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Determination of Anti-Tumor Constitute Mollugin from Traditional Chinese Medicine *Rubia cordifolia*: Comparative Study of Classical and Microwave Extraction Techniques

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Abstract: *Rubia cordifolia* is a common traditional Chinese medicine (TCM) used for centuries for the treatment of cough, inflammation of the joints, uterine hemorrhage, and uteritis. Mollugin is a major active component present in *R. cordifolia* and recognized as a potential anti-tumor compound. In this work, a microwave-assisted extraction (MAE) method has been developed for extracting mollugin from *R. cordifolia*. Several variables that can potentially affect the extraction yield, namely extracting solvent, microwave power, extraction time, and solid-liquid ratio were optimized. The separation and quantitative determination of mollugin was carried out by HPLC with UV detection at 254 nm. Under appropriate MAE conditions, such as extraction time of 4 min, ethanol concentration of 70% (v/v), microwave power of 460 W, and solid-liquid ratio of 1:20 (g/mL), the extraction yield of mollugin from *R. cordifolia* with MAE was higher than conventional extraction methods such as Soxhlet extraction, heat reflux extraction, and ultrasonic-assisted extraction. Due to the considerable saving in time and its higher extraction yield, the proposed MAE procedure was obviously a more rapid and effective sample preparation technique.

Keywords: HPLC, microwave-assisted extraction, mollugin, *Rubia cordifolia*, traditional Chinese medicines

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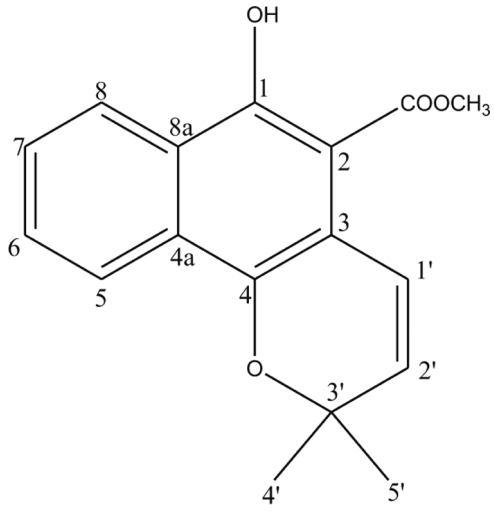
INTRODUCTION

Because of the high pharmacological activity, low toxicity, and rare complication, Traditional Chinese Medicines (TCMs) have played more and more important roles in clinical therapy (1). TCMs have quite a long history, dating back several thousands of years. There have been 12,806 medical resources of TCMs found in China, including 11,145 medicinal plants, 1581 medicinal animals, and 80 medicinal minerals. Also, more than 3214 products have been compiled in the Pharmacopoeia of the People's Republic of China (2005 edition) (2,3). Recently, interest in the TCMs-related subject has increased, especially in the quantitative analysis of active components in TCMs.

Rubia cordifolia, known as Qiancao in Chinese, is a common TCM which has been cultivated mainly in northern China for centuries. It is officially listed in the Chinese Pharmacopoeia and people often use it for the treatment of cough, inflammation of the joints, uterine hemorrhage, uteritis, chronic bronchitis, trauma, and certain cancers (3–5). Recently, it was found that the roots of *R. cordifolia* have antibacterial, antioxidant, and anti-inflammatory activities (6–8). It has been reported that mollugin is a major constituent isolated from *R. cordifolia* and demonstrated to strongly suppress the secretion of hepatitis B surface antigen on human hepatoma cells (5,9). Additionally, it has been recognized as a potential anti-tumor compound and attracted much attention on the research of its bioactivity and total synthesis (10–12). In our previous work, we have isolated and purified mollugin from *R. cordifolia* using high-speed countercurrent chromatography (13). The chemical structure of mollugin is shown in Fig. 1.

Therefore, it is very interesting to extract the major active component mollugin from *R. cordifolia*. The most conventional method for extraction of mollugin from *R. cordifolia* is heat-reflux extraction (HRE) (14). The heat-reflux extraction process is time-consuming, laborious, and solvents-wasting. Other techniques such as supercritical carbon dioxide extraction, pressurized fluid extraction (PFE), ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE) have also become of interest as alternatives for the conventional methods. Among them, MAE is the simplest and the most economical technique for extraction organic compounds from plant materials and foods (15). Recently, MAE has been represented many advantages such as short extraction time and lower consumption of solvents to extract biologically active compounds from different matrices (16), such as extraction of glycyrrhizic acid from licorice root (17), extraction of essential oil from *Eupatorium cannabinum* subsp. *corsicum* (L.) (18), rosemary essential oil from *Rosmarinus officinalis L.*(19), extraction of solanesol from tobacco leaves



mollugin

Figure 1. The chemical structure of mollugin.

(20), extraction of isoflavonoids and saponins from Radix Astragali (21), extraction of lignan from *Magnolia biondii* Pamp (22), extraction of vanillin from *Vanilla planifolia* (23), extraction of paclitaxel from *Taxus baccata* L. (24), etc.

In this paper, we developed an analytical method for determination of mollugin from TCM *R. cordifolia.* Extraction solvent, microwave power, extraction time, and solid-liquid ratio were optimized respectively. HPLC with UV detection was applied for analysis and quantification of mollugin and the operation conditions were examined. What is more, the extraction yield of mollugin by MAE was compared with conventional extraction methods, such as Soxhlet extraction, heat reflux extraction, and ultrasonic-assisted extraction.

EXPERIMENTAL

Materials and Reagents

All the organic solvents used for MAE were of analytical grade and purchased from Huadong Chemicals, Hangzhou, China. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, Bedford, MA, USA) was used for all the solutions and dilutions. Methanol used for HPLC analysis was of chromatographic grade and purchased from Merck, Darmstadt,

Germany. The dried roots of *R. cordifolia* were purchased from a local drug store (Hangzhou, China) and authorized by Prof. H. X. Sun (College of life science, Zhejiang University). A voucher specimen was kept at Department of Chemistry, Zhejiang University, P. R. China. The standard mollugin was prepared by high-speed countercurrent chromatography in our lab (13).

Apparatus

A household microwave-assisted extraction unit (Glanz WP700, Shunde, China) equipped with a 2450 MHz magnetron was used for extraction of the active ingredients from medicinal herbs. It was modified in our laboratory with the addition of a water condenser. The whole system was run at atmospheric pressure and could be employed at the maximum power of 700 W.

An Agilent 1100 liquid chromatography consisting of a G1311A QuatPump, a G1322 Degasser, a G1314A variable wavelength detector (VWD), a model 7725i injection valve with a 20 μL loop, and an Agilent ChemStation for data treatment, was used for analysis and quantification of the mollugin. An Agilent Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm i.d., 5 μm, 120 Å) was used as the LC analytical column.

Ultrasonic bath was KQ50B, which was produced by Kunshan ultrasonic apparatus Co. Ltd., Jiangshu, China.

Microwave-Assisted Extraction

One gram sample was extracted by MAE using 20 mL of different organic solutions. The extraction time was 4 min and extraction power was 280 W for MAE. The optimum concentration of selected organic solution was also studied in detail. The MAE conditions were optimized according to using different extraction times, extraction powers and solid-liquid ratios. The extracts obtained were diluted to 50 mL with the same solutions, and then were filtered through a 0.45 μm membrane prior to HPLC analysis.

Conventional Extraction Methods

Heat reflux extraction (HRE) using a water-bath was performed with a 1.0 g sample and 20 mL 70% ethanol in a flask (100 mL) and the suspensions were made to boil for 2 h. A mechanical stirrer was used during the extraction. The extracts obtained were diluted to 50 mL with the same solutions, and then were filtered through a 0.45 μm membrane prior to HPLC analysis.

Ultrasonic-assisted extraction (UAE) using an ultrasonic bath (KQ50B, Kunshan ultrasonic apparatus Co. Ltd., Jiangshu) was performed. Root of *R. cordifolia* (1.0 g) was mixed with 20 mL 70% ethanol. The suspensions were sonicated with continuous power for 60 min. The extracts obtained were diluted to 50 mL with the same solutions, and then were filtered through a 0.45 μm membrane prior to HPLC analysis.

Soxhlet extraction was carried out using a 10 g sample placed inside the Soxhlet apparatus (250 mL) and 200 mL 70% ethanol for 4 h. The extracts obtained were diluted to 500 mL with the same solutions, and then were filtered through a 0.45 μm membrane prior to HPLC analysis.

Analysis and Quantitative Determination of Mollugin by HPLC

HPLC analysis of the extracts was performed with an Agilent Zorbax Eclipse XDB-C8 (150×4.6 mm i.d., 5 μm, 120 Å). The mobile phase was methanol (solvent A) and water (solvent B) at gradient: A from 45 to 95% and B from 55 to 5% for 50 min. The solvent flow rate was 0.6 mL/min, and the effluent was monitored at 254 nm. The injection volume was 20 μL, and the temperature used for HPLC was ambient. The extraction yield of mollugin was defined as follows:

$$\text{extraction yield (mg/g)} = \frac{\text{mass of mollugin in extraction solution (mg)}}{\text{mass of sample (g)}}$$

The mass of mollugin in extraction solution (one-step extraction) was analyzed by RP-HPLC.

RESULTS AND DISCUSSION

Optimization of MAE

A series of preliminary experiments were performed to determine the optimum operation conditions such as solvent, extraction time, extraction power, and solid-liquid ratio of the microwave-assisted extraction step.

Selection of Solvent

Several kinds of organic solvents, such as methanol, ethanol, acetone, ethyl acetate, and water were studied as the extractants for MAE of

mollugin from *R. cordifolia*. The extractions were carried out at 280 W for 4 min using 20 mL of solvent with 1.0 g crude sample. The result showed that the extraction yield of ethanol was the highest (4.40 mg/g) and the extraction yield of water was the lowest (0 mg/g). What is more, ethanol was nontoxic and volatile. So we chose ethanol as the organic solvent to optimize the parameters affecting the microwave-assisted extraction of mollugin from *R. cordifolia*.

Selection of the Concentration of Ethanol

It has been proven that, in many instances, the addition of small quantities of water to the extraction solvent improves their extracting properties (25). To find out the optimum ethanol concentration for microwave-assisted extraction of mollugin, extraction was carried out in ethanol water solution of different concentrations (from 40 to 100%). The extraction conditions were the same as those employed for the selection of the optimum extraction solvent.

Figure 2a shows that, the addition of water to the solvent improved the extraction properties of ethanol. The extraction yield increases when the ethanol content in water increases from 40 to 70%, reaching the highest value at 70%, and then starts to decrease. Because water can easily penetrate into the histiocyte of *R. cordifolia*, it can enhance the histiocyte to absorb microwave energy to reach a high temperature. The mass transfer of mollugin from *R. cordifolia* to the solvent was enhanced. Therefore, 70% (v/v) ethanol was the optimum ethanol concentration.

Selection of the Microwave Power

Microwave power controls energy supplied to the sample. It is related with the inside temperature of the microwave oven. It affects interactions and equilibrium rate and controls partition of analytes between sample and solvent. To examine the effect of microwave power on the extraction yield, extractions were carried out with 20 mL 70% ethanol for 4 min at four different microwave powers of 280, 460, 600, and 700 W respectively. The extraction yield was shown in Fig. 2b. It revealed that when microwave power was increased sequentially, the extraction yield of mollugin was increased, and the highest efficiency was obtained when the sample was extracted with 70% ethanol at 460 W. However, high microwave power might make the oven temperature overly high, and decrease of the extraction yield when the microwave power was more than 460 W. So the optimum microwave power was selected to be 460 W for further experiments.

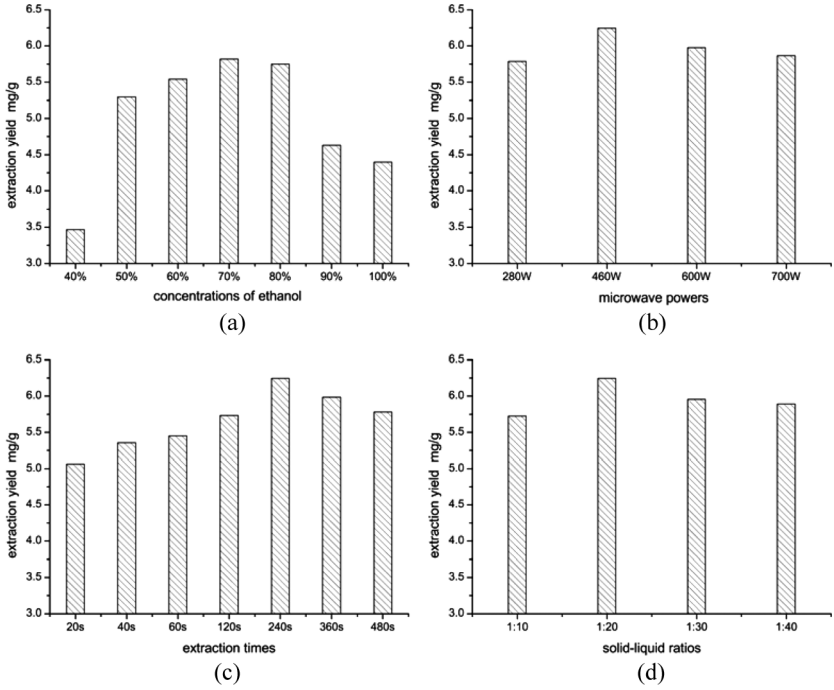


Figure 2. Optimization of MAE by selection of concentration of ethanol (a), microwave power (b), extraction time (c), and solid-liquid ratio (d). The conditions were: (a), Sample, 1.0 g; microwave power, 280 W; extraction time, 4 min; solid-liquid ratio, 1:20. (b), Sample, 1.0 g; solvent, 70% ethanol; extraction time, 4 min; solid-liquid ratio, 1:20. (c), Sample, 1.0 g; solvent, 70% ethanol; microwave power, 460 W; solid-liquid ratio, 1:20. (d), Sample, 1.0 g; solvent, 70% ethanol; microwave power, 460 W; extraction time, 4 min.

Selection of the Extraction Time

Generally speaking, the quantity of analytes extracted is increased with the increasing of the extraction time at the same power, although degradation may occur. To optimize extraction time, extractions were carried out at 460 W for different times using 20 mL 70% ethanol as the extraction solvent. The results obtained were given in Fig. 2c. As confirmed in Fig. 2c, a clear increase of extraction yield of mollugin was obtained when the extraction time increased from 20 s to 240 s. The efficiency could reach its maximum in 240 s during the MAE process. If the MAE time was more than 240 s, the extraction yield declined. With the further increase of extraction time, the mollugin was kept at high

temperature for a longer period of time, which probably led to the thermal decomposition of mollugin. Therefore, 240 s was selected as the optimum extraction time for further experiments.

Selection of the Solid-Liquid Ratio

The solid-liquid ratio is a factor that should be studied to increase the extraction yield of mollugin from *R. cordifolia*. Larger solvent volumes can make the procedure complex and wasteful, while smaller volumes can make the target extraction incomplete. To evaluate the effect of the solid-liquid ratio, a series of extractions were carried out with different solid-liquid ratios (1:10, 1:20, 1:30, and 1:40 g/mL). The extraction conditions were: 460 W of power, 4 min of time, 1.0 g of sample, and 70% ethanol as extraction solvent. The results were shown in Fig. 2d. The extraction yield of mollugin increased with the increase of amount of solvent before the solid-liquid ratio reached 1:20 at which the efficiency was highest, and then it fell down. So the variable of solvent volume was 20 mL.

Based on the above experiment, the optimum MAE conditions were found to be: ethanol water solution of 70% (v/v) as extraction solvent, microwave power of 460 W, extraction time of 4 min, solid-liquid ratio of 1:20 (g/mL).

Comparison of Different Extraction Procedures

In order to compare the extraction yield of MAE with other conventional extraction techniques, heat reflux extraction (HRE), ultrasonic-assisted extraction (UAE), and Soxhlet extraction were carried out to extract mollugin from *R. cordifolia*. The comparison results were listed in Table 1. MAE gave higher efficiency, while heat reflux, ultrasonic, and

Table 1. Comparison of proposed MAE procedures with conventional extraction methods

		Extraction yield (mg/g)		Recovery (%)	
Methods	Times (min)	Mean	SD	Mean	SD
MAE (n = 5)	4	6.24	0.17	99.5	4.5
UAE (n = 3)	60	5.86	0.19	96.1	3.6
HRE (n = 3)	120	5.80	0.35	97.2	4.1
Soxhlet (n = 3)	240	5.10	0.32	100.7	4.0

MAE was processed under the optimum conditions.

Soxhlet extraction yielded lower efficiencies. On extraction time, MAE was the fastest extraction method, and just needs 4 min. Heat reflux extraction needs 120 min, ultrasonic-assisted extraction 60 min, and Soxhlet extraction 240 min, respectively. On extraction yield, MAE gave the highest efficiency. Soxhlet extraction is not so fit for extracting mollugin because of low extraction yield and long extraction time.

The mechanism of MAE leads to its higher extraction yield (15). When we use MAE method, microwaves directly heat solvents and sample, the direct interaction of microwaves with free water molecules present in the cells resulted in the subsequent rupture of the cells and release of intracellular products into the solvent. So when compared with conventional techniques, MAE could obtain higher extraction yield by using less solvents at short extraction time, which indicated that MAE was a more rapid and the most effective sample preparation technique among these four mainstream techniques.

Method Validation

Mollugin was identified using liquid chromatography by its retention time in comparison with that of the standard compound. The chromatograms of the extract and the standard mollugin were showed in Fig. 3. The retention time of mollugin was 41.0 min.

To evaluate the proposed MAE approach, some parameters such as linearity, reproducibility, and recovery were determined under the above optimized conditions. Linearity was investigated over a concentration range from 2.70 to 429 mg/L by HPLC. The linearity plotting at 254 nm was $y = 120.29\ x - 725.89$, where x was mollugin concentration as mg/L and y was the peak area. The limits of detection (LODs) and the limits of quantification (LOQs) expressed as the mass of analyte that gives a signal that is 3σ and 10σ, respectively, above the mean blank signal, where σ is the standard deviation of the blank signal for 10 measurements. The LOD of mollugin is 4.489 μg/L and the LOQ is 14.96 μg/L. The correlation coefficient of the regression line is 0.9992.

The reproducibility study was carried out on repeated extractions of the plant sample, with both the proposed and the conventional methods. As can be seen in Table 1, among the four methods, MAE has the highest reproducibility (RSD = 2.74%) and HRE has the lowest (RSD = 6.27%). Finally, some samples were spiked with known quantities of standards. The recovery using the MAE method was compared with those obtained using the conventional methods. Table 1 showed that the recoveries of the four methods gave no obvious difference (between 96.1% and 100.7%

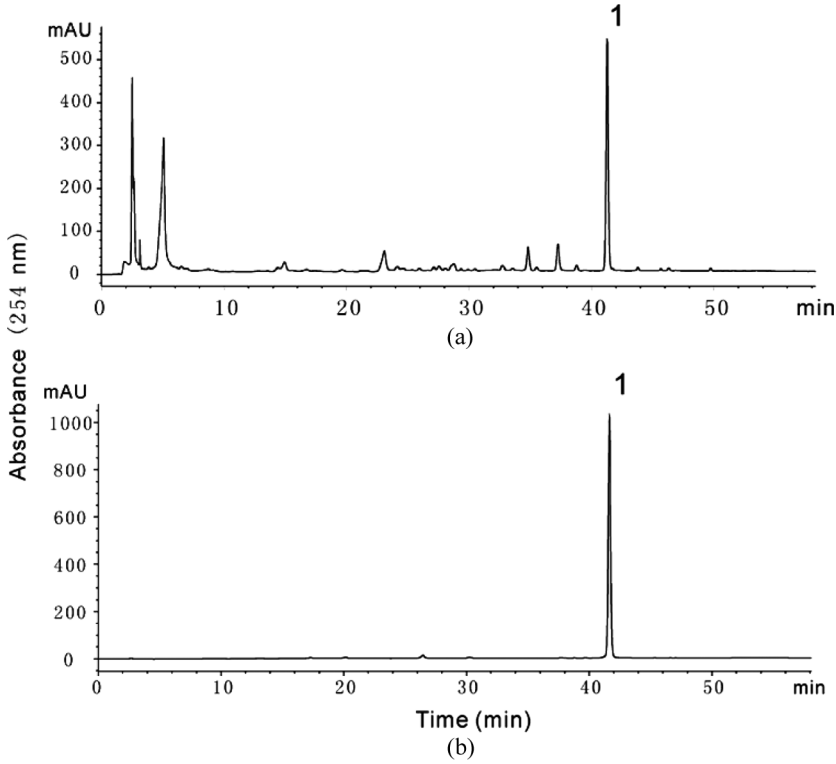


Figure 3. The chromatograms of crude extract by the optimal conditions (a) and standard solution of mollugin (b). Peak 1: mollugin. HPLC conditions: column, Agilent Zorbax Eclipse XDB-C8 (150mm × 4.6 mm i.d., 5 μm, 120 Å); gradient, A (methanol) from 45 to 95% and B (water) from 55 to 5% for 50 min; flow rate, 0.6 mL/min; column temperature, room temperature; UV wavelength, 254 nm.

with RSD lower than 4.5%). The method validation studies indicated that the present method provides good recovery and reasonable precision.

CONCLUSION

An efficient MAE method has been developed for the extraction and quantification of mollugin from TCM *R. cordifolia*. The optimum MAE conditions were found to be: ethanol water solution of 70% (v/v) as extraction solvent, microwave power of 460 W, extraction time of 4 min, solid-liquid ratio of 1:20 (g/mL). The results indicate that this extraction procedure is efficient and precise. Compared to the conventional

techniques such as heat reflux extraction (HRE), ultrasonic-assisted extraction (UAE), and Soxhlet extraction, the proposed MAE procedure reduced extraction time and obtained high efficiency of mollugin, which showed great potential for efficient sample preparation and large-scale industrial application in the near future.

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