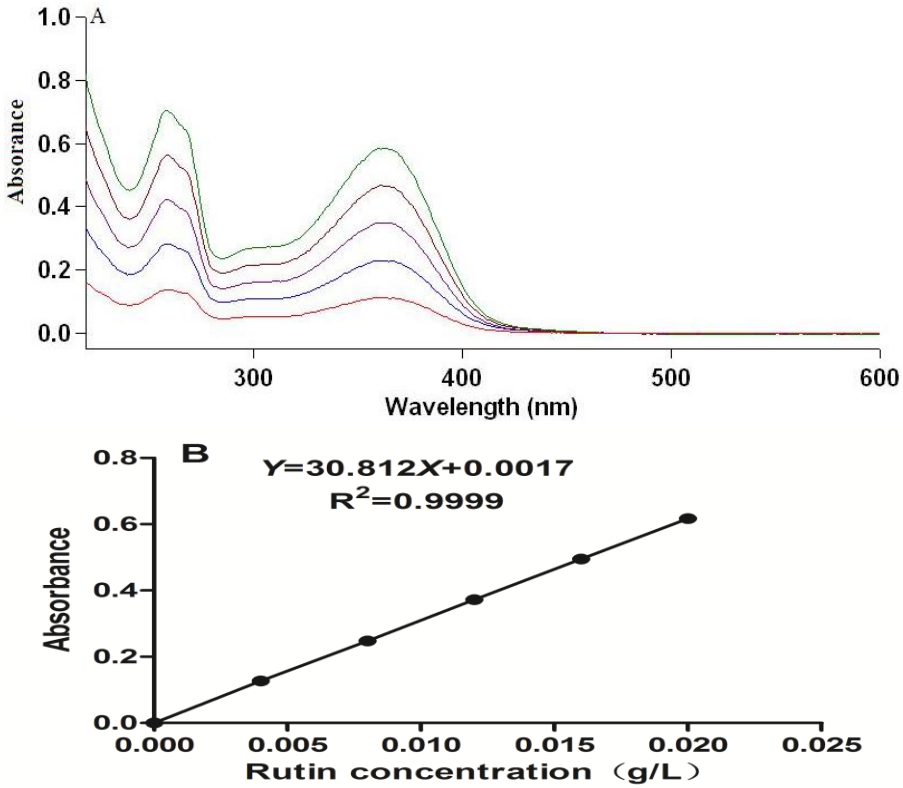

Figure 1. Calibration and linearity of the rutin. 90% ethanolic solution containing rutin corresponding to the concentrations of 0, 0.0042, 0.0084, 0.0168, 0.0336 and 0.0672 mg/ml was prepared.λmax265 nm was used for the ultraviolet scanning. A: The scan curve of rutin. B: The standard curve of absorption capacity of standard rutin. Y=30.812X+0.0017, R^2=0.9999 with a good linearity in the range of 4.2-67.2 μg/ml

polarities which can lead to different component types and quantities of extracts yield. Thus, the selection of a suitable solvent and its concentration is an important step for an optimal extraction yield. In this study, different solvents of methanol, ethanol, n-buty alcohol were tested as extraction solvents. As shown in Figure 2A, the flavonoid yield in methanol solution was the highest among the three solvents and the extraction efficiency increased in parallel with the increment of the methanol concentration within the scope of 0 ~ 40%, got to steady level within the scope of 40 ~ 60%, and then declined if the methanol concentration further rose. Thus, 50% (v/v) methanol solution was selected as the solvent for the exaction in the subsequent experiments (Figure 2B).

Effect of solid-to-liquid ratio on flavonoid yield

The solid-to-liquid ratio is another important factor which greatly influenced the flavonoid yield. Figure 3 shows that when the *F. pueraria* quality and methanol concentration were kept fixed, the extraction yield reached plateau as the solid-to-liquid ratio increased to 1: 30. This was probably because the increment in the solid-to-liquid ratio enhanced the solvent to penetrate and dissolve the target components, and a greater volume of solvent caused sufficient swelling of the material. But more solvent consumption may have negative effects on energy conservation and cost reduction. Our results showed that 1: 30 is the most appropriate ratio of solid-to-liquid for

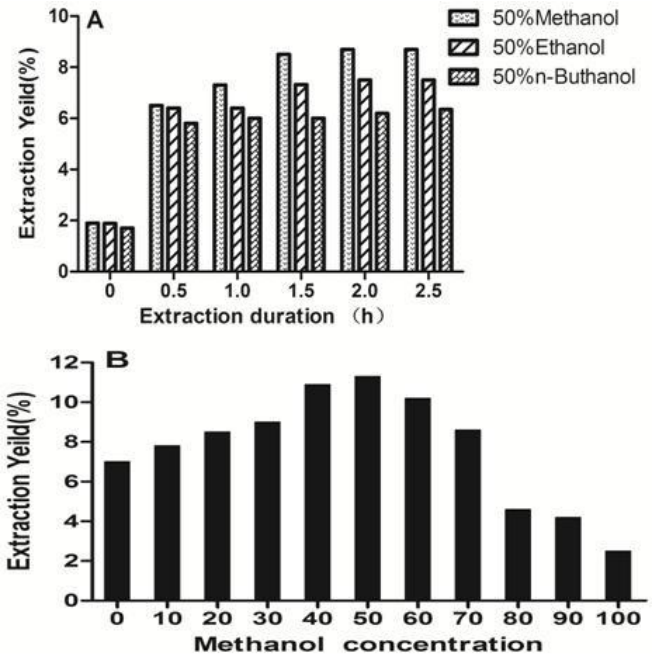

Figure 2. Effect of suitable solvent and its concentration on flavonoid yield. A: Effects of different solvents of methanol, ethanol, n-buty alcohol on the extraction yield; B: Effects of different methanol solution concentrations on the extraction yield. (amount of flowers of Pueraria lobata: 10g; solid-to-liquid ratio: 1:30; extraction duration: 2.0 h; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

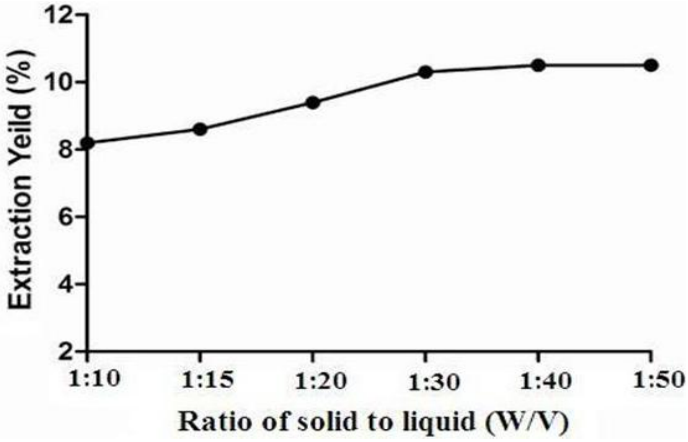

Figure 3. Effect of solid-to-liquid ratio on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; extraction duration: 2.0 h; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

flavonoid yield.

Effect of extraction duration on flavonoid yield

As shown in Figure 4, the flavonoid yield from *F. pueraria* increased a lot within 1.5 h but very little when the extraction time exceeds 1.5 h under the same conditions. One reason may be the flower petals are very thin so that the flavonoids are rapidly released and reached equilibrium. On the other hand, more flavonoids is oxidized and degraded when the extraction duration

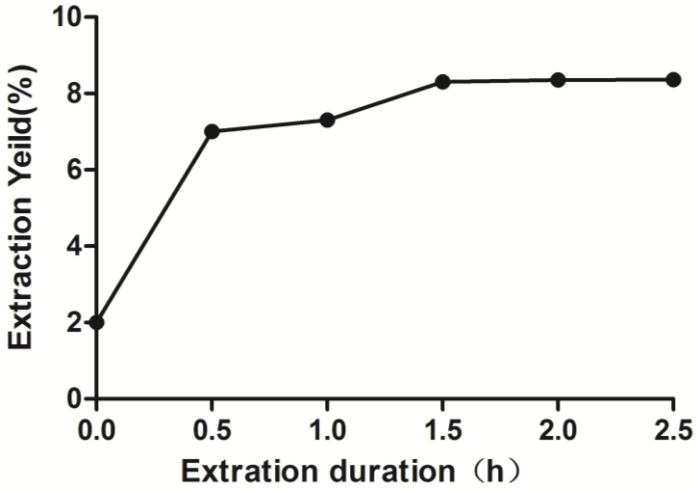

Figure 4. Effect of extraction duration on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; solid-to-liquid ratio: 1:30; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

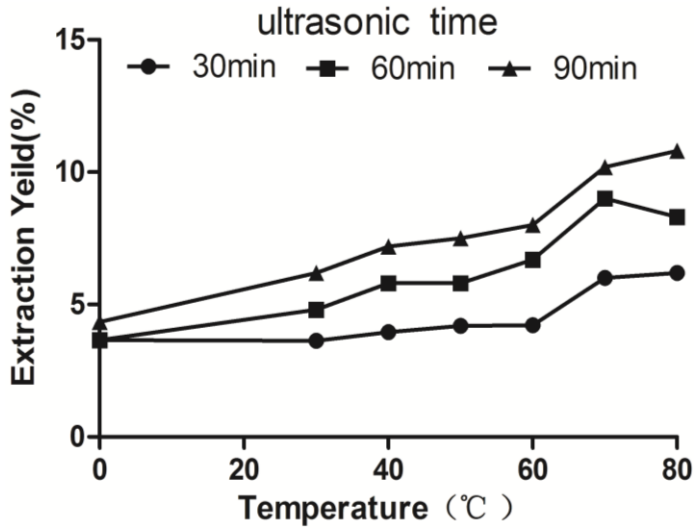

Figure 5. Effect of temperature with different ultrasonic time on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; solid-to-liquid ratio: 1:30; extraction duration: 2.0 h)

extended. Thus, 1.5 h was chosen as the ultrasound-assisted extraction time in subsequent experiments.

Effect of temperature with different ultrasonic time on flavonoid yield

The effect of temperature and different ultrasonic time on flavonoid yield was also evaluated in the present study under the optimal conditions described. Ultrasound can cause temperature increase in the extraction. As shown in Figure 5, the total flavonoid yields consistently elevated during extraction when ultrasonic time was 30, 60 and 90 min, respectively. It suggested that ultrasound and increased temperature leads to accelerated diffusion of active ingredients and increased extraction efficiency, and the solvent viscosity and surface tension would decrease as the temperature increased, which is contributed to the sample wetting and matrix penetration. The flavonoid solubility also increased as a result of the temperature improvement. But once the extraction temperature exceeded 70°C, the extraction efficiency changed slightly.

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

n	Concentration (%) A	Solid/liquid (g/ml) B	Duration (h) C	Temperature (°C) D	Yield (%)
1	40	1/20	1.0	50	10.12
2	40	1/30	1.5	60	14.83
3	40	1/40	2.0	70	16.94
4	50	1/20	1.5	70	15.26
5	50	1/30	2.0	50	14.21
6	50	1/40	1.0	60	13.27
7	60	1/20	2.0	60	14.87
8	60	1/30	1.0	70	15.02
9	60	1/40	1.5	50	11.33
k_1	13.963	13.417	12.803	11.887	-
k_2	14.247	14.687	13.807	14.323	-
k_3	13.740	13.847	15.340	15.740	-
R	0.507	1.270	2.537	3.853	-

Table 3. Variance analysis of orthogonal test.

Origins	SS	v	F	Critical value	P
A	0.387	2	1.000	19.000	-
B	2.503	2	6.468	19.000	-
C	9.792	2	25.302	19.000	P<0.05
D	22.792	2	58.894	19.000	P<0.05
SD	0.39				

Table 4. The proof experiment for optimum process of ultrasound-assisted extraction.

Times	Extraction yield (%)
1	17.41
2	17.54
3	17.48
$\overline{X}$	17.48
RSD (%)	2.17

It may be due to enhanced degradation or conversion along with the higher temperature in the extraction. Hence, 90 min for ultrasound time and the temperature 70°C were the most appropriate to achieve total flavonoid yield via UAE.

Orthogonal design

According to single factor experiment results, and in accordance with the $L_9(3^4)$ orthogonal experiment design for the four factors (solvent concentration, solid-to-liquid ratio, extraction time, extraction temperature) and three levels, the optimal extraction condition was obtained (Tables 1 and Table 2). As shown in Table 3, there is significant difference between factors C and D on the extraction efficiency ($P < 0.05$). No significant difference was found between factor A and factor B ($P > 0.05$). Thus, the most optimal conditions for total flavonoids extraction were as follows: $A_2B_2C_3D_3$, that is: methanol solution concentration of 50%, solid-to-liquid ratio of 1:30, extraction duration of 2 h, at temperature of 70°C, ultrasound 3 times and 30 min each time, and the validation experiments of 3 batches confirmed that the total flavonoids yield rate was (17.48 ± 2.17)% under these optimum conditions (Table 4).

Purification of total flavone

The total flavonoids yield with the optimum extraction conditions of UAE were further isolated by silica gel column chromatography. Combined with the melting point and the color reaction, UV scan, HPLC, IR, electron impact: mass spectroscopy (EI-MS), five isolated monomer compounds were identified as: irisolidone, genistein, daidzein, kakkalide, and puerarin (Bai et al., 2010; Kim et al., 2003; Yasuda et al., 1995) (Figure 6).

Conclusion

Through this optimization study, an efficient UAE method was developed to extract total flavonoids from *F. pueraria*. Under the most optimal conditions of 50% (v/v) methanol as the solvent, solid-to-liquid ratio of 1: 30, extraction duration of 2.0 h, ultrasound 3 times and 30 min each time, at the temperature of 70° C, the total flavonoids yield rate was almost up to 17.5% in the extracts.

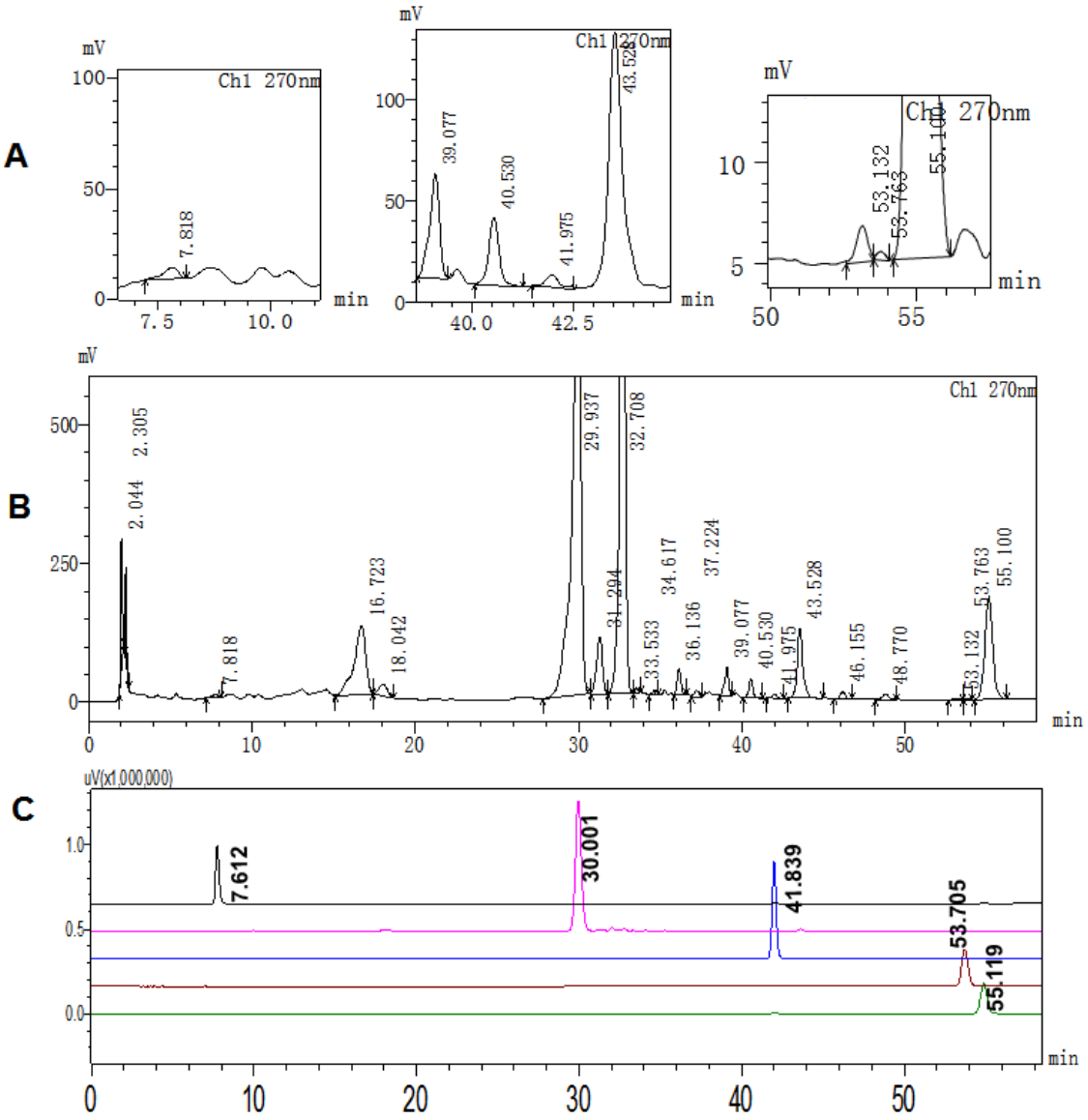

Figure 6. HPLC chromatogram for five isolated monomer compounds identified in the final extract as irisolidone, genistein, daidzein, kakkalide, and puerarin. A: Sample enlarge; B: Sample; C: Reference substance; $t_{puerarin}$=7.612min; $t_{kakkalide}$=30.001min; $t_{genistein}$=41.839min; $t_{irisolidone}$=53.705min; $t_{daidzein}$=55.119min

ACKNOWLEDGEMENTS

This work was financially supported by Colleges and Universities of Hubei Province Outstanding Young Scientific and Technological Innovation Team Plan (No.T201213), Hubei Province Natural Science Foundation Project of Outstanding Young Talents (No.2012FFA005).

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