Optimization on Preparation Condition of Propolis Flavonoids Liposome by Response Surface Methodology and Research of Its Immunoenhancement Activity

Ju Yuan,¹ Yu Lu,² Saifuding Abula,³ Yuanliang Hu,¹ Jiaguo Liu,¹ Yunpeng Fan,¹ Xiaojuan Zhao,¹ Deyun Wang,¹ Xu Liu,¹ and Cui Liu¹

¹ Institute of Traditional Chinese Veterinary Medicine, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China
² National Research Center of Veterinary Biologicals Engineering and Technology, Jiangsu Academy of Agricultural Science, Nanjing 210014, China
³ College of Veterinary Medicine, Xinjiang Agricultural University, Urumchi 830052, China

Correspondence should be addressed to Deyun Wang; dywang@njau.edu.cn

Received 23 October 2012; Revised 26 January 2013; Accepted 31 January 2013

Academic Editor: Zenon Czuba

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The aim of this study is to prepare propolis flavonoids liposome (PFL) and optimize the preparation condition and to investigate further whether liposome could promote the immunoenhancement activity of propolis flavonoids (PF). PFL was prepared with ethanol injection method, and the preparation conditions of PFL were optimized with response surface methodology (RSM). Moreover, the immunoenhancement activity of PFL and PF in vitro was determined. The result showed that the optimal preparation conditions for PFL by response surface methodology were as follows: ratio of lipid to drug (w/w) 9.6:1, ratio of soybean phospholipid to cholesterol (w/w) 8.5:1, and speed of injection 0.8 mL/min. Under these conditions, the experimental encapsulation efficiency of PFL was 91.67 ± 0.21%, which was close to the predicted value. Therefore, the optimized preparation condition is very reliable. Moreover, the results indicated that PFL could not only significantly promote lymphocytes proliferation singly or synergistically with PHA, but also increase expression level of IL-2 and IFN-γ mRNA. These indicated that liposome could significantly improve the immunoenhancement activity of PF. PFL demonstrates the significant immunoenhancement activity, which provides the theoretical basis for the further experiment in vivo.

1. Introduction

Natural products are a promising source for the discovery of new pharmaceuticals. In the last decades, several works dealing with propolis composition and biological properties have been published, revealing the interest of researchers on this bee product and its potential for the development of new drugs as well [1–4]. Propolis has been employed extensively since ancient times in Egypt, Greece, Roman Empire, and so on. Its use continues today as a popular remedy and is available in either in pure form or combined with other natural products in cosmetics and as a constituent of healthy foods.

Propolis presents plenty of biological and pharmacological properties, such as immunomodulatory, antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and antiparasite activities, among others [5–10]. Propolis mechanisms of action have been widely investigated in the last years, using different experimental models in vitro and in vivo. Researchers have been interested in the investigation of isolated compounds responsible for propolis action and find flavonoids are one of the most important groups [11]. Propolis flavonoids (PF) are responsible for many of its biological and pharmacological activities [12]. Although PFs have good effect as antioxidant, antitumor, immunomodulatory, and so forth, they are easy to be oxidized [13], which makes the
flavonoids themselves be not stable. The storage, use, and function of propolis were affected with its instability.

Liposome is a synthetic bilayer membrane vesicle with phosphorus and has good affinity on cell membrane. For their biodegradability, biocompatibility, low toxicity, and their ability to entrap both lipophilic and hydrophilic drugs [14], liposomes are known for their potential and actual uses in targeted drug delivery. If PFs are encapsulated with liposome, their stability will be increasingly promoted. However, the effective factors on preparation of propolis flavonoids liposome (PFL) are various, and the preparation conditions need to be optimized. In additional, it is also important whether the biological activity is changed with liposome encapsulation.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables [15–17]. In many processes, the relationship between the response and the independent variables is usually unknown; therefore the first step in RSM is to approximate the function (response) in terms of analyzing variables (independent variables). Usually, this process employs a low-order polynomial equation in a predetermined region of the independent variables, which is later analyzed to locate the optimum values of independent variables for the best response [17].

Therefore, in the present study, RSM was employed to optimize the preparation conditions of PF, and the biological activity of PF was compared between preencapsulation and postencapsulation. The aim of this strategy is to optimize the best preparation condition of PFL and observe immunoenhancement activity in vitro, which provide the theoretical basis for the further experiment in vivo to study whether PFL could promote immune response.

2. Materials and Methods

2.1. Materials. Propolis was purchased from Dahua Chinese Traditional Medicine Company in Nanjing, Jiangsu Province. PFs were prepared in our laboratory (briefly, propolis was extracted with 95% ethanol for three times, and the ethanol solution was retrieved. Then, the precipitation was extracted with ethyl acetate for three times, and then the ethyl acetate was retrieved. Finally, the precipitation was dried in vacuum, and PF was obtained.). Soybean phospholipid (number 20110908) was manufactured by Shanghai Taiwei Pharmaceutical Co., Ltd, and Cholesterol (number 20110706) purchased from Anhui Tianqi Chemical Technology Co., Ltd. Protamine (Sigma, P4380) was dissolved by physiological saline to 10mgmL−1.

2.2. Preparation of PF Liposome. Propolis flavonoids (PF) liposome was prepared with ethanol injection method [18]. Lecithin, cholesterol, and propolis flavonoids were dissolved in about 10 mL of ethanol, and the ethanol was injected into the buffer (40°C, PBS) with a slow speed and continued to thermostatic mixing. Liposomes formed spontaneously after further evaporation of the residual ethanol. The resulting mixture was homogenized with ultrasonication for 30 min to form the small single-chamber liposome (Ultrasonic Cleaner KQ5200B, Kunshan Sonicatic equipment Inc. China) [18]. Ultimately, the solution was filtered with 0.8μm, 0.45μm, and 0.22μm millipore membrane successively [19].

2.2.1. Entrapment Efficiency (EE) of PFL Array. 0.8 mL of PFL was added in 10 mL centrifuge tube and mixed with 0.8 mL of protamine solution (10 mg·mL−1). After 3 min, 3.0 mL of physiological saline was added. After adequate mixing, this suspension was centrifuged at 3000×r·min−1 at the room temperature for 30 min. All supernatant was taken from centrifuge tube, setting the volume to 10 mL with physiological saline, 5 mL was taken to assay the content of PF by ultraviolet spectrophotometry method with Rutin standard substance [20], and the content of PF was called the content of free drug. The precipitation remaining in centrifuge tube was dissolved by 3.0 mL of Triton X-100 and then setting the volume to 10 mL with physiological saline. Five millilitres of the solution were taken to assay the content of PF by Rutin standard method and called the content of encapsulated drug. The formula to calculate liposome encapsulation efficiency was EE% = (1 − Cf/Ci), Cf: the content of free drug, Ci: the total content of drug [21–23].

2.2.2. Optimization of PFL Preparation. Based on the single-factor test, three factors, ratio of lipid to drug w/w (A), ratio of soybean phospholipid to cholesterol w/w (B), and speed of injection/mL·min−1 (C), were selected to optimize the preparation conditions of PF liposome. The selected factors were subjected to response surface methodology (RSM) with a three-factor three-coded level Box-Behnken design (BBD) to optimize the preparation conditions of PFL. The range and the levels of experimental variables investigated in this study are presented in Table I. In addition, several verification experiments were done according to the optimal conditions.

2.3. Immunoenhancement Activity of PFL In Vitro

2.3.1. T Lymphocyte Proliferation Assay. Blood samples were collected from nonimmunized White Roman chickens at 60
Table 1: Factors and levels of Box-Behnken experimental design.

| Factors                        | Code | Range and levels |
|--------------------------------|------|------------------|
| A (ratio of lipid to drug, w/w) | X₁   | 5:1 10:1 15:1    |
| B (ratio of soybean phospholipid to cholesterol, w/w) | X₂   | 6:1 8:1 10:1    |
| C (speed of injection, mL·min⁻¹) | X₃   | 0.3 0.6 1.2      |

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2.4. Statistical Analysis. Data of optimization of PFL preparation are analyzed by Design-Expert 7.0 software (Stat-Ease, Inc.), second-order polynomial equation, and ANOVA of the quadratic regression model, and the optimal conditions were showed. Data of immunoenhancement activity experiment are expressed as mean ± standard errors (S.E.). Duncan, LSD’s multiple range tests and t-tests were used to determine the
Table 2: Response surface Box-Behnken design and experimental encapsulation efficiency (%).

| Number | $X_1$ | $X_2$ | $X_3$ | Practical acquired EE | Predicted acquired EE |
|--------|-------|-------|-------|-----------------------|-----------------------|
| 1      | 5:1   | 8:1   | 0.3   | 76.00                 | 74.99                 |
| 2      | 5:1   | 10:1  | 0.6   | 82.60                 | 82.88                 |
| 3      | 15:1  | 6:1   | 0.6   | 76.90                 | 76.62                 |
| 4      | 15:1  | 8:1   | 1.2   | 72.10                 | 73.11                 |
| 5      | 10:1  | 10:1  | 1.2   | 82.04                 | 81.41                 |
| 6      | 15:1  | 10:1  | 0.6   | 80.15                 | 79.78                 |
| 7      | 10:1  | 6:1   | 0.3   | 76.40                 | 77.03                 |
| 8      | 15:1  | 8:1   | 0.3   | 78.25                 | 77.89                 |
| 9      | 10:1  | 6:1   | 1.2   | 77.00                 | 76.27                 |
| 10     | 10:1  | 8:1   | 0.6   | 92.60                 | 91.30                 |
| 11     | 10:1  | 8:1   | 0.6   | 90.78                 | 91.30                 |
| 12     | 10:1  | 10:1  | 0.3   | 80.02                 | 80.75                 |
| 13     | 10:1  | 8:1   | 0.6   | 91.00                 | 91.30                 |
| 14     | 10:1  | 8:1   | 0.6   | 91.54                 | 91.30                 |
| 15     | 5:1   | 6:1   | 0.6   | 76.80                 | 77.17                 |
| 16     | 10:1  | 8:1   | 0.6   | 90.58                 | 91.30                 |
| 17     | 10:1  | 8:1   | 0.6   | 90.58                 | 91.30                 |

Table 3: Estimated regression model of relationship between response variables (EE) and independent variables ($X_1$, $X_2$, and $X_3$).

| Source | Sun of squares | df | Mean square | $F$ value | $P$ value prob $> F$ |
|--------|---------------|----|-------------|-----------|---------------------|
| Model  | 700.32        | 9  | 77.81       | 75.33     | <0.0001             |
| A      | 6.66          | 1  | 6.66        | 6.45      | 0.0387              |
| B      | 39.21         | 1  | 39.21       | 37.96     | 0.0005              |
| C      | $6.612 \times 10^{-3}$ | 1 | $6.612 \times 10^{-3}$ | $6.402 \times 10^{-3}$ | 0.9385 |
| AB     | 1.63          | 1  | 1.63        | 1.57      | 0.2499              |
| AC     | 22.33         | 1  | 22.33       | 21.61     | 0.0023              |
| BC     | 0.50          | 1  | 0.50        | 0.49      | 0.5073              |
| $A^2$  | 225.61        | 1  | 225.61      | 218.42    | <0.0001             |
| $B^2$  | 99.76         | 1  | 99.76       | 96.58     | <0.0001             |
| $C^2$  | 241.12        | 1  | 241.12      | 233.44    | <0.0001             |
| Residual | 7.23       | 7  | 1.03        |           |                     |
| Lack of fit | 4.60     | 3  | 1.53        | 2.34      | 0.2150              |
| Pure error | 2.63      | 4  | 0.66        |           |                     |
| Cor total | 707.55     | 16 |             |           | $R^2 = 0.9898$      |

$R^2_{adj} = 0.9766$

The results showed that the maximum EE value (92.60%) was found in conditions of $X_1 = 10:1$, $X_2 = 8:1$, and $X_3 = 0.6\text{mL.min}^{-1}$. The values of regression coefficients were calculated, and the response variable and the test variables were related by the following second-order polynomial equation:

$$EE = 91.30 - 0.91X_1 + 2.21X_2 - 0.029X_3 - 0.64X_1X_2 - 2.36X_1X_3 + 0.36X_2X_3 - 7.32X_1^2 - 4.87X_2^2 - 7.57X_3^2.$$  

(1)

The statistical significance of the regression model was checked by $F$-test and $P$ value, and the analysis of variance (ANOVA) for the response surface quadratic model was shown in Table 3. The determination coefficient ($R^2 = 0.9898$), showed by ANOVA of the quadratic regression model, indicating that the model was highly significant and adequate for prediction within the range of experimental difference among groups. $P$ values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Statistical Analysis and the Model Fitting of PFL Preparation Optimization. There were 17 experimental runs for optimizing the three individual parameters in the Box-Behnken design (BBD), and the experimental conditions and the EE of PFL according to the factorial design were shown in Table 2. The results showed that the maximum EE value (92.60%) was found in conditions of $X_1 = 10:1$, $X_2 = 8:1$, and $X_3 = 0.6\text{mL.min}^{-1}$. The values of regression coefficients were calculated, and the response variable and the test variables were related by the following second-order polynomial equation:

$$EE = 91.30 - 0.91X_1 + 2.21X_2 - 0.029X_3 - 0.64X_1X_2 - 2.36X_1X_3 + 0.36X_2X_3 - 7.32X_1^2 - 4.87X_2^2 - 7.57X_3^2.$$  

(1)
variables. The $P$ value was used as a tool to check the significance of each coefficient, and the smaller the $P$ value was, the more significant the corresponding coefficient was. In this table the linear coefficients ($X_1, X_2$), a quadratic term coefficient ($X_1^2, X_2^2$, and $X_3^2$), and the interaction coefficient ($X_1 \times X_2$) were found significantly ($P<0.05$). The other term coefficients were not significant ($P>0.05$). By the Design-Expert software to further optimize the preparation conditions, the optimum for preparation of PFL conditions obtained was as follows: ratio of lipid to drug (w/w) 9.6:1, ratio of soybean phospholipid to cholesterol (w/w) 8.5:1, and speed of injection 0.8 mL-$\text{min}^{-1}$.

### 3.2. Verification of Predictive Mode

The suitable of the model equation for predicting the optimum response value was tested by using the selected optimal conditions. The maximum predicted and experimental value of EE was given in Table 4. To ensure the predicted result was not biased toward the practical value, experiment rechecking was performed by these modified optimal conditions: ratio of lipid to drug (w/w) of 9.6:1, ratio of soybean phospholipid to cholesterol (w/w) of 8.5:1, and speed of injection 0.8 mL-$\text{min}^{-1}$. A mean value of $91.67 \pm 0.21\% (n=3)$ obtained from real experiments, demonstrated the validation of the RSM model, indicating that the model was adequate for the preparation process.

### 3.3. Effect of PFL on T Lymphocyte Proliferation In Vitro

#### 3.3.1. Effect of T Lymphocyte Proliferation in Single Stimulation of Drugs

The results are listed in Figure 1. At 60–15 $\mu\text{g}\cdot\text{mL}^{-1}$, the $A_{570}$ values of PFL group were the highest in those four groups and significantly higher than those of PF, BL, and cell control groups ($P<0.05$). At 15–3.75 $\mu\text{g}\cdot\text{mL}^{-1}$, the $A_{570}$ values of PF groups were significantly higher than those in BL and cell control group ($P<0.05$).

#### 3.3.2. Effect of T Lymphocyte Proliferation in Synergistical Stimulation of Drugs with PHA

The results are shown in Figure 2. At 60–3.75 $\mu\text{g}\cdot\text{mL}^{-1}$, the $A_{570}$ values of PFL group were the highest in those five groups. At 60–15 $\mu\text{g}\cdot\text{mL}^{-1}$, the $A_{570}$ values of PFL group were significantly higher than those in PF, BL, PHA, and cell control groups ($P<0.05$). At 7.5–3.75 $\mu\text{g}\cdot\text{mL}^{-1}$, the $A_{570}$ values of PFL and PF groups were significantly higher than those in BL, PHA, and cell control groups ($P<0.05$).

#### 3.3.3. Effect of PFL on Expression Level of IL-2 mRNA

The results are listed in Figure 3. IL-2 mRNA level of PFL and

### 3.4. Effect of PFL on Expression Level of IFN-γ mRNA

The results are showed in Figure 4. IFN-γ mRNA level of PFL and PF groups at 60–15 $\mu\text{g}\cdot\text{mL}^{-1}$ were significantly higher than those of BL and PHA groups ($P<0.05$), and these of PFL group being significantly higher than those of PF groups ($P<0.05$). IL-2 mRNA level of PFL group at 60 $\mu\text{g}\cdot\text{mL}^{-1}$ was significantly higher than those of PFL at 30 $\mu\text{g}\cdot\text{mL}^{-1}$ and 15 $\mu\text{g}\cdot\text{mL}^{-1}$ groups, and at 30 $\mu\text{g}\cdot\text{mL}^{-1}$ being significantly higher than at 15 $\mu\text{g}\cdot\text{mL}^{-1}$ group.

### 4. Discussion

Encapsulation efficiency is critical factor to appraise the quantity of liposome drugs and is affected with many factors. In order to achieve high encapsulation efficiency of PFL, in the experiment the preparation conditions of PFL must be optimized.

Traditionally, optimization in analytical chemistry has been carried out by monitoring the influence of one factor at a time on an experimental response. Its major disadvantage is that it does not include the interactive effects among the variables studied. As a consequence, this technique does not

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**Table 4: Predicted and experimental values of the responses at optimum conditions.**

|                          | Ratio of lipid to drug (w/w) | Ratio of soybean phospholipid to cholesterol (w/w) | Speed of injection/(mL$\cdot\text{min}^{-1}$) | EE (%)       |
|--------------------------|-----------------------------|-----------------------------------------------|---------------------------------|--------------|
| Optimum conditions       | 9.630 :1                    | 8.470 :1                                       | 0.760                           | 91.59        |
| Modified conditions      | 9.6 :1                      | 8.5 :1                                         | 0.8                             | 91.67 $\pm$ 0.21 |

**Figure 1: Changes of T lymphocyte proliferation in single stimulation with drugs ($A_{570}$ values). $^a$ Bars in the same day without the same superscripts differ significantly ($P<0.05$).**

PF groups at 60–15 $\mu\text{g}\cdot\text{mL}^{-1}$ were significantly higher than those of BL and PHA groups ($P<0.05$), and these of PFL group being significantly higher than those of PF groups ($P<0.05$). IL-2 mRNA level of PFL group at 60 $\mu\text{g}\cdot\text{mL}^{-1}$ was significantly higher than those of PFL at 30 $\mu\text{g}\cdot\text{mL}^{-1}$ and 15 $\mu\text{g}\cdot\text{mL}^{-1}$ groups, and at 30 $\mu\text{g}\cdot\text{mL}^{-1}$ being significantly higher than at 15 $\mu\text{g}\cdot\text{mL}^{-1}$ group.
The evidence-based complementary and alternative medicine study demonstrates a significant increase in T lymphocyte proliferation (A_{570} values) in the presence of drugs with PHA compared to the control groups (Figure 2). In vitro, the immunomodulatory activity of PFL was compared to PF and PHA control, with PFL showing significantly higher values in certain concentrations (15–60 μg·mL^{-1}) compared to PF (Figures 1 and 2). Furthermore, the results suggested that concentrations of 15–60 μg·mL^{-1} of IL-2 mRNA and IFN-γ mRNA in PFL group were significantly higher than those in PF group (Figure 3 and Figure 4). In addition, the increases of expressions of IL-2 mRNA and IFN-γ mRNA in PFL group were significant from 15 μg·mL^{-1} to 60 μg·mL^{-1}. These indicated that the immunomodulatory activity of PFL was related with concentrations, when the higher the concentration was, the better the immunomodulatory activity was. Which indicated that the immunomodulatory activity of PF was obviously promoted after PF was encapsulated with liposome, and higher encapsulation efficiency played important role in the immunomodulatory activity of PFL.

In conclusion, PFL was prepared by ethanol injection method, and the preparation conditions of PFL were optimized with RSM. The optimal preparation conditions for PFL was as follows: ratio of lipid to drug (w/w) 9.6 : 1, ratio of soybean phospholipid to cholesterol (w/w) 8.5 : 1, and speed of injection 0.8 mL·min^{-1}. Under these conditions, the experimental encapsulation efficiency of PFL was 91.67 ± 0.21%. In vitro, PFL not only could significantly promote T lymphocyte proliferation, but also significantly increased the expressions of IL-2 mRNA and IFN-γ mRNA in PFL group compared to PF group (Figures 1 and 2). Therefore, PFL not only could significantly promote T lymphocyte proliferation, but also significantly increased the expressions of IL-2 mRNA and IFN-γ mRNA in PFL group compared to PF group (Figures 1 and 2).
lymphocytes proliferation singly or synergistically with PHA, but also could increase the expression levels of IL-2 and IFN-γ mRNA, demonstrated the stronger immunoenhancement activity, which provides the theoretical basis for the further experiment in vivo.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| PF | Propolis flavonoids |
| PFL | Propolis flavonoids liposome |
| RSM | Response surface methodology |
| PHA | Phytohemagglutinin |
| ELISA | Enzyme-linked immunosorbent assay |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| CMF | Calcium and magnesium-free |
| PBS | Phosphate-buffered saline |
| DMSO | Dimethyl sulfoxide |
| EE | Entrapment efficiency |
| BBD | Box-Behnken design |
| BL | Blank liposomes |
| BC | Blank control |
| IL-2 | Interleukin-2 |
| IFN-γ | Interferon-γ |

**Authors’ Contribution**

J. Yuan and Y. Lu contributed equally to this work.

**Acknowledgments**

The project was supported by National Natural Science Foundation of China (Grant no. 30901085), the Fundamental Research Funds for the Central Universities (Grant no. KYZ2011I7), Special Fund for Agro-scientific Research in the Public Interest (Grant no. 201303044), and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). The authors are grateful to all other staff in the Institute of Traditional Chinese Veterinary Medicine of Nanjing Agricultural University for their assistances in the experiments.

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