Optimization of the Extraction Process of Anti-*Vibrio parahaemolyticus* Active Substances from Coptidis Rhizoma (Huanglian)

Xintong Wang\(^{1,2,3}\), Siya Guo\(^{1,2,3}\), Zongyi Zhang\(^{1,2}\), Qian Xu\(^{1,2,3}\) and Lei Guo\(^{1,2,3*}\)

1 Jiangsu Key Laboratory of Marine Bioresources and Environment, Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang 222005, China
2 Jiangsu Key Laboratory of Marine Biotechnology, Jiangsu Ocean University, Lianyungang 222005, China
3 Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening, Jiangsu Ocean University, Lianyungang 222005, China
Email: guol@jou.edu.cn

**Abstract.** The purpose of this study is to optimize the extraction process of the anti-*Vibrio parahaemolyticus* active substances from Coptidis rhizoma (Huanglian). Single factor experiment, Box-Behnken experimental design combined with response surface methodology were used to optimize the extraction process. The obtained optimal extraction conditions followed as: extraction solvent is 100% methanol, extraction temperature of 86 °C, liquid/material ratio of 21 mL/g, extraction time of 116 min. The actual value of the inhibition zone of the extract can reach 15.62± 0.16 mm, which is not significantly different from the predicted value of 15.12 mm. This indicates that the above method is feasible to optimize the extraction process of the anti-*V. parahaemolyticus* active substances from Coptidis rhizoma.

**1. Introduction**

*Vibrio parahaemolyticus* is a gram-negative halophilic bacterium and a high incidence of food-borne pathogens. It is the main cause of food-borne diarrhea and global aquatic product-related infectious diarrhea, which often exists in coastal environments and aquatic products [1,2]. *V. parahaemolyticus* was first discovered in a food poisoning outbreak in Osaka in 1950. In Taiwan, Japan, Southeast Asia and other regions, food poisoning incidents caused by *V. parahaemolyticus* account for more than half of all food poisoning incidents [3]. In addition, the outbreak of *V. parahaemolyticus* infection in the United States has increased. It is estimated that about 30,000 people are hospitalized for gastrointestinal diseases caused by *V. parahaemolyticus* each year, and about 5,000 of them die from *V. parahaemolyticus* infection [4]. At present, food-borne diseases caused by *V. parahaemolyticus* have surpassed *Salmonella*, ranking first on the list of food-borne pathogens, becoming one of the most widely distributed and common diseases in the world, and it is one of the public health safety issues that are highly valued at home and abroad [5]. Nowadays, the treatment of foodborne diseases caused by *V. parahaemolyticus* mainly depends on the use of antibiotics, but the long-term use of antibiotics can lead to the emergence of drug resistance in the human body and seriously endanger human health and life safety [6]. Therefore, it is particularly important to find high-efficiency, low-toxicity, safe and green antibiotic substitutes in today's society.

Coptidis rhizoma, also known as "Huanglian" in Chinese, is a traditional Chinese medicine, which is the dried rhizomes of *Coptis chinensis* Franch., *C. deltoidea* C. Y. Cheng et Hsiao, or *C. teeta* Wall.
Huanglian has antibacterial, antiviral, antioxidant, hypoglycemic, anti-inflammatory and cholinesterase inhibition effects [7-10]. In the present study, the purpose is to optimize the extraction process of the anti-\textit{V. parahaemolyticus} active substances from \textit{Coptidis rhizoma} (Huanglian). Based on the single factor experiment, the extraction process conditions were optimized by Box–Behnken experimental design (BBD) combined with response surface methodology (RSM).

2. Materials and Methods

2.1. Materials and Chemicals

\textit{Coptidis rhizoma} (Huanglian) was purchased from Bozhou Zhongyitang Traditional Chinese Medicine Co., Ltd. \textit{Vibrio parahaemolyticus} is preserved in the Laboratory of Marine Natural Products Chemistry, Jiangsu Ocean University (Lianyungang, China). Methanol (HPLC grade) and all other chemicals (analytical grade) were purchased from Sinapharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Single-Factor Experiment Assay

Accurately weigh 1 g of Huanglian powder that has been crushed and passed through a 40-mesh sieve to extract antibacterial active substances, and investigate the effects of single factor on the anti-\textit{V. parahaemolyticus} activity of the extract. The single factor experiment settings are as follows:

1. Effect of extraction solvent on the antibacterial activity of the extract. Put 1 g of Huanglian powder into a 250 mL conical flask, add 20 mL of different extraction solvents (distilled water, methanol, 50% methanol, ethanol and 50% ethanol), and extract for 120 min in a water bath at a constant temperature of 80 °C.

2. Effect of different concentrations of methanol. Put 1 g of Huanglian powder into a 250 mL conical flask, add 20 mL of different concentrations of methanol (60%, 70%, 80%, 90%, 100%), and extract for 120 min in a water bath at a constant temperature of 80 °C.

3. Effect of extraction temperature. Put 1 g of Huanglian powder into a 250 mL conical flask, add 20 mL of 100% methanol, and extract at different temperatures (60 °C, 70 °C, 80 °C, 90 °C, 100 °C) for 120 min.

4. Effect of extraction time. Put 1g of Huanglian powder into a 250 mL conical flask, add 20 mL of 100% methanol, and extract at 90 °C for different times (60 min, 90 min, 120 min, 150 min, 180 min).

5. Effect of liquid/material ratio. Put 1 g of Huanglian powder into a 250 mL conical flask, add different volumes (10 mL, 20 mL, 30 mL, 40 mL, 50 mL) of 100% methanol, and extract at 90 °C for 120 min.

After each step of extraction is completed, the extract is filtered with a Buchner funnel, and the supernatant obtained is diluted or concentrated to 20 mL. Then, the diameter of the inhibition zone against \textit{V. parahaemolyticus} is measured to select the single factor conditions for further optimization.

2.3. Extraction Process Optimization Assay

BBD and RSM were adopted to determine the optimal process conditions for the extraction of antibacterial active substances from Huanglian. Design Expert 8.0.0 (Stat-Ease, Minneapolis, USA) was used for data analysis and model building [11]. BBD consists of 12 factorial points and 5 center points. Extraction temperature ($X_1$), extraction time ($X_2$) and liquid/material ratio ($X_3$) are used as independent variables, and the diameter of the inhibition zone of the extract against \textit{V. parahaemolyticus} is used as the dependent variable ($Y$, mm). Table 1 lists the ranges and levels of the three independent variables, and the values of the dependent variable.

The extraction process is optimized by the software to achieve the purpose of obtaining the largest diameter of the inhibition zone. The model of the system is evaluated by the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_i X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

(1)
Where $Y$ represents the predicted response, $\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are constant coefficients, and $X_i$ and $X_j$ are independent variables.

2.4. Antibacterial Activity Assay
The Oxford cup method was used to evaluate the inhibitory activity of the active extract against V. parahaemolyticus [12]. 20 mL of MH broth medium (2.0 g beef powder, 1.5 g soluble starch, 17.5 g acid hydrolyzed casein, 20 g agar powder) was added into the Petri dish. After the medium has solidified, 200 µL of V. parahaemolyticus suspensions ($1 \times 10^6$ cfu/mL) was spread evenly on the plate, then the Oxford cups were placed on the culture medium and 200 µL of the extracts were added into the cup. The Petri dishes were incubated at 32 °C for 24 h, then the diameters of inhibition zone were measured by the electronic vernier caliper.

3. Results and Discussion

3.1. The Effects of Single Factor on the Anti-V. Parahaemolyticus Activity of the Extract
This experiment studied the antibacterial effect of the traditional Chinese herbal medicine Coptidis rhizoma (Huanglian) on the food-borne pathogen V. parahaemolyticus. Before optimizing the experiment, the key factors that independently affect the antibacterial activity of the extract were studied, including extraction solvent, solvent concentration, extraction temperature, extraction time and liquid-to-material ratio.

The diameters of the inhibition zone of the extracts with different extraction solvents (distilled water, methanol, 50% methanol, ethanol and 50% ethanol) against V. parahaemolyticus were 10.40±0.16 mm, 14.86±0.64 mm, 12.40±0.20 mm, 14.66±0.30 mm and 11.85±0.82 mm, respectively. Therefore, the effect of different concentrations of methanol on the antibacterial activity of the extract was further studied (Fig. 1). It shows that when 100% methanol is used as the extraction solvent, the extract has the best antibacterial activity, which indicates that the active substances have a certain degree of hydrophobicity.

Fig. 2 shows the influence of different temperatures on the diameter of the bacteriostatic zone of the extract, showing that the diameter of the bacteriostatic zone of the extract increases with the increase of temperature from 60 °C to 90 °C. It shows that the increase in temperature will reduce the viscosity of the extract and increase the solubility of the effective substances. However, too high temperature may cause the decomposition of active substances and reduce the antibacterial effect, so 90 °C is chosen as the center point of RSM.

Fig. 3 shows the effect of different extraction times on the diameter of the bacteriostatic zone of the extract. It can be seen that the diameter of the bacteriostatic zone rises faster when it rises from 90 min to 120 min, and the bacteriostatic effect is slightly reduced at the longer time. Therefore, 120 min is chosen as the RSM center point.

Fig. 4 shows the effect of different liquid/material ratios on the diameter of the inhibitory zone of the extract. It can be seen that the diameter of the inhibition zone increases when the liquid/material ratio enhances from 10:1 mL/g to 20:1 mL/g. After that, the diameter of the inhibition zone no longer increases with the increase of the liquid/material ratio, so 20:1 mL/g is selected as the RSM center point.
3.2. Optimization of the Extraction Conditions

According to the results of the single factor test, the BBD was performed, and the extraction temperature ($X_1$), extraction time ($X_2$) and liquid/material ratio ($X_3$) were selected as independent variables for further optimization. The operating parameter codes and response values of BBD are shown in Table 1. Based on the analysis of the parameters, RSM can be used to estimate the empirical relationship between the response variable and the test variables. Through the multiple regression analysis of the experimental data, the equation (2) is established as follows:

$$Y = 15.14 - 0.19X_1 - 0.0037X_2 + 0.074X_2X_3 + 0.12X_1X_3 - 0.48X_2X_3 - 0.9X_1^2 - 0.37X_3^2$$

(2)

Table 2 shows the analysis of variance (ANOVA) of the results of the BBD experiment. The coefficient of determination $R^2$ (0.9799) of the model is very close to 1, which shows that the regression model fully defines the real behavior of the system [13]. $P$ value $< 0.0001$ indicates that the fitness of the model is significant, that is, the relationship between the independent variables and the dependent variable of the regression model is significant. The lack of fit value of the model is 0.3541, indicating that the difference is not significant, and it is concluded that the degree of fitting of the quadratic polynomial regression equation is relatively good, which has statistical significance. At the same time, it is pointed out that the interaction of extraction temperature and time, the interaction of time and liquid/material ratio, and the secondary effect of the three factors are very significant ($P < 0.01$), which shows that the relationship between each factor and the response value is not a simple linear relationship.
Through the prediction of the model, the best extraction parameters were obtained: \( X_1 = 86 \, ^\circ C \), \( X_2 = 116 \, \text{min} \), \( X_3 = 21 \, \text{mL/g} \), and the predicted value of the inhibition zone diameter was 15.18 mm. Using the above optimized conditions for the verification test, the average value of the inhibition zone was 15.62± 0.16 mm (n = 3), which was not significantly different from the predicted value. This reveals that RSM has a good feasibility for the extraction optimization of anti-\textit{V. parahaemolyticus} active substances from Coptidis rhizoma (Huanglian).

Table 1. Coding (actual) levels of operating parameters and observed values by BBD.

| No. | \( X_1 \) (\(^\circ C\)) | \( X_2 \) (h) | \( X_3 \) (mL/g) | \( Y \) (mm) |
|-----|-----------------|-------------|----------------|----------|
| 1   | –1 (60)         | –1 (120)    | 0 (10)         | 14.37    |
| 2   | +1 (100)        | –1 (90)     | 0 (20)         | 13.41    |
| 3   | –1 (80)         | +1 (150)    | 0 (20)         | 13.77    |
| 4   | +1 (100)        | +1 (150)    | 0 (20)         | 14.17    |
| 5   | –1 (80)         | 0 (120)     | –1 (10)        | 14.73    |
| 6   | +1 (100)        | 0 (120)     | –1 (10)        | 14.01    |
| 7   | –1 (80)         | 0 (120)     | +1 (30)        | 14.67    |
| 8   | +1 (100)        | 0 (120)     | +1 (30)        | 14.44    |
| 9   | 0 (90)          | –1 (90)     | –1 (10)        | 13.44    |
| 10  | 0 (90)          | +1 (150)    | –1 (10)        | 14.18    |
| 11  | 0 (90)          | –1 (90)     | +1 (30)        | 14.52    |
| 12  | 0 (90)          | +1 (150)    | +1 (30)        | 13.32    |
| 13  | 0 (90)          | 0 (120)     | 0 (20)         | 15.15    |
| 14  | 0 (90)          | 0 (120)     | 0 (20)         | 15.03    |
| 15  | 0 (90)          | 0 (120)     | 0 (20)         | 15.03    |
| 16  | 0 (90)          | 0 (120)     | 0 (20)         | 15.13    |
| 17  | 0 (90)          | 0 (120)     | 0 (20)         | 15.34    |

Table 2. Analysis of variance for response surface quadratic model \(^a\).

| Source     | Sum of Squares | df | Mean Square | F Value | Prob > F | Sig.  |
|------------|----------------|----|-------------|---------|----------|-------|
| Model      | 6.56           | 9  | 0.73        | 37.99   | < 0.0001 | **    |
| \( X_1 \)  | 0.29           | 1  | 0.29        | 14.87   | 0.0062   | **    |
| \( X_2 \)  | 0.011          | 1  | 0.011       | 0.59    | 0.4687   |       |
| \( X_3 \)  | 0.044          | 1  | 0.044       | 2.27    | 0.1756   |       |
| \( X_1 X_2 \) | 0.46          | 1  | 0.46        | 24.12   | 0.0017   | **    |
| \( X_1 X_3 \) | 0.06          | 1  | 0.06        | 3.13    | 0.1201   |       |
| \( X_2 X_3 \) | 0.94          | 1  | 0.94        | 49.08   | 0.0002   | **    |
| \( X_1^2 \) | 0.39           | 1  | 0.39        | 20.33   | 0.0028   | **    |
| \( X_2^2 \) | 3.42           | 1  | 3.42        | 178.6   | < 0.0001 | **    |
| \( X_3^2 \) | 0.57           | 1  | 0.57        | 29.95   | 0.0009   | **    |
| Lack of Fit | 0.07           | 3  | 0.023       | 1.45    | 0.3541   |       |

\(^a\) *P < 0.05, **P < 0.01.

4. Conclusion
In conclusion, based on the results of single factor experiments, the Box–Behnken experimental design combined with response surface methodology was used to determine the optimal extraction parameters of anti-\textit{V. parahaemolyticus} active substances from Coptidis rhizoma (Huanglian) followed as: the extraction solvent was 100% methanol, the extraction temperature was 86 \(^\circ C\), the...
liquid-to-material ratio was 21 mL/g, and the extraction time was 116 min. The results provided a basis for further chemical identification and application of anti-*V. parahaemolyticus* active substances from Coptidis rhizoma (Huanglian).

5. Acknowledgments
This work financially supported by the Natural Science Foundation of Jiangsu Higher Education Institutions of China (19KJB350007), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and Postgraduate Research & Practice Innovation Program of Jiangsu Province (SJCX19_1001).

6. References
[1] Deepanjali A, Kumar HS, Karunasagar I, and Karunasagar I 2005 *Appl. Environ. Microbiol.* 71 3575-3580
[2] Martinez-Urtaza J, Lozano-Leon A, Varela-Pet J, Trinanes J, Pazos Y, and Garcia-Martin O 2008 *Appl. Environ. Microbiol.* 74 265-274
[3] Pan TM, Wang TK, Lee CL, Chien SW, and Horng CB 1997 *J. Clin. Microbiol.* 35 1260-1262
[4] Yeung PS, and Boor KJ 2004 *Foodborne Pathog. Dis.* 1 74-88
[5] Wang W, Li M, and Li Y 2015 *J. Food Sci.* 80 R10-19
[6] Nicolaou KC, and Rigol S 2018 *J. Antibiot.* 71 153-184
[7] Cao TQ, Ngo QT, Seong SH, Youn UJ, Kim JA, Kim J, Kim JC, Woo MH, Choi JS and Min BS 2018 *Bioorg. Chem.* 77 625-632
[8] Lin Y, Guo HC, Kuang Y, Shang ZP, Li B, Chen K, Xu LL, Qiao X, Kim JC, Liang H, and Ye M 2020 *Fitoterapia* 141 104464
[9] Yang Y, Li Y, Yin D, Chen S, and Gao X 2016 *J. Med. Food* 19 593-600
[10] Chen J, Wang F, Liu J, Lee FS, Wang X, and Yang H 2008 *Anal. Chim. Acta.* 613 184-195
[11] Guo L, Guo J, and Xu F 2017 *3 Biotech* 7 377
[12] Guo L, Zhang F, Wang X, Chen H, Wang Q, Guo J, Cao X, and Wang L 2019 *3 Biotech* 9 14
[13] Guo L, Zhu W, Xu, F, Liu M, Xie Y, and Zhang J 2014 *CyTA – J. Food* 12 32-39