Optimization of Compound Coptis Ointment Extraction through the Integration of Pharmacodynamic Indices and Orthogonal Test

Jin ZHOU, Xiuli ZHOU, Yu ZHOU, Ming MEI, Qiang GAO, Xinguo LIU*

Department of Pharmacy, Wuhan No.1 Hospital, Wuhan 430022, China

Abstract [**Objectives**] To determine the optimal reflux extraction process conditions for Compound Coptis Ointment. [**Methods**] The study employed the orthogonal experimental design method and drug-sensitive disc agar diffusion method to evaluate the extraction rate, berberine hydrochloride content, and bacteriostatic ring diameter of the extract as comprehensive indices, and optimized the extraction process conditions of Compound Coptis Ointment using ethanol reflux. [**Results**] Based on the results of comprehensive indices, the optimal reflux extraction conditions for Compound Coptis Ointment were determined to be soaking in 1.5 times the amount of 70% ethanol for 2 h, followed by two extractions with 6 times the amount of 70% ethanol for 1 h each time. Three verification tests were conducted under the optimal process conditions. The yield of the extract was 28.32% ±0.53%, the content of berberine hydrochloride was 4.68% ±0.45%, and the diameter of the bacteriostatic ring was (2.5 ±0.2) cm. [**Conclusions**] The extract had higher drug content and exhibited better antibacterial effects. The optimized extraction process is simple, stable, and reliable, and can be effectively used to optimize the extraction process of Compound Coptis Ointment.

Key words Compound Coptis Ointment, Orthogonal test, Antibacterial test, Extraction process

1 Introduction

Skin ulcer is a common clinical disease in dermatology, characterized by localized skin or mucosal defects that can extend to the dermis or deeper layers, primarily caused by infection, injury, tumors, vasculitis, and other factors[1-5]. Compound Coptis Ointment, a classic prescription in traditional Chinese medicine for treating skin ulcer disease, has a long history of application and widespread use in various departments of Chinese hospitals[6]. The main ingredients in Compound Coptis Ointment are Rhizoma Coptidis and Cortex Phellodendri. Rhizoma Coptidis is rich in berberine, jatrorrhizine, and other components, which exhibits strong antibacterial, anti-endotoxin, antiviral, anti-inflammatory, and anti-diarrheal effects[7-9]. Cortex Phellodendri contains berberine, jatrorrhizine, magnolia, phellodendrine and other compounds, which possesses antibacterial, anti-inflammatory, immune-enhancing, and anti-ulcer properties. Therefore, the extraction rate of berberine hydrochloride directly influences the content of active ingredients and the therapeutic effect of the medication[9-10].

Moreover, previous experimental studies have indicated that the mechanism of traditional Chinese medicine's external application in treating skin ulcers might be associated with its antibacterial and anti-inflammatory effects, improvement of wound ischemia and hypoxia, promotion of wound blood circulation, augmentation of wound fibroblast count, and regulation of type I and III collagen functions[11-14]. In this study, we utilized the extraction rate, berberine hydrochloride content, and antibacterial activity of the extract as comprehensive indices. Additionally, we employed the orthogonal test design method to systematically assess the impact of process conditions on the reflux extraction effectiveness of the ointment.

2 Materials and methods

2.1 Instruments High-performance liquid chromatography, Shimadzu Company; Analytical column (250 mm × 4.6 mm, 5 μm), Shimadzu International Trading (Shanghai) Co., Ltd.; Pure water treatment system, Millipore Company; Electronic analytical balance (Max 210 g, d = 0.01 mg), Sartorius Company; Electric heating sleeve, Beijing Yongguangming Medical Instrument Co., Ltd.; Digital display constant temperature water bath pot, Changzhou Aohua Instrument Co., Ltd.; Ultrasonic cleaner, Kunshan Ultrasonic Instrument Co., Ltd.; Isolated constant temperature incubator, Shanghai Qixin Scientific Instrument Co., Ltd.; Clean bench, Suzhou Xiangyu Purification Technology Co., Ltd.

2.2 Reflux extraction methods In the prescription, Cortex Phellodendri, Rhizoma Coptidis, Rhizoma Curcumae and other medicinal materials contain more liposoluble components. Therefore, different concentrations of ethanol were used to extract medicinal materials. Preliminary experiments showed that ethanol concentration, solvent dosage, extraction time, and extraction times had a significant influence on the extraction effect of decoction pieces. Hence, the above four factors were chosen as the research targets. We accurately weighed 240 g of Compound Coptis Ointment powder with a particle size less than 0.178 mm and added 1.5 times the amount of solvent for soaking for 2 h. The $L_9(3^4)$ orthogonal experiment was designed with 90 ℃ water bath

Received: June 10, 2023 Accepted: September 5, 2023

Supported by Key Research Project of Wuhan Municipal Health Commission (WZ21A08).

Lead Author: Jin ZHOU (1983 -), male. Contact: Phone: 18627015425, E-mail: 52483033@qq.com.

*Corresponding Author: Xinguo LIU (1980 -), male. Contact: Phone: 13628661063, E-mail: lxgdata@126.com.

reflux as the fixed condition. The reflux extraction process was optimized using extraction rate, berberine hydrochloride content and bacteriostatic ring diameter as indices. The factor level design of the orthogonal experiment is presented in Table 1.

Table 1 Orthogonal test factor level of reflux extraction process of Compound Coptis Ointment

Level	Factor			
	Ethanol concentration A//%	Solvent amount B//mv	Extraction time C//min	Extraction times D
1	50	1∶6	30	1
2	60	1∶8	45	2
3	70	1∶10	60	3

2.3 Extraction rate determination Experiments were conducted following the conditions listed in Table 1, and reflux extraction solutions under different extraction conditions were obtained. These reflux extractions were concentrated to 400 mL, and 100 mL of the concentrated extraction solution was accurately taken. The concentrated extraction solution was placed in an evaporator with constant weight and dried in a water bath. The concentrate was then transferred to a vacuum drying oven at 105 ℃ for 6 h. After reaching constant weight, it was precisely weighed, and the extract yield (%) was calculated as follows:

Extract yield (%) = [(Weight of pan + Dry extract) − Weight of pan] × (400/100)/Total weight of slices × 100%

2.4 Determination of berberine hydrochloride content

2.4.1 Preparation of reference solution. An appropriate amount of berberine hydrochloride reference substance was taken, precisely weighed, and dissolved in methanol to make a 1 mL solution containing 60.2 μg of berberine.

2.4.2 Preparation of test solution. One gram of dry extract was precisely weighed and dissolved in methanol to a volume of 100 mL in a flask. The solution was shaken uniformly, and then filtered through a 0.45 μm microporous membrane to obtain the filtered solution.

2.4.3 Preparation of negative control solution. Medicinal materials, except Rhizoma Coptidis and Cortex Phellodendri, were weighed and prepared using the same method as the negative control samples of Rhizoma Coptidis and Cortex Phellodendri. Negative control solutions of Rhizoma Coptidis and Cortex Phellodendri were prepared according to the method in Section **2.4.2**.

2.5 Conditions for HPLC Chromatographic column: octadecyl silane bonded silica gel column (250 mm × 4.6 mm, 5 μm); mobile phase: acetonitrile-0.03 mol/L sodium dihydrogen phosphate-phosphoric acid solution (23∶77∶0.1); flow rate: 1.0 mL/min; column temperature: 30 ℃; injection volume: 10 μL; detection wavelength: 265 nm; theoretical plate number should not be less than 3 000 according to berberine hydrochloride.

2.6 Determination of antibacterial activity *in vitro*[4-5]

2.6.1 Preparation of bacterial liquid. Using sterile techniques, a small amount of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* were selected from the inoculation ring and inoculated on the slant of nutrient agar medium. After smearing the bacterial solution evenly, the bacteria were cultured in a 37 ℃ thermostat for 24 h. Four to five bacterial colonies were selected in sterile 0.9% sodium chloride solution test tubes, and the concentration was diluted to the same concentration as a 0.5 McFarland turbidity tube (1.5×10^8 CFU/ mL).

2.6.2 Preparation of drug sensitive paper. Filter paper with strong water absorption was selected to make several circular paper sheets with a diameter of 6 mm. A clean test tube was sterilized at 121 ℃ for 20 min and then placed into a constant temperature drying oven. Several filter paper pieces were taken with sterilized tweezers and immersed in the extract of Compound Coptis Ointment, 0.9% sodium chloride solution, and chlorhexidine acetate solution, respectively. After the drug solution was completely absorbed by the paper, the multi-drug solution was gently brushed off. The drug-sensitive paper was then saved.

2.6.3 Determination of antibacterial activity. Three types of bacteria were swabbed on the ultra-clean table using sterilized cotton swabs. After rotating the inner wall of the tube to extrude the bacterial solution, the plate was evenly coated on the surface of an MH agar plate three times, rotating the plate by 60° each time to spread the bacterial solution as evenly as possible. Finally, a circle was spread along the inner wall of the plate for 5 min. The drug-containing filter paper was placed on the agar surface of the petri dish with sterile tweezers, and the name of the drug solution was marked outside the petri dish. The plate was then covered, inverted in a 35 ℃ incubator, and cultured. Penicillin and ceftazidime solution were used as positive controls, and 0.9% sodium chloride solution was used as a negative control. After 24 h, the growth of bacteria was observed, and the diameter of inhibition zone was measured.

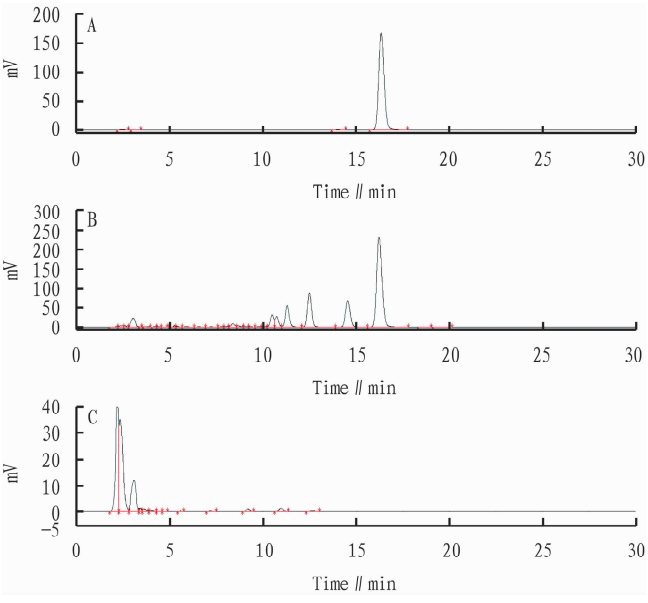
2.7 Calculation of index comprehensive score The extract yield, berberine hydrochloride content, and inhibition zone diameter in the concentrated extraction were determined under various conditions of orthogonal test.

Comprehensive score was calculated according to the following formula: Comprehensive score = (Extract yield/ Maximum extract yield) × 25% + (Berberine hydrochloride content/Maximum berberine hydrochloride content) × 25% + (Bacteriostatic ring diameter score/Maximum bacteriostatic ring diameter score) × 50%. And the comprehensive score was statistically analyzed.

3 Results and analysis

3.1 Berberine content determination Fig. 1 showed that both berberine hydrochloride reference substance and test solution exhibited a berberine chromatographic peak at about 16 min, while the negative control had no interference. The methodological results demonstrated that berberine exhibited a good linear relationship within the range of 0.602 − 6.020 μg, with a linear regression equation (R^2 = 0.999 9). The precision *RSD* of this method was 0.61% (n = 6), and the recovery rate was 98.4%.

3.2 Antimicrobial susceptibility test *in vitro* The results of *in vitro* antibacterial rate test are presented in Fig. 2. The Compound Coptis Ointment extract had a significant inhibitory effect on *S. aureus*, *E. coli* and *P. aeruginosa*.

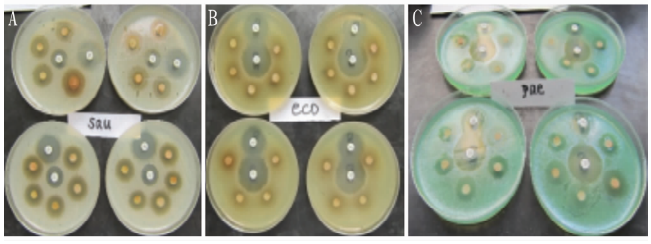


Note: A. Berberine hydrochloride reference solution; B. Sample solution; C. Negative control solution.

Fig. 1 HPLC diagram for the specificity of the determination method of berberine hydrochloride content

3.3 Optimum results of alcohol extraction process

$L_9(3^4)$ orthogonal experimental design was employed to investigate the alcohol extraction process of drugs. The results are shown in Table 2.



Note: A. Antibacterial effect of *Staphylococcus aureus*; B. Antibacterial effect of *Escherichia coli*; C. Antibacterial effect of *Pseudomonas aeruginosa*.

Fig. 2 Antibacterial effect of Compound Coptis Ointment extract *in vitro*

The data in Table 2 were processed to obtain Table 3. Using the comprehensive score as the evaluation index, the range R and variance analysis showed that the order of factors affecting the reflux effect was C > A > D > B. Therefore, the experimental conditions of $A_3B_1C_3D_2$ were determined to be better. According to the results of comprehensive indices, the optimal reflux extraction conditions of Compound Coptis Ointment were determined as follows: 1.5 times the amount of 70% ethanol for 2 h, and then 6 times the amount of 70% ethanol for 2 times, 1 h each time.

Table 2 Alcohol extraction orthogonal test

Test No.		A	B	C	D	Extraction rate//%	Determination of berberine hydrochloride//%	Inhibition zone diameter (fraction)	Conflation
1		1	1	1	1	25.61	1.42	2.6	44.88
2		1	2	2	2	26.22	3.15	3.8	61.09
3		1	3	3	3	28.02	2.64	5.9	72.02
4		2	1	2	3	27.74	3.41	5.4	72.87
5		2	2	3	1	26.96	4.89	6.6	86.55
6		2	3	1	2	24.40	2.51	4.3	53.35
7		3	1	3	2	26.37	3.33	8.8	90.55
8		3	2	1	3	25.46	2.79	4.1	60.28
9		3	3	2	1	24.15	3.18	6.3	73.60
Comprehensive score	K_1	177.99	208.30	164.19	205.03				
	K_2	218.45	207.92	207.56	210.67				
	K_3	224.43	204.65	249.12	205.17				
	R	46.440	3.650	84.93	5.64				

Note: The diameter of bacteriostatic ring (0 – 3 cm) is uniformly divided into 0 – 10 points.

Table 3 Analysis of variance of comprehensive score

Source of error	Square sum of deviations	F	P
A	425.494 0	158.504 9	<0.01
B	2.684 4	1.000 0	–
C	1 202.366 2	447.905 0	<0.01
D	6.897 7	2.569 5	>0.05

3.4 Validation test of optimum experimental conditions

Three verification tests were carried out according to the final extraction process, and the extract yield, berberine hydrochloride content and bacteriostatic ring diameter were determined. The verification test showed that the optimized process had good repeatability. The yield of extract was 28.32% ±0.53%, the content of berberine hydrochloride was 4.68% ±0.45%, and the diameter of bacteriostatic ring was (2.5 ±0.2) cm. The bacteriostatic effect was remarkable.

4 Discussion

The treatment of skin ulcers in western medicine mainly focuses on wound infection prevention and treatment and wound protection, which is insufficient compared to the treatment of traditional Chinese medicine. For instance, long-term use of systemic antibiotics can lead to drug resistance. In China, there are few clinical studies and applications on the external treatment of skin ulcers,

especially for large-scale trauma. Surgical debridement and skin grafting are often used for patients with intractable pain and high economic costs. The use of various dressings and long treatment courses can lead to poor clinical efficacy.

Compound Coptis Ointment is a traditional Chinese medicine with dermatological characteristics. In clinical practice, it is commonly used to treat skin diseases such as siltation dermatitis, eczema, herpes disease, frostbite, severe drug eruption, gangrenous pyodermatitis, radioactive dermatitis, and bedsore. The clinical efficacy has been unanimously accepted by patients. Rhizoma Coptidis and Cortex Phellodendri have the effect of clearing away heat-evil, eliminating dampness, and detoxification. Modern medical studies have shown that Rhizoma Coptidis and Cortex Phellodendri have strong inhibitory effects on *S. aureus*, *E. coli*, *B. anthracis*, and *S. hemolyticus*[15-16]. Recent research shows that angelica has the function of regulating platelets and anticoagulation, and fresh rehmannia has anti-inflammatory and anti-allergic effects and inhibits the growth of various fungi[17]. Radix Angelica Sinensis and Radix Rehmanniae have antibacterial, anti-inflammatory, moisturizing, and astringent effects, and their components are mainly condensed tannins, which are clinically safe. Curcumin has such effects as lowering lipids, inhibiting platelet aggregation, and enhancing the activity of plasminogen[18-19]. Curcumin and angelica can promote blood circulation and remove blood stasis. Modern pharmacological studies have shown that drugs promoting blood circulation and removing blood stasis can improve the microcirculation of ulcer surfaces and promote wound healing[20-21].

Currently, the active ingredients in medicinal powder are not only low in content but also difficult to control microorganisms, leading to unstable quality standards and inconvenient dressing changes. To better apply Compound Coptis Ointment to clinical practice, this study deeply analyzed the effective components contained in medicinal materials and combined them with efficacy indices to objectively compare the effects of different reflux conditions on the extraction of effective components. Further pharmacological and pharmacodynamic studies were carried out to develop the traditional prescription into a new Chinese medicine with high efficiency, stability, and convenience.

5 Conclusions

The optimized extraction process is rational and reliable, suitable for preparation production. The multi-index design, combining chemical and biological activities, provides a methodological reference for the optimization of the extraction process of classical herbal prescriptions.

References

[1] YANG H, WANG WS, TAN Y, *et al.* Investigation and analysis of the characteristics and drug sensitivity of bacteria in skin ulcer infections[J]. Chinese Journal of Traumatology (English Edition), 2017, 20(4): 194-197.

[2] TAKIKAWA M, NAMBU M, YAMAMOTO N, *et al.* Radiation-induced skin injury on the upper arm following cardiac interventional radiology: A review and case report[J]. Wounds a Compendium of Clinical Research & Practice, 2012, 24(4): 91-98.

[3] LEE HC, NIU KC, CHEN SH, *et al.* Hyperbaric oxygen therapy in clinical application. A report of a 12-year experience[J]. Zhonghua Yi Xue Za Zhi (Taipei), 1989, 43(5): 307-316.

[4] NOBUHIRO U, TOMONORI T, TAKESHI M, *et al.* An unexpected skin ulcer and soft tissue necrosis after the nonconcurrent combination of proton beam therapy and pazopanib: A case of myxofibrosarcoma[J]. Auris Nasus Larynx, 2017, 44(4): 484-488.

[5] ASHIDA A, MURATA H, MIKOSHIBA Y, *et al.* Successful treatment of rheumatoid vasculitis-associated skin ulcer with a TNF-α antagonist[J]. International Journal of Dermatology, 2014, 53(2): e154-e156.

[6] ZHANG XF, SUN GF, CHEN YF, *et al.* Effects of Huanglian ointment on wound healing of mice with full-thickness skin defect and the related mechanism[J]. Zhonghua shao shang za zhi = Zhonghua shaoshang zazhi = Chinese journal of burns, 2016, 32(12): 714-720.

[7] MULUYE RA, BIAN Y, ALEMU PN. Anti-inflammatory and antimicrobial effects of heat-clearing Chinese herbs: A current review[J]. Journal of Traditional and Complementary Medicine, 2014, 4(2): 93-98.

[8] WU J, LUO Y, DENG D, *et al.* Coptisine from Coptis Chinensis exerts diverse beneficial properties: A concise review[J]. Journal of Cellular and Molecular Medicine, 2019, 23(12): 7946-7960.

[9] ALESSRA, BAGGIONI, ARRIGO, *et al.* Berberine and its role in chronic disease[J]. Advances in Experimental Medicine & Biology, 2016 (928): 27-45.

[10] UCHIYAMA T, KAMIKAWA H, OGITA Z. Anti-ulcer effect of extract from phellodendri cortex[J]. Yakugaku Zasshi Journal of the Pharmaceutical Society of Japan, 1989, 109(9): 672-676.

[11] LIN Y, HE X, XIE X, *et al.* Huiyang shengji extract improve chronic nonhealing cutaneous through the TGF-β1/Smad3 signaling pathway[J]. Evidence-based Complementary and Alternative Medicine, 2021: 8881565.

[12] KIM M, LEE HJ, RANDY A, *et al.* *Stellera chamaejasme* and its constituents induce cutaneous wound healing and anti-inflammatory activities [J]. Scientific Reports, 2017(7): 42490.

[13] MA J, YIN C, MA S, *et al.* Shensong Yangxin capsule reduces atrial fibrillation susceptibility by inhibiting atrial fibrosis in rats with post-myocardial infarction heart failure[J]. Drug Design, Development and Therapy, 2018(12): 3407-3418.

[14] MA J, MA SY, YIN CX, *et al.* Shengmai San-derived herbal prevents the development of a vulnerable substrate for atrial fibrillation in a rat model of ischemic heart failure[J]. Biomedicine & pharmacotherapy, 2018(100): 156-167.

[15] SUN XD, LIU ZX, FAN M, *et al.* Study on the antibacterial effect of compound *Phellodendron amurense* liquid *in vitro*[J]. Electronic Journal of Clinical Medical Literature, 2020, 7(9): 9-10

[16] WANG HF, SHANG ZQ, GUI ZH. Antibacterial activity *in vitro* of traditional Chinese herb coptidis rhizoma on four common clinical drug-resistant strains[J]. China Pharmacist, 2018, 21(9): 1662-1664.

[17] ZHANG LB, LU JL, LIU JW. Phthalide derivatives with anticoagulation activities from *Angelica sinensis*[J]. Journal of Natural Products, 2016, 79(7): 1857-1861.

[18] LIN Z, TIWARI JK, DONG J, *et al.* Traditional Chinese medicine for senile dementia[J]. Evidence-based Complementary and Alternative Medicine, 2012, 2012:692621.

[19] CAO R, ZHANG H, GUO J, *et al.* A novel pharmacological method to study the Chinese medicinal formula Hua-Zheng-Hui-Sheng-Dan [J]. Evidence-Based Complementray and Alternative Medicine, 2015 (2015): 1-11.

[20] CHEN T, ZHU Z, DU Q, *et al.* A skin lipidomics study reveals the therapeutic effects of Tanshinones in a rat model of acne[J]. Frontiers in pharmacology, 2021(12): 675659.

[21] NIA JJ, NICOLA I, VICKY E, *et al.* Neuromuscular electrostimulation on lower limb wounds[J]. British Journal of Nursing: BJN, 2018, 27 (20): S16-S21.